BIOREFINERY of PROTEINS from RUBBER PLANTATION RESIDUES

Widyarani

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Chapter 1

Introduction

1.1 Biobased economy

For millennia of human history, we depended on biomass as our energy source. The major shift towards fossil fuels started around 250 years ago with the use of coal during the emerging industrial revolution[1]. Today fossil fuels have become our main energy source (Figure 1.1). We also depend on fossil resources for machinery, heat, and fertiliser in agriculture, and as feedstocks for the petrochemical industry. Coupled with advances in science and technology, the use of fossil resources has brought unprecedented wealth and prosperity.

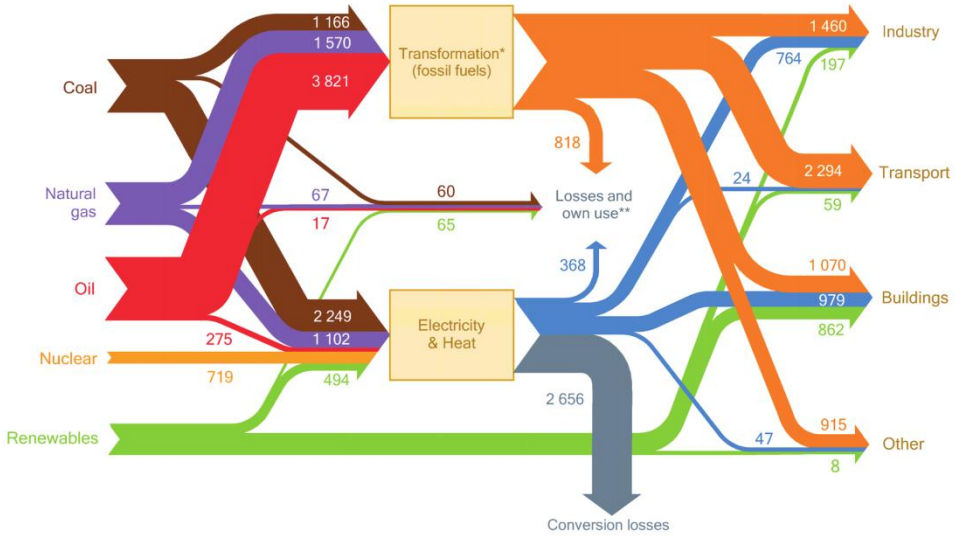

Figure 1.1 The global energy system, 2010[2].
Numbers in million tonnes of oil equivalent (Mtoe).
Other includes agriculture, feedstocks for the petrochemicals industry, and non-energy uses.
* Transformation of fossil fuels from primary energy into a form that can be used in the final consuming sectors.
** Includes losses and fuel consumed in oil and gas production, transformation losses and own use, generation lost or consumed in the process of electricity production, and transmission and distribution losses.

Despite its many benefits, the fossil-based economy also has several drawbacks. Fossil fuels use contributes to the increase of greenhouse gases in the atmosphere, leading to anthropogenic climate change. Population growth and the still-increasing wealth, particularly in non-OECD countries, increases energy demand by 1% annually[2]. The increasing use of fossil resources causes depletion of (cheap) oil resources, and in some parts of the world disrupts the security of energy supply that results in geo-political

tensions. In the last couple of years, new techniques such as horizontal drilling and hydraulic fracturing are introduced to extract oil and natural gas in previously unattainable reservoirs. These techniques open new oil and natural gas reserves, as well as reduce the fuel price significantly. However, they are also considered posing bigger risk of surface and groundwater contamination compared to conventional techniques[3]. Oil extraction in permafrost area, like the Arctic, disrupts the ecosystem balance with contamination and temperature increase from drilling activities[4].

As an alternative approach, biobased economy can potentially avoid or minimise the drawbacks of fossil-based economy. Furthermore, the switch towards biobased economy also offers potential advantages such as employment opportunities and rural development[5]. Biobased economy does not mean a complete abandonment of fossil fuels use, as this will hurt the economy and our daily life, and is unlikely to happen in the near future. Instead, it is envisioned as the "technological development that leads to a significant replacement of fossil fuels by biomass in the production of pharmaceuticals, chemicals, materials, transportation fuels, electricity and heat"[5]. This also means that biobased economy is not limited to biofuels, but also to a range of products that can be produced from biomass. Two aspects of the biobased economy are discussed as follows.

1.1.1 Food vs. fuel

Even though fossil fuels are currently our main energy sources, Figure 1.1 shows that still 13% of our energy comes from renewable sources, including biomass. Biomass use for energy in 2010 was estimated to be 1277 Mtoe[2], equivalent to the energy content of 2970 million tonnes of wood. However, more than 50% of the biomass for energy use is traditional biomass: firewood, charcoal, animal manure, and agricultural residues. This type of biomass has limited applications and relatively low efficiency, and its use is expected to decrease in the coming years. On the other hand, the use of transportation biofuels is expected to increase from 66 Mtoe in 2010 to 230 Mtoe in 2035[2].

Current allocations of biomass for biofuel and its expected increase in the coming years generate concerns whether the shift towards biofuel will threaten food security, often dubbed as food vs. fuel debate. With the world population estimated to increase to 9.6 billion in 2050[6], we will need to provide 70% to 100% more food than we do today[7,8].

To determine whether biomass use for energy (and other non-food applications) poses a threat to food security, an understanding of our agricultural system is required. Currently around 37% of the total ice-free land area is used for agriculture, either for cropping or pasture (Figure 1.2a). With the aid of fertiliser and irrigation (calculated as the yield

increase in carbon equivalent), the net primary productivity from cropland is 8400 million tonnes carbon/year (Figure 1.2b). About half of this value goes to harvest and residue, the remainder goes to roots and losses due to pests and weeds. From the total harvest (2400 million tonnes carbon/year), the highest fraction is used for livestock feed while only a small fraction goes directly to food. Losses occur from harvest account for 30-40% of food fraction, mainly due to inefficient processing and lack of proper storage in developing countries and from waste by distributors and consumers in developed countries[10,11]. These facts suggest that our current food production and consumption systems are rather inefficient. Next to food usage, around 240 million tonnes carbon/year was used for energy[9], which equals one-third of the fraction used for food, but is relatively a small fraction from the total harvest.

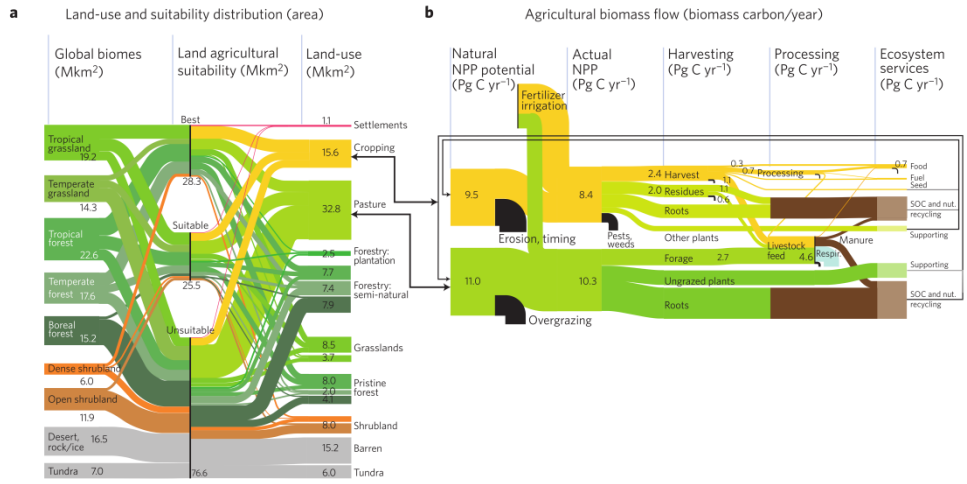

Figure 1.2 Distribution of major terrestrial ecological communities (biomes), land agricultural suitability and land use (a) and its connection to the global agricultural annual biomass flows (b) for 2009[9].

NPP = net primary productivity; SOC = soil organic carbon. 1 Pg = 1000 million tonnes

Next to cropland, Figure 1.2b also shows the use of pasture land is still not optimal. Only about half of forage is grazed by the livestock, the other half is either unsuitable for fodder or inaccessible. Furthermore there is still around 25% grassland that is not used for pasture that also represents underutilised biomass source.

To increase food production, expansion of agricultural land is theoretically feasible (Figure 1.2a), however most arable land that is currently not used for agriculture belongs to tropical forests that should be preserved due to their critical role for life on Earth.

Therefore in the last few decades, the increase in food production is led by cropland yield increase via improvement in agricultural practices and the use of fertiliser, pesticide, and irrigation. To double global food production in 2050, cropland yield must increase at the rate of 2.4% per year. However, several studies projected that cropland yield currently increases at a lower rate or even reaches plateau[7,12,13]. This is probably because major yield increases have been achieved in the past, particularly during green revolution in the late 1960s, and more recently some crops are already reaching their maximum (theoretical) yields. Some resources, e.g. water, have become limiting, although this problem can partially be solved by irrigation[7]. On the other hand, cropland yields still generally vary between regions and particularly for developing countries, technology improvement is still expected to contribute to yield increase[8].

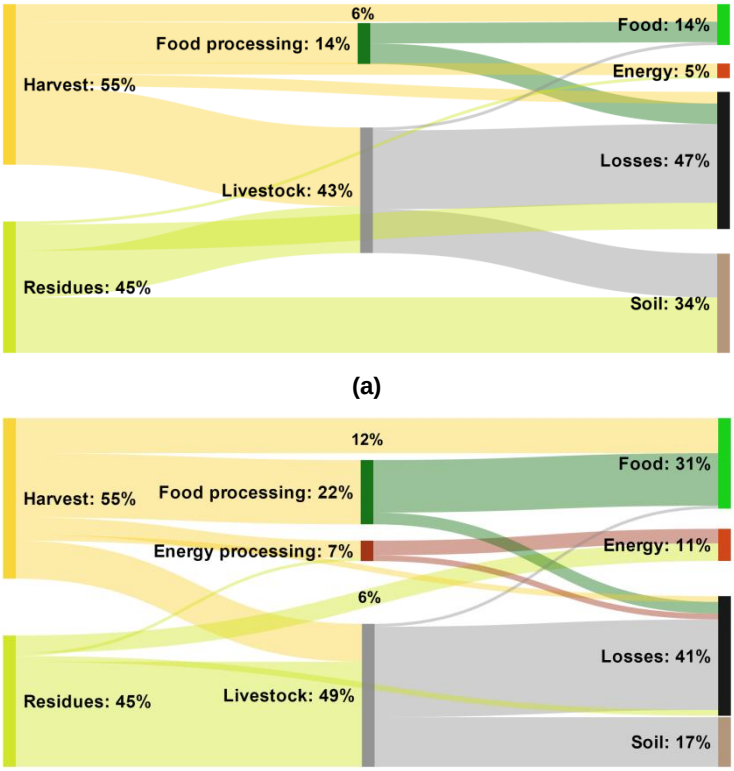

Figure 1.3 Distribution of harvest and above-ground residues to different uses.
(a) Current distribution[9]; (b) Envisaged distribution.
Numbers are based on carbon weight.

When cropland yield increase is limited, the other approach that can be taken is optimising the yield by redistribution of biomass fractions to their optimal uses. Figure 1.3a shows current distribution of cropland yield (harvest and above-ground residues); in total 68% is used or processed for food, feed (livestock), and energy[9]. The overall biomass use efficiency for food (including livestock products) and energy only accounts for 19% of the initial biomass fraction or 28% of the used fractions.

If cropland yield can be redistributed to their optimal uses, more fractions can be used. Figure 1.3b assumes 96% of biomass fractions can be used and redistributed: harvest allocation for food is doubled, less harvest is used for feed, and more fractions from residues can be used for feed and energy. No residues are allocated directly to the soil, however, the roots (below ground residues) that are available at roughly the same amount as the above-ground residues can still provide nutrients for the soil. With this approach, the overall biomass use efficiency for food and energy is now more than doubled to 42% of the initial biomass fraction.

Table 1.1 Cropland yield, biomass use efficiency, and land use efficiency.

Scenario	Cropland yield index[a]	Biomass use efficiency[b] (%-carbon weight)	Land use efficiency index[c]
Constant yield	100	19	19
	100	42	42
Low increase[d]	121	19	23
	121	42	51
High increase[d]	182	19	35
	182	42	76
Low yield increase,	121	45[e]	54
optimised	121	64[f]	77

[a] Cropland yield consists of harvest and above-ground residues. Index 2008 = 100.

[b] The use of cropland yield for food and energy.

[c] Calculated as cropland yield multiplied by biomass use efficiency.

[d] Low and high yield increase based on projection of four key global crops: maize, rice, wheat, and soybean[13], assuming increase in harvest is proportional to overall cropland yield.

[e] Efficiency includes the increase of food produced from the same input of feed.

[f] Efficiency based on reduced feed allocation to produce the same amount of food, reallocation of all harvest to food, and reallocation of surplus feed from residue to energy (Figure 1.5).

As shown in Table 1.1, the increase of biomass use efficiency from 19% to 42% without changes in yield already gives higher increase in land use efficiency compared to low yield increase without increase in biomass use efficiency. Combination of low yield increase and higher biomass use efficiency may be more beneficial and gives less impact to the environment than higher yield increase and no change in biomass use efficiency. This shows that increasing food production and gradual shifting towards biofuels are both challenges that can and should be handled simultaneously, and one of the approaches that can be taken is increasing land use efficiency by a more efficient biomass use.

1.1.2 Protein-based biorefinery

Increasing biomass use efficiency can be done by allocating biomass fractions to applications that optimise their values. Based on Figure 1.3, this means allocating more harvest to food, optimising nutrient recycling, and reducing losses. Additionally, residues and grass can be fractionated into protein, fibre, and energy; optimal use can be allocated for each fraction. The use of non-traditional feedstocks e.g. agricultural residues and grass may increase land use efficiency, but asks for a new technological approach. The latter can be in the form of new conversion technologies or new applications of existing technologies[5].

Biorefinery is the sustainable processing of biomass into a spectrum of biobased products and bioenergy. The term is analogous to petroleum refinery, in which crude oil is fractionated and refined into different products. With a biorefinery approach, the existing use of biomass for food, feed, traditional biofuels (e.g. firewood, charcoal), and biomaterials (e.g. paper, clothes, timber) is expanded to biobased chemicals, advanced biomaterials, and transportation biofuels[14].

Proteins are essential components in human diet. Global protein consumption for food is estimated to increase from 355 million tonnes/year in 2005 to 748 million tonnes per year in 2050[8]. The increase is not only driven by population growth, but also by increasing wealth that shifts consumption patterns. This is more evident in developing countries, where population is estimated to increase from 5.9 billion in 2013 to 8.25 billion in 2050, and meat consumption is estimated to increase from 28 kg/person/year in 2007 to 42 kg/person/year in 2050. In developed countries, on the other hand, population is only estimated to increase from 1.25 billion in 2013 to 1.3 billion in 2050, while meat consumption is still estimated to increase from 80 kg/person/year in 2007 to 91 kg/person/year in 2050[6,7]. The increase in animal protein consumption adds significant pressure to the whole food system, as only less than 3% of the carbon input (from harvest,

crop residue, and pasture forage) for livestock ends up as food, including eggs and dairy (Figure 1.2b). In 2008, 150-170 million tonnes protein were used in compound feed, sourced almost entirely from oilseed meals[15].

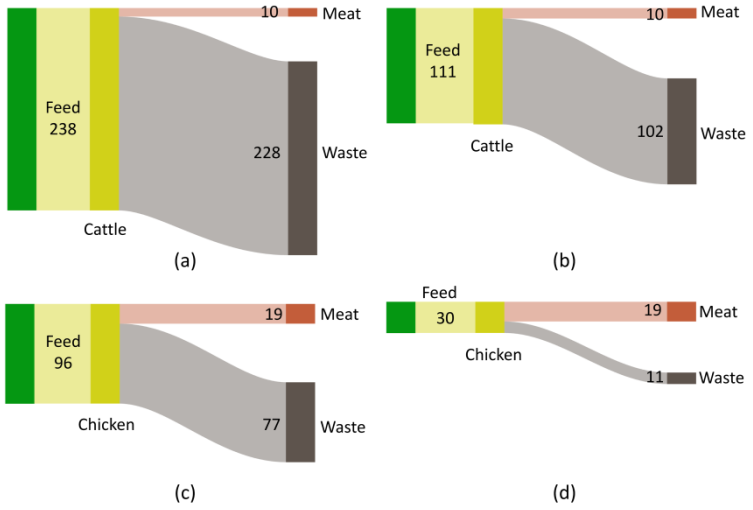

Figure 1.4 Estimated feed protein requirements for beef cattle and chicken based on protein conversion efficiency (a, c) and daily protein intake recommendation (b, d).
Numbers in million tonnes of protein.
Meat production and livestock population were for year 2013[16]. Meat protein content and protein conversion efficiency were calculated from Smil[17]. Daily protein intake recommendation were estimated from NRC[18,19].

Figure 1.4a and 1.4c illustrate that only 4% of feed protein for beef cattle and 20% of feed protein for chicken are converted into meat protein[17]. Based on daily protein intake recommendation (NRC[18,19]), the total protein requirements were estimated as 374 kg protein for a 1.5-year old cattle and a 0.5 kg protein for a 9-week old chicken. Therefore to feed the same amount of animal and produce the same amount of meat protein, less than half of feed protein is required (Figure 1.4b and 1.4d) The reason why a lot more protein should be fed to the livestock than it is required is to compensate for non-ideal protein profile in the feed, whether it is the protein digestibility, the losses by the microbial conversion of the proteins in the rumen, or the amino acid profile in the poultry diet.

Based on Figure 1.4 and assuming protein use is proportional to carbon use, if proteins with the ideal profile can be provided, the allocated biomass for feed in Figure 1.3b can produce 2-3 times the amount of food from the livestock. This is estimated to further increase biomass use efficiency from 42% to 45%. At a low increase in cropland yield, the

biomass use efficiency results in a land use efficiency index of 54, almost three times the base case with current yield and biomass use (Table 1.1).

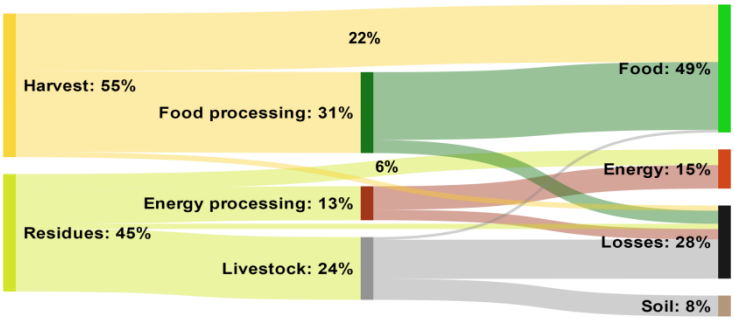

Figure 1.5 Distribution of harvest and above-ground residues to different uses with optimised biomass allocation for feed.

Alternatively, the same amount of food from the livestock can be produced from half the amount of feed with the ideal protein profile (Figure 1.5). This means more harvest can be allocated for food. Furthermore, since also less residues is allocated for feed, the remainder can be allocated for other uses e.g. energy. If this can be done efficiently, the harvest previously allocated for energy can also be allocated for food. This can increase biomass use efficiency to 64% and land use efficiency index to 77 (Table 1.1). However, this may require a shift in diet because, even though the absolute amount of food from livestock may be the same, the relative amount compared to total food decreases from 5% in the base case to only 2% in this scenario.

Biorefinery for protein, particularly using agricultural residues, can increase protein availability from non-food sources for multiple applications (Figure 1.6). Agricultural residues include biofuels production residues, leaves, grass, stover, microalgae, and animal slaughter waste. First generation bioethanol or biodiesel production, for instance, yields residues that contain up to 52% protein[20]. With the increasing use of biofuels, this type of residue will be abundantly available.

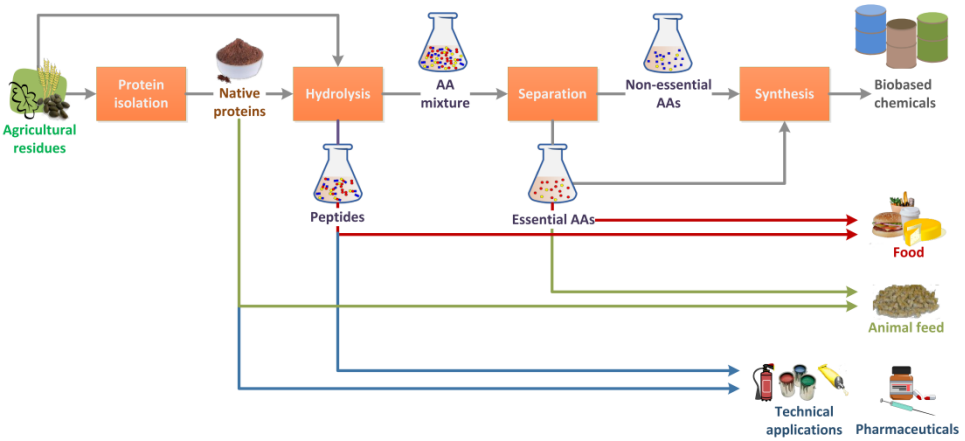

Figure 1.6 Idealised non-fermentative routes for protein-based biorefinery from agricultural residues.

By definition, agricultural residues are not used directly as food, but they can be used indirectly as animal feed. Residues with 50% protein, e.g. soybean meal, can be used directly as animal feed. Residues with lower protein content may be used as feed ingredient, but need several processing steps to get optimum applications (Figure 1.6). Processing also can increase protein conversion efficiency of the feed. Digestibility can be increased via alkaline treatment or hydrolysis with protease[21,22]. Hydrolysis of proteins from agricultural residues also can produce short peptides or essential amino acids for both food and feed applications[23].

Proteins also can be used for food and non-food applications based on their technical properties, e.g. as emulsifier, foaming agent, and adhesives[24]. Purified proteins or peptides of high quality might be applied for cosmetics or pharmaceuticals[23,25].

Amino acids from proteins also can be used to produce nitrogen-containing chemicals. Figure 1.7 illustrates that 1,2-ethanediamine, a bulk chemical that is used in e.g. corrosion inhibitors, anti-scaling agents, and lubricants[28], can be produced via ethanolamine from both petrochemical and biobased sources. In the petrochemical route, ethanolamine is produced via oxidation of ethylene and addition of ammonia. In the biobased route, ethanolamine can be produced via decarboxylation of serine, an amino acid. The latter requires less process steps and energy than the petrochemical route. Ammonia is only added at the last step, from ethanolamine to 1,2-ethanediamine, because serine already has one $-NH_2$ group[27].

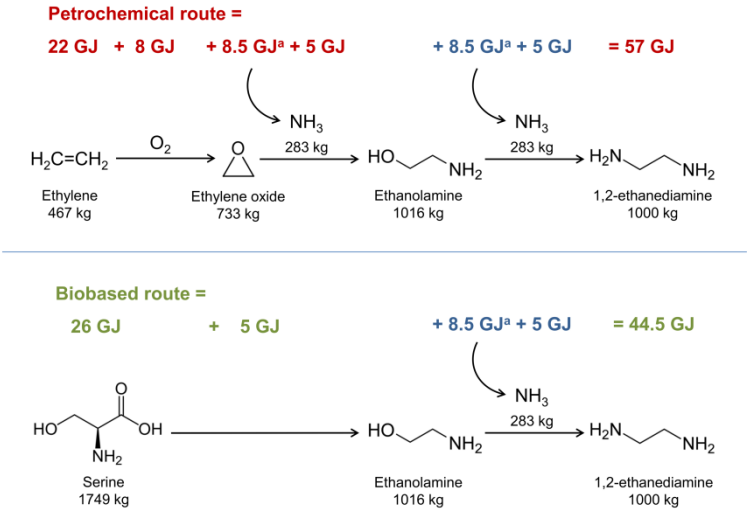

Figure 1.7 Production of 1,2-ethanediamine from ethylene and serine[26,27].
a = energy for ammonia production

In this illustration (Figure 1.7), the calorific value of ethylene and serine are based on heat of combustion while the energy to produce either serine or ethylene is not taken into account. Steam cracking of naphtha to produce ethylene requires 20.4 GJ/tonne energy input[29]. With equivalent comparison, energy consumption to obtain serine from biomass should be less than 33 GJ/tonne to make biobased route favourable. Currently, serine is industrially produced via fermentation using *Methylobacterium sp.*[30]. Available data on energy consumption in production of serine (or amino acids in general) is scarce, but from current industrial practice for glutamic acid and lysine production, it is estimated at 24 GJ/tonne[31].

Next to fermentation, the alternative to produce amino acids is via hydrolysis of proteins, as illustrated in Figure 1.6 (simplified). Protein hydrolysis was widely applied before 1950, but is currently only applied mostly for cyst(e)ine and proline productions due to limited raw material availability[30]. With the foreseen abundance of protein-rich agricultural residues, protein hydrolysis may also gain renewed significance. Amino acids from hydrolysis, however, are present as a mixture containing multiple amino acids. Due to these heterogeneous properties and the aqueous system the amino acids are present in, it is still difficult to isolate single amino acids from this mixture. Therefore, the challenge in using proteins for biobased chemicals production lies not only in converting the amino acid to the desired product, but also in obtaining amino acids from the biomass in a cost-efficient and energetically-efficient way.

1.2 Agriculture beyond food

The (envisioned) switch towards biobased economy is influencing land use practices. With growing agricultural production, Indonesia is one of the countries that experience rapid changes in rural landscape and farmers' livelihood. For instance in Riau, Sumatera between the years 2002 and 2009, 15% of the small scale rice fields were converted to other uses, almost half of them to oil palm plantations. This brought positive consequences, such as increased income, but also disturbed rice sufficiency; rice being the staple food for the majority of the Indonesian population[32]. "Agriculture Beyond Food" is an interdisciplinary research programme launched in 2009 to study the opportunities and constraints of the implementation of biobased economy in Indonesia. The research in this thesis was a part of the cluster "Breakthrough in biofuels: Mobile technology for biodiesel production" that studied the technologies to use agricultural waste streams in remote rural areas. Central Kalimantan was selected as the study area because it was also the location of the short-lived Mega Rice Project that resulted in severe environmental damages. This will be discussed in subchapter 1.2.3.

1.2.1 Biobased economy in Indonesia

Indonesia is an archipelago with total 191 million hectares of land, of which most is forest area. Arable land and permanent crops comprise of 25% of the total land area[16]. Located in the equator, the agricultural sector has the advantage of year-round sunshine and warm climate. Rice is the most important commodity, both in terms of quantity and in net production value (Figure 1.8). To fulfil domestic needs, Indonesia still imports around 5% of its rice logistic[16].

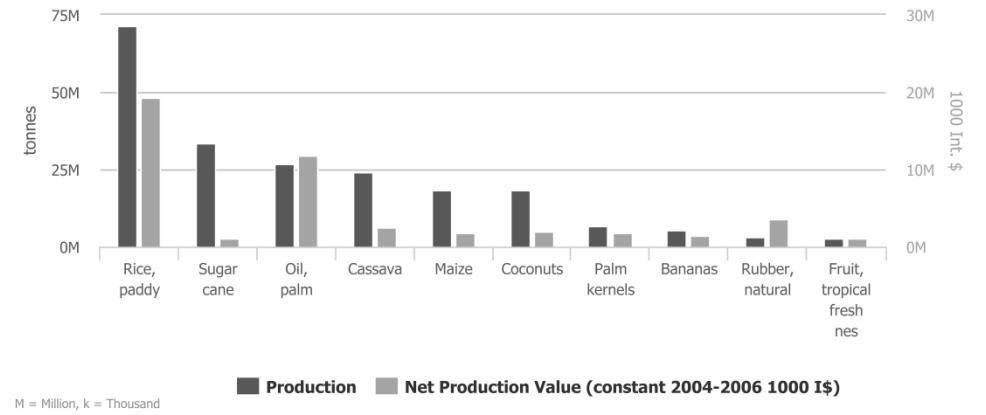

Figure 1.8 Ten most important food and agricultural commodities in Indonesia, 2013[16].

In 2013, the contribution of the agricultural sector to Indonesian gross domestic production was USD 125 billion, of which more than 80% of the output was allocated to food, including livestocks and fisheries[33,34]. Forestry contribution to the gross domestic product was USD 5.5 billion[33], however it was estimated that 0.6 to 8.7 billion is lost annually to illegal logging[35]. Indonesia has one of the highest deforestation rates in the world, partly due to logging and recently more due to land clearing, particularly for oil palm[35,36].

For estate crops, oil palm and rubber have the highest net production value (Figure 1.8). Indonesia produced 33 million tonnes palm oil in 2014, nearly half the world production, of which 70% was exported. For domestic use, two-thirds was used in food applications and one-third was used in industrial applications, including biodiesel and oleochemicals[37]. The share of industrial applications is expected to increase with increasing demand—along with capacity—of biodiesel production. Palm oil biodiesel is currently the most important transportation biofuel, followed by bioethanol from cassava and sugarcane that are still produced at much lower volumes[38]. The other biobased products, e.g. pharmaceuticals and biochemicals, are still of minor value[39].

1.2.2 Rubber industry in Indonesia

Rubber trees or *Hevea brasiliensis* (Figure 1.9a) are harvested for their latex, which is used to produce natural rubber. Indonesia has the world's largest rubber harvesting area of 3.6 million hectares[16], which is mostly (85%) owned by smallholder farmers[40]. The productivity of these plantations is low, on average 0.9 tonne dry-rubber/ha[16].

(a) (b)

Figure 1.9 Rubber trees in an agroforest (a) and latex tapping process (b).

Latex is harvested from rubber tree by tapping—leaking latex from the bark by making an incision to expose latex vessels (Figure 1.9b)—and the leaked latex is collected for 6-8 hours, sometimes longer. The latex can be transferred to the processing plant as liquid latex or slab (coagulated latex); the latter is commonly produced by smallholder farmers in villages and remote areas. The price received by farmers is fluctuating, often determined by middlemen who collect the slabs and bring them to the plant[41].

Next to latex, rubber wood has economic value as construction material[42]. In large plantations, a small amount of good quality seeds are used for propagation.

1.2.3 Study area

In this study, biomass originating from Central Kalimantan, Indonesia, was investigated. Central Kalimantan was the location of the Mega Rice Project initiated in 1996 by the Indonesian government to increase national rice production. The project aimed to convert one million hectares of peatlands into paddy fields, relying on land clearing by fire, building of deep drainage and irrigation channels, and on a transmigration programme involving farmers from outside the area. The opening of the peatlands led to illegal logging and altered the ecosystem. Fires occurred partly because of the application of fire clearance, and were enhanced by draught episodes and the drainage system. The project failed to meet its objective. By the end of the project in mid-1998, the peatlands destruction was irreversible and the risk of fire was still imminent in the following years (Figure 1.10)[43].

Figure 1.10 The Ex-Mega Rice Project area, 2004[44].

The local livelihood in the ex-mega rice project area relies on rotating rice cultivation, smallholder plantations, forest timber extraction, collection of non-timber forest products, and fishing. Rubber has gained importance since the high rubber price in 2005[45]. Rubber plantation area in Central Kalimantan is 450 thousand hectares, and has not changed much in the last five years. The area comprises mostly of small plantations and agroforests. Recently there is a huge increase in oil palm plantations, from 129 thousand hectares in 2011 to 881 thousand hectares in 2013[46].

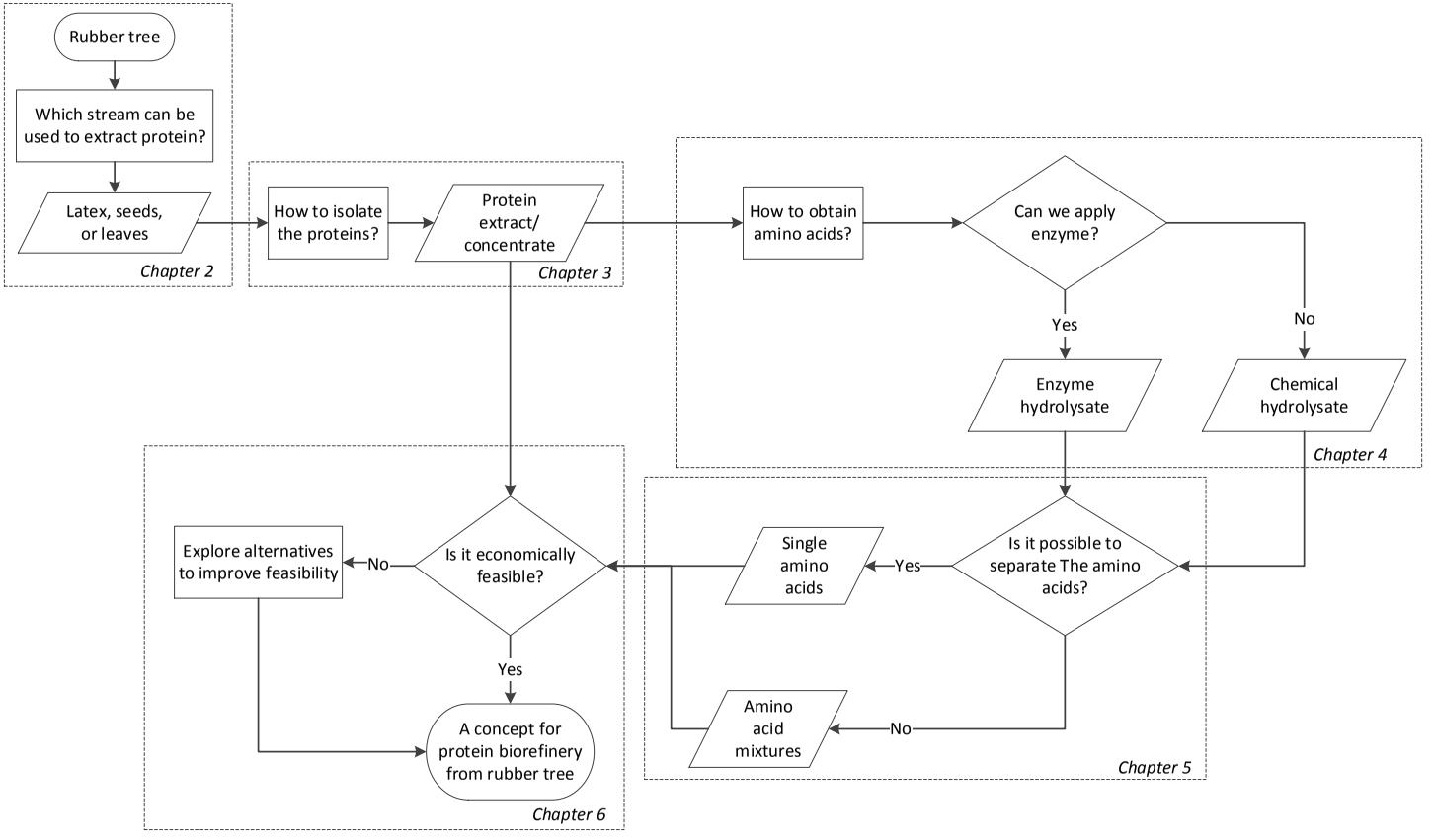

Figure 1.11 Research approach to valorise protein from rubber trees.

1.3 Research questions

The objective of this research was to design a process for the recovery of proteinaceous fractions from rubber trees for applications that are suitable for local use. In order to achieve this objective, the following research questions needed to be answered:

1. Which stream(s) can be used to obtain proteins from the rubber tree?
2. How to efficiently isolate proteins from rubber seed and its press cake?
3. Which method is most suitable in a biorefinery framework to obtain amino acids from the proteins?
4. Is it possible to separate amino acids in the protein hydrolysate?
5. What are the possible applications of the proteinaceous fractions for rural and industrial conditions? In particular, which application has the highest value in rural economies for the Indonesian case?

1.4 Thesis outline

The approach taken in this research is depicted in Figure 1.11. **Chapter 2** will discuss the availability, possible applications, and economic potential of proteins that are present in different fractions of the rubber tree. **Chapter 3** will discuss methods to obtain protein and oil from the rubber seeds. The focus is on optimising protein recovery, therefore the envisaged process should have the highest protein recovery and a reasonable oil recovery, taking into account both protein and oil quality. **Chapter 4** will discuss methods to increase the value of rubber seed protein by enzymatic protein hydrolysis. Specifically, the selective hydrolysis of hydrophobic amino acids will be discussed. **Chapter 5** will discuss separation of amino acids from the hydrolysate, using precipitation with ethanol as the anti-solvent. The application to other mixtures that are rich in amino acids will also be discussed. **Chapter 6** will summarize the findings in Chapter 2-5 and discuss the feasibility of the process from the perspective of local economy and sustainability.

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Chapter 2

Valorisation of Proteins from Rubber Tree

* This chapter was submitted as: Widyarani; Coulen, S.; Sanders, J. P. M.; Bruins, M. E. Valorisation of proteins from rubber tree.

Abstract

Rubber tree is primarily grown for its latex that is used in rubber production. Indonesia has the largest rubber plantation area that is mostly owned and run by smallholder farmers. Using non-latex fractions from the rubber tree may generate additional income, and increase the economics of rubber plantations in general. Proteins from non-food sources are important biobased feedstock since they can be used in several applications: food, feed, or biochemicals, with no or little competition with food production. Several biomass streams from the rubber tree and subsequent latex processing were investigated. Based on the amount of available proteins, latex waste streams, seeds, and leaves were considered to have the highest potential, and processes to isolate proteins from these streams were proposed. Protein isolation from latex requires complex (and expensive) separation processes, therefore it is only economically feasible when specific use of the protein(s) for high value applications can be identified. A biorefinery concept can be applied to obtain multiple products from the seeds and leaves, and protein extraction can be performed with available knowledge and technology. In these cases, small scale processing can be more beneficial for the farmers, especially if the products are used locally for feed.

Keywords: biorefinery, protein, rubber latex, rubber seeds, rubber leaves, Indonesia

2.1 Introduction

Rubber tree (*Hevea brasiliensis*) is an industrial crop currently planted mainly to produce natural rubber. The tree belongs to *Euphorbiaceae* family and grows in tropical climates. In the forest, the tree can reach up to 40 m height. In plantations, however, tree height is usually less than 25 m[1]. Indonesia has 3.5 million hectares rubber trees harvesting area, the largest in the world[2]; 85% is run by smallholder farmers in traditional plantations and agroforests[3].

Currently, studies related to rubber tree are focused on optimising latex production as its main product. Utilisation of other fractions from rubber tree has received less attention. Using protein fractions from the rubber tree may increase the overall economics of rubber plantations. Proteins from non-food sources are important biobased feedstock since they can be used in several applications: food, feed, or biochemicals. The objective of this study was to identify the availability, possible applications, and economic potential of proteins that are present in different parts of the rubber tree.

2.2 Methods

Data on Indonesian rubber production were compiled from literature and interviews with researchers at Rubber Technology Research Centre, West Java; plant and plantation managers at PTPN 8 Cikumpay processing plant and plantation, West Java; and smallholder farmers at Subang (West Java), Palangka Raya and Pulangpisau (Central Kalimantan), and Banjarbaru (South Kalimantan). As a case study, we also gathered data from a pilot seed refinery program in Palangka Raya, Central Kalimantan.

Latex, crumb rubber, waste water, bark, and leaves samples were collected from PTPN 8 Cikumpay. Protein content of these samples was determined by Kjeldahl[4], using the Gerhardt Kjeldahltherm and Gerhardt Vadopest system.

2.3 Identification of protein fractions from rubber tree

Currently there are two material streams from rubber tree that are considered having (economical) importance, namely latex and wood. Small quantity of seeds with selected breed and quality are used for propagation. There is a growing interest in using the seeds for oil production. Another stream that has considerable amount of proteins, but is often overlooked, are the leaves of the rubber tree. The bark of the tree trunk is also discussed,

due to its availability and ease of collection. The overview of these streams is presented in Figure 2.1, and each stream is discussed separately as follows.

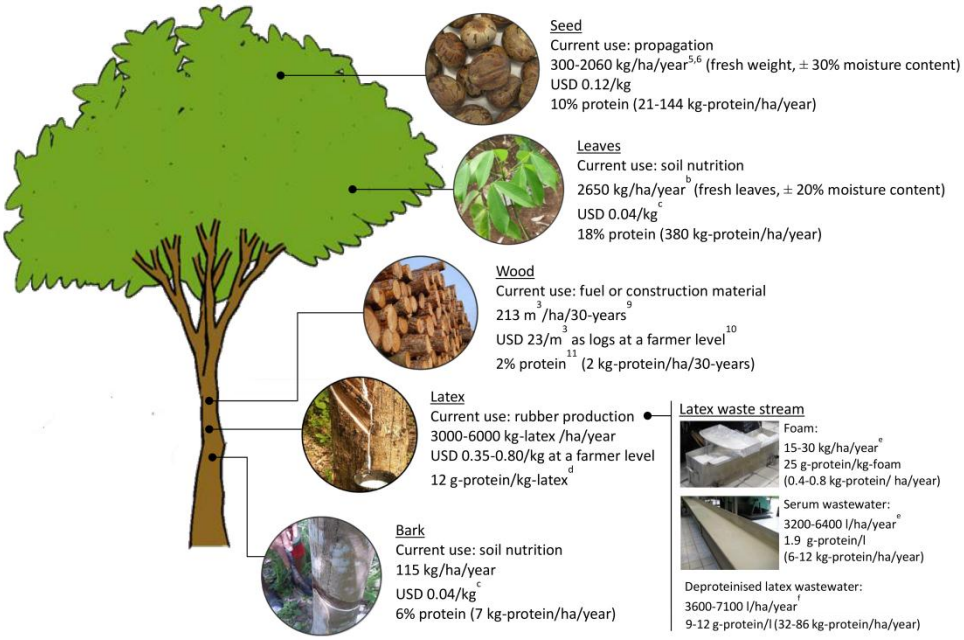


Figure 2.1 Overview of mass streams from rubber tree, current use, and potential for protein[a].
[a] Data from interviews and own measurements, unless otherwise specified.
[b] Assuming leaf area index of 5, leaf mass area of 88 g/m^2, 80% dry weight[7], and 60% collection.
[c] Price for organic fertiliser[8].
[d] Fresh latex with 35-35% dry rubber content[12].
[e] Assuming all latex is processed into ribbed smoked sheet (RSS).
[f] Assuming all latex is processes into deproteinised latex.
Protein contents are in %-dry weight unless otherwise specified. USD 1 = IDR 13,000.

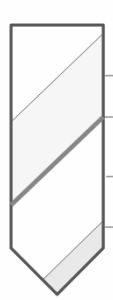
2.3.1 Latex

The latex of the rubber tree can be processed into a variety of rubber products, and currently is the main commercially applied fraction. Latex tapping usually starts when the tree is 5-7 years old. The maximum latex yield is reached for trees between 15-22 years old, after which the yield decreases. When the trees are 25-30 years old, latex yields only reach 50-67% of their previous maximum[9,13]. Latex tapping is performed by making an

incision in the bark of the rubber tree to expose latex vessels in the bark to start the leaking of latex. The latex is collected in a cup that is attached to the tree. After 6-8 h, the latex in the collection cup is transferred into a larger container and brought to the processing plant. Ammonia is often added to prevent pre-coagulation of the latex.

2.3.1.1 Latex yield and properties

The latex yield of the rubber tree is influenced by tree clone, tree age, seasons, climate, and soil conditions. Yields range from 24-32 g-fresh latex per tree/tapping in Nigeria[14] to 75-120 g-fresh latex per tree/tapping in Thailand[15]. In Indonesia, the yield can vary between 25-110 g-fresh latex per tree/tapping (interview with farmers), amounting to an annual yield of 4-6 tonnes-fresh latex per hectare for plantations and 3 tonnes-fresh latex per hectare for agro-forests. Plantations can give higher yields because they use better clones and apply artificial fertiliser. Also, tree spacing in plantations is optimised for better yields while in agro-forests the tree spacing is mostly arbitrary and sometimes too packed, making nutrition absorption not optimal. In agro-forests, fertilising is rare to none, and sometimes old trees are still used as long as they still produce latex, albeit small.



	Volume[12] (%)	Protein content[16,17] (% of protein in latex)
Rubber phase	30-45	25-27
Frey-Wyssling particles	2-3	-
Serum (C-serum)	40-50	43-48
Bottom fraction	10-20	25-32

Figure 2.2 Fractionation of latex after ultracentrifugation.

Fresh latex can be separated by ultracentrifugation at 44000-59000x*g*, and the resulting fractions are presented in a simplified form in Figure 2.2. Fresh latex contains 1-2% of protein that is distributed between rubber phase, serum, and bottom fraction; no protein is present in the phase containing Frey-Wyssling particles[16,17].

The proteins in the rubber phase are mostly insoluble. They are attached to the rubber particles and stabilise their surface. Two proteins from the rubber phase with 14.6 and 23 kDa molecular mass are identified as allergenic proteins[12,17,18].

Serum is the aqueous phase that makes up 40-50% of the latex volume and contains a variety of proteins at different concentrations. The most abundant protein is an acidic protein with an isoelectric point of 4.7 and a molecular weight of 40 kDa. This protein is important in preventing latex coagulation[19]. Free amino acids are present in the serum at total concentration of 16 mmol/l-latex, mostly consisting of alanine (26%), and aspartic acid, glutamic acid, and glutamine (18-19% each)[20].

The bottom fraction is viscous and has a yellowish colour; it contains 9% rubber particles and 2% protein[21]. The majority (50-70%) of proteins in this fraction consists of hevein[17,22], a 5 kDa protein that contains 18% cysteine and is soluble in the presence of neutral salts[22–24]. The allergenic and antifungal properties of hevein are well identified[23,25]. A previous study showed that most of the hevein from the latex is conserved after isolation from rubber factory effluent, obtaining a concentration of 0.7 g/l and suggesting that the effluent can be a source of proteins with antifungal properties[26]. The other proteins that are identified in the bottom fraction are 1,3-β-glucanase and hevamine; the latter shows high chitinase/lysozyme activity[22,27].

A 43 kDa protein that is partially homologue to patatin, the main storage protein in potato, was also found in the bottom fraction and serum[22,28]. The amount of this protein is 1%-w of the bottom fraction[28].

2.3.1.2 Latex processing

Rubber latex can be processed into various types of rubber products: crumb rubber, ribbed smoked sheet, concentrated latex, deproteinised rubber, air dried sheet, crepe, etc. Each of these products has different specifications and end-products. Most Indonesian smallholder farmers produce coagulated latex (lump), either by acid addition or natural coagulation at the plantation. The coagulated lump is further processed into crumb rubber (CR) in rubber processing plants. Some of these plants also process liquid latex into ribbed smoked sheet (RSS) or concentrated latex. Simplified process of RSS and CR production is presented in Figure 2.3. More than 80% of Indonesian rubber products are in the form of CR because, unlike RSS processing, the lump is easier to produce and store by the farmers themselves[3].

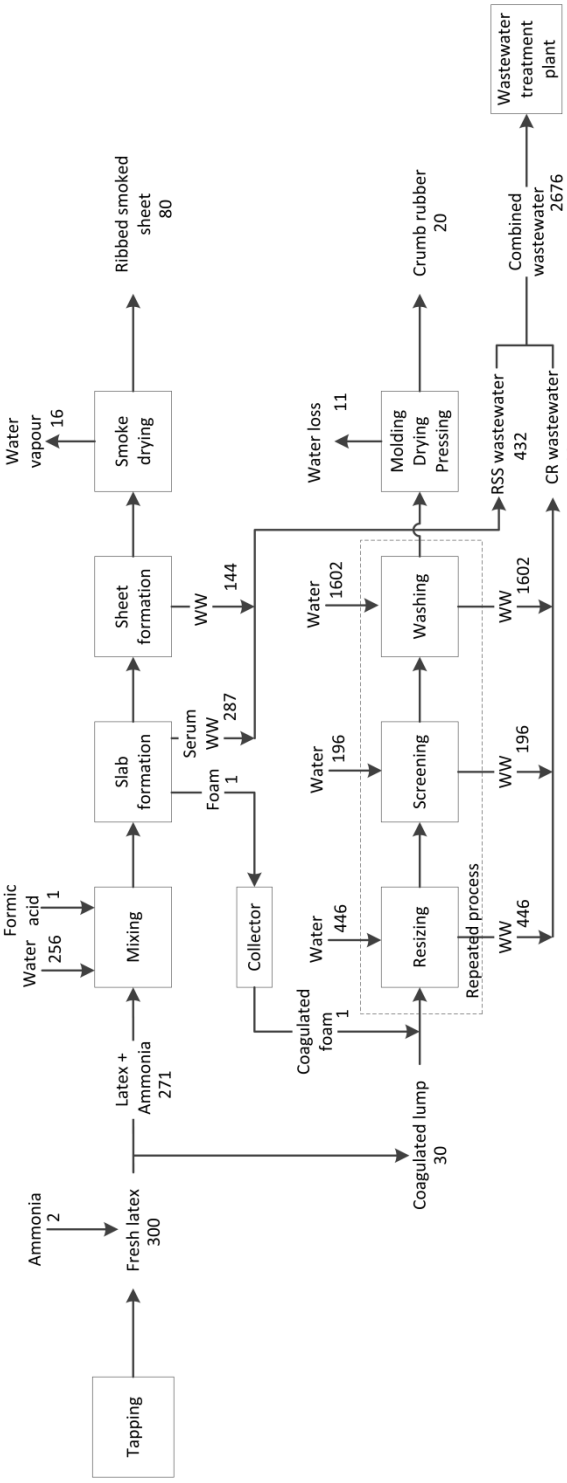


Figure 2.3 Simplified process for producing crumb rubber (CR) and ribbed smoked sheet (RSS).
Numbers indicate mass streams in tonne for producing 80 tonnes RSS and 20 tonnes CR. Numbers were calculated based on data from interview with plant manager at PTPN 8 Cikumpay, for the case of latex with 30% dry rubber content. Water use was calculated from Leong *et al.*[29].

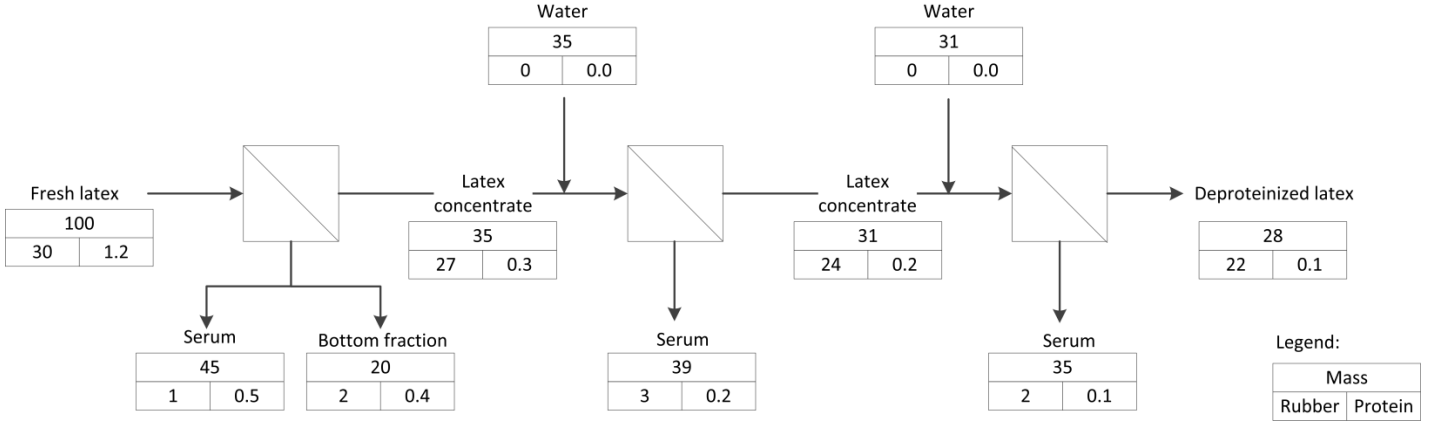


Figure 2.4 Production of deproteinised latex by centrifugation and washing.
Numbers indicate mass streams in tonne for processing 100 tonnes of latex (see Legend). Material balance calculations were based on Yeang *et al.*[17] and Perrella and Gaspari[30].

Table 2.1 Comparison of processes for deproteinised rubber production.

Method	Current stage of application	Results	Ref.
Multiple centrifugation steps and washing	Industrial	Rubber particles are concentrated. The separated proteins are present as native proteins in the liquid stream. The loss of rubber particles is ±10% for every centrifugation step. Only 50-75% protein is separated. Protein stream also contains rubber particles.	30
Solubilisation with urea and/or surfactant	Industrial	Up to 100% separation of protein is possible. (Denatured) proteins are present in the liquid stream, including water-insoluble proteins.	31
Solubilisation and hydrolysis with protease	Industrial	Up to 98% separation of protein; allergenicity can be totally removed. Hydrolysed proteins are present in the liquid stream.	32, 33
Coagulation and precipitation	Industrial	Up to 98% separation of protein. Proteins are precipitated together with Frey-Wyssling particles and components from bottom fraction.	34, 35
Ion exchange	Patented process	Up to 98% separation of protein is possible. Proteins are attached to resin and can be recovered by washing. Possible coagulation of rubber particles on resin.	36

Protein in latex is attached to rubber particles in the end-products and may cause allergenic reactions. Therefore reduction of protein in the latex is beneficial, especially for latex used for products that come into contact with human skin e.g. gloves or mattress. Several processes have been designed and applied to produce deproteinised rubber (Table 2.1). The most common is centrifugation and washing (Figure 2.4); the process can be combined with urea/surfactant or protease solubilisation[31,32].

Based on current latex processing (Figure 2.3, Figure 2.4, Table 2.1), three potential streams were considered for protein extraction (Figure 2.1): foam, serum wastewater, and the waste stream from deproteinised latex production. The other streams from current processes, e.g. RSS or CR wastewater (Figure 2.3), were not of interest because their protein contents are too low.

- **Serum wastewater.** Serum wastewater is obtained during slab formation in RSS production (Figure 2.3). When rubber slabs are collected, serum wastewater is left in the vessels and then discarded into wastewater treatment, therefore it can be collected easily. When collected directly from the vessel, this wastewater contains 0.5 g-N/l. Only 50% of the total nitrogen in the serum are proteins and amino acids[37], the rest is ammonia that is added to prevent pre-coagulation during collection. Based on this estimate, 1.9 g-protein/l is present in serum wastewater, the highest in all latex wastewater streams from RSS/CR production.

- **Foam.** Foam is formed during the mixing of latex with acid to form slab in RSS production (Figure 2.3). It is unwanted in the process because foam makes air columns in the slab, therefore the foam is removed from the mixing vessels, collected, and coagulated. The foam that is already coagulated has similar properties with dry latex and is usually used in CR line without any pre-treatment. Uncoagulated foam contains 5%-dw protein. However, only less than 1 kg of foam with 49% water content can be collected per 100 kg processed latex.

- **Waste streams from deproteinised rubber production.** A combination of multiple centrifugation and washing steps is the most applied process to produce deproteinised rubber. The combined liquid streams from this process contain 9-12 g-protein/l (Figure 2.4; Hatamoto *et al.*[38]).

2.3.2 Rubber wood

In rubber plantations, regular replacement of old and unproductive trees is necessary to maintain latex production. The wood from the old trees is currently used as additional fuel, particularly in RSS production. However, there is a growing interest in using rubber wood as timber, particleboard, or fibreboard. Rubber wood has excellent physical properties, can be processed into various products, and is considered an eco-friendly source of timber because its production does not need a new land opening[39,40]. At the end of a 30 years period, 213 m^3/ha rubber wood can be produced[9]. Rubber wood price at a farmer level is IDR 300,000 (USD 23) per cubic metre as logs[10], while the international market price is around USD 250/m^3 for hardwood logs and USD 500/m^3 for fibreboard[41,42].

Rubber wood is a typical lignocellulosic material with protein content of only 2%-dw[11]. These two properties present several challenges in protein extraction that render it not feasible. Furthermore, the recent use of rubber wood already presents a potential profit[9].

2.3.3 Seed

The flowering of rubber trees occurs one month after defoliation and coincides with the peak of solar radiation intensity. This is followed by fruit formation; each rubber fruit contains 3-4 seeds. After 4-5 months, the fruits will dehiscence and the seeds inside will fall to the ground and are available for collection[14,43]. The annual yield of rubber seeds can vary between 300 and 2060 kg/ha[5,6]. GT1, a clone of Indonesian origin and one of the most widely used varieties, produces 397000 seeds/ha per year[44], corresponding to roughly 1900 kg of fresh material. In Indonesia the seeding season varies between regions but generally occurs between July and January. The seeding season coincides with the rainy season, therefore moisture content of the rubber seeds is relatively high (Table 2.2). High moisture content makes the seeds prone to fungal contamination and deterioration, both in the plantation and during storage.

Table 2.2 Composition of rubber seed[5,45].

Parameter	Unit	Range	Average
Whole Seed			
Weight (fresh)	g	3.1 – 6.3	4.8
Hull fraction	%-w	32 – 53	40
Kernel fraction	%-w	47 – 64	60
Kernel			
Moisture (fresh)	%-w	28 – 50	36
Oil content	%-dw	40 – 50	49
Protein content	%-dw	17 – 20	18
Hull			
Moisture (fresh)	%-w		4
Oil content	%-dw		1
Protein content	%-dw		3
Crude fibre	%-dw		69

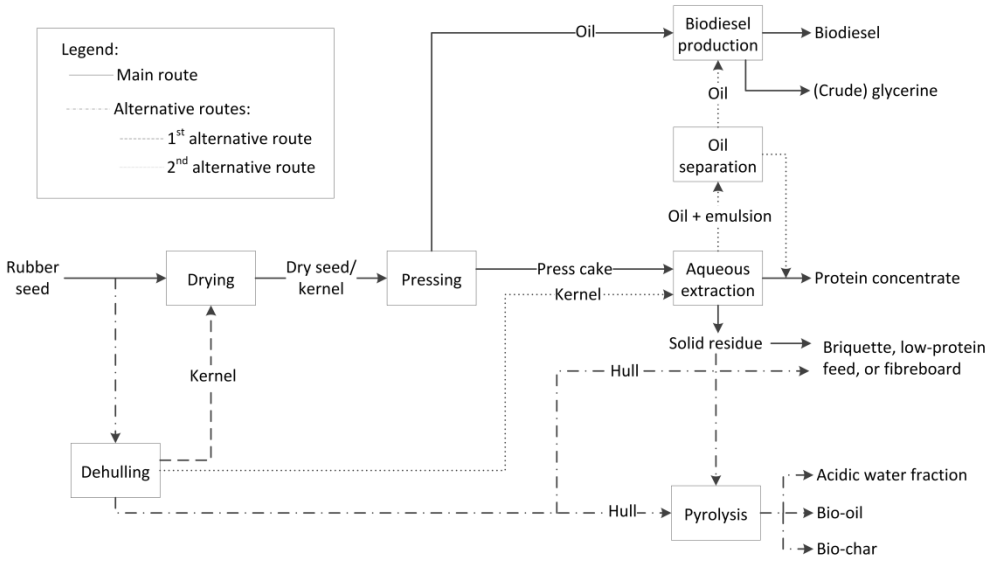


Figure 2.5 Proposed concept for rubber seeds biorefinery.

In most plantations, the seeds of rubber trees are currently left on the ground to become humus. A small amount of good quality seeds can be used for propagation. The oil, being one of the components that is present in the highest amount, is an interesting product that is currently getting more attention mainly as an alternative feedstock for biodiesel production[6,46]. Valorisation of oil alone, however, may not be economically feasible[46]. Therefore, separation and use of all fractions to get better value are envisaged. Pressing the kernel for the oil results in press cakes with 20-28%-dw protein content. Oil pressing followed by protein extraction from the press cake is proposed as the optimal process to obtain both oil and protein from the rubber seed[45]. A proposed biorefinery concept is presented in Figure 2.5.

There is still limited information on proteins that are present in the rubber seed. Amino acid analysis of the proteins in the kernel showed high number of aspartic acid, glutamic acid, arginine, valine, and leucine[45], and overall 34% essential amino acids that suggest the proteins can be used for feed applications. Direct application of the seeds or kernels as protein source, however, is not possible due to the presence of some anti-nutritional factors, most notably cyanide. Fresh rubber seed kernels contain the equivalent of 1640 mg-HCN/kg-dw, but the concentration is reduced to 42 mg/kg after three months of storage[5]. Application of high temperature, including during screw pressing, can reduce 61-93% of the initial cyanide content[5,47].

Rubber seed protein concentrate has a similar amino acid profile as the kernel, and is soluble in water at pH up and above 8.5, with isoelectric point between 4 and 5[48].

2.3.4 Leaves

During the dry season, mature rubber leaves enter a senescent phase for two months, which ends with one month of partial or complete defoliation. The tree can be leafless for 2-4 weeks, after which refoliation occurs during one month (Figure 2.6)[49–51]. The amount of leaves varies between clones, age, and time of the year. In an 8-year old monoculture plantation, the leaf area index is 0.5 m^2/m^2 during the dry season and 5 m^2/m^2 during the rainy season[52]. In a mature plantation, a leaf area index of 7 m^2/m^2 was observed[53].

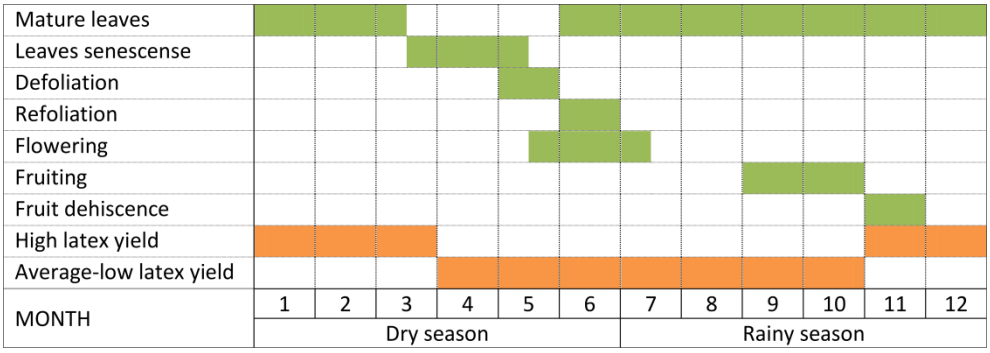


Figure 2.6 Development phases of rubber tree[14,49].
Numbers indicate months counted from the beginning of the dry season, which vary between regions, and do not correspond directly to months order in the Gregorian calendar.

We measured the crude protein content of fallen fresh leaf as 18%-dw. Similar values of 14-21%-dw have also been reported[7]. Protein content changes with leaf age. The total protein content in the mature leaves increases during growth and reaches a peak right before the senescent phase, after which the protein content decreases significantly. Some of these proteins have been identified as antioxidative enzymes[49]. Proteins with molecular weights of 13 and 55 kDa were identified in the leaves[54], the latter being especially abundant. Both proteins may be RuBisCo small units[55]. Rubber leaves have been reported as part of the diets of proboscis monkeys and lesser short-nosed fruit bats[56,57], and the leaf protein concentrate was used in rabbits diet without adverse effect[58]. Integration of sheep grazing with rubber plantation had been implemented[59], even though there is a concern that rubber leaves (and seeds) might cause metabolic problems due to the

presence of anti-nutritional factors. Similar to the seeds, mature rubber leaves contain cyanide equivalent to 1300 mg-HCN/kg-dw[60]. The leaves also contain 7%-dw tannins out of 11% total phenols[57].

To harvest rubber leaves for their protein, it is important that leaf harvesting does not result in lower latex yield. Artificial defoliation using herbicide has been applied as a method to control leaf fall disease that is often found in rubber plantation[61]. Based on this finding, leaf harvesting might even present a benefit in plantation management. The optimum harvesting time still needs to be considered for influence on latex yield, the amount of available leaves, and the leaf protein content. In addition, rubber leaves cyanide content is influenced not only by leaf age, but also by latex tapping activities and sunlight exposure; young leaves harvested in the shade or during the night have the highest cyanide content[62]. Based on the development phases of rubber trees (Figure 2.6), we propose to harvest the leaves before the mid of dry season; that is before the leaves enter the senescent phase. It is expected that protein content in this period is still high, while latex yield is not severely influenced. Assuming a leaf area index of 5 m^2/m^2, leaf mass area of 88 g/m^2, 80% dry weight[7], and 60% collection, 2650 kg fresh leaves/ha can be collected, which is equivalent to 2100 kg leaf-dry biomass or 380 kg crude protein (Figure 2.1).

2.3.5 Bark

The bark of rubber trees is obtained during the latex tapping, but is not collected and left on the ground. We estimated that for every 400 trees tapped (daily average number per worker), 1.5 kg of fresh bark can be collected easily. However, this will only amount to 115 kg of dry bark/ha/year (Figure 2.1), which is very low considering it has to be collected and stored year-round. Furthermore, the protein content of the bark (6 %-dw) is too low and its high lignocellulosic content might pose difficulty in protein extraction. Protein recovery is therefore less feasible than from the other streams.

2.4 Isolation of protein-rich products

Based on the protein contents and their availability, only latex residual streams, seeds, and leaves were considered interesting, and isolation of proteins from these streams is discussed as follows.

2.4.1 Latex

Three potential streams were considered for protein isolation from latex, namely foam, serum wastewater, and the waste stream from deproteinised latex production (Figure 2.1). In general, at least two difficulties arise: dilute streams and attachment to rubber particles. The dilute streams mean that protein recovery from latex should be integrated into the current rubber production process instead of a standalone process, as processing outside the current plants will require transportation of large volumes of water. In practice, the most feasible process to obtain value from latex processing waste stream at present are coagulation-precipitation to recover rubber and anaerobic digestion to produce methane[38]. Considering the fungicidal properties of rubber latex proteins, it might be possible to use the wastewater directly as fungicide, e.g. in the nursery for rubber trees between 1-3 years old. Further investigation is needed to study the feasibility of this option. A possible drawback could be the remaining rubber particles in the wastewater, which might form a white-sticky layer in the spraying apparatus and on the leaf and soil.

As the proteins are present in dilute streams, the isolated proteins should have specific application and economic value to make the process feasible. According to our current knowledge, the protein with the most prospective application is hevein for antimicrobial or antifungal agents[23,63]. The other protein with potential application is the 43 kDa patatin-homologue[22,28], due to its similarity with patatin. Patatin is currently investigated for food application as emulsifier, gelling agent, and foaming agent[64–67], and synthesis of monoacylglycerols[68].

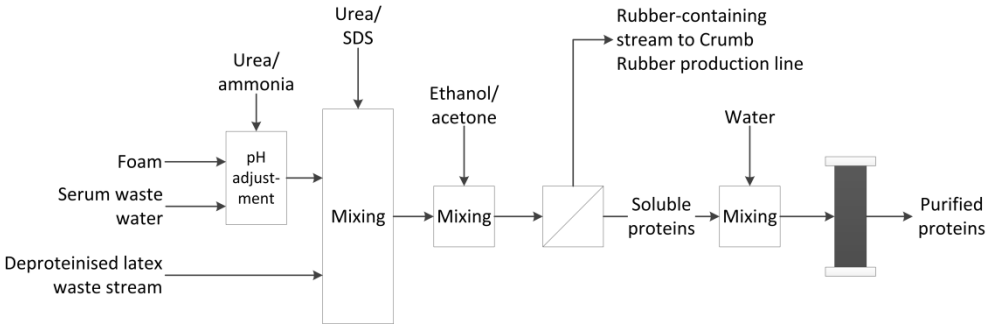


Figure 2.7 Conceptual process design to isolate protein from latex processing waste stream.

Once the target protein has been identified, a conceptual process design as illustrated in Figure 2.7 is proposed to obtain the protein. A crucial step is separating the proteins from rubber particles, as the presence of rubber in the protein stream reduces its quality and may even attach to the separation equipment and create blockages. The use of additives, e.g. urea or SDS[31], is required to solubilise the proteins that may be attached to rubber particle surfaces, mostly in serum wastewater and foam. After solubilisation, the protein-containing fraction is separated from the rubber-containing stream via precipitation[31,69]. Acetone was shown to be effective to separate protein from aqueous stream during deproteinised rubber processing[31]. To isolate the proteins and obtain the final product(s), chromatography and/or membrane filtration can be used. By using membrane with molecular weight cut-off of 30 kDa, the total solid of latex wastewater was concentrated from 39 g/l to 154-275 g/l[70]. Ultra- or nano-filtration can also be used to separate hevein, which is relatively small (5 kDa), from the rest of the protein stream. Another alternative is using expanded bed adsorption chromatography, which is also used to isolate native potato proteins from potato juice, followed by ultrafiltration to concentrate the protein fractions and remove anti-nutritional factors[65]. The highest component cost is the purification via chromatography, with estimated processing cost of USD 184/kg-product[71]. Consequently this process is only feasible if the product has a high value application, e.g. pharmaceutical.

2.4.2 Seed

Alkaline extraction followed by isoelectric precipitation is commonly used to get protein from oilseed press cakes (Figure 2.8). Alkaline conditions (0.1 M NaOH) can be used to extract protein from rubber seed kernel, press cake, and hexane-extracted meal, and 50-81% protein from rubber seed kernel can be recovered in the extract[45]. The process may need to be adjusted to remove cyanide that is still present in the press cake. Using high(er) temperature for extraction and drying may aid in removing the cyanide. Higher extraction temperature, however, may result in lower protein purity because more non-protein compounds can also be extracted. The use of high temperature also increase energy consumption and may cause protein denaturation. An overall process optimisation is still needed by taking all these factors into account.

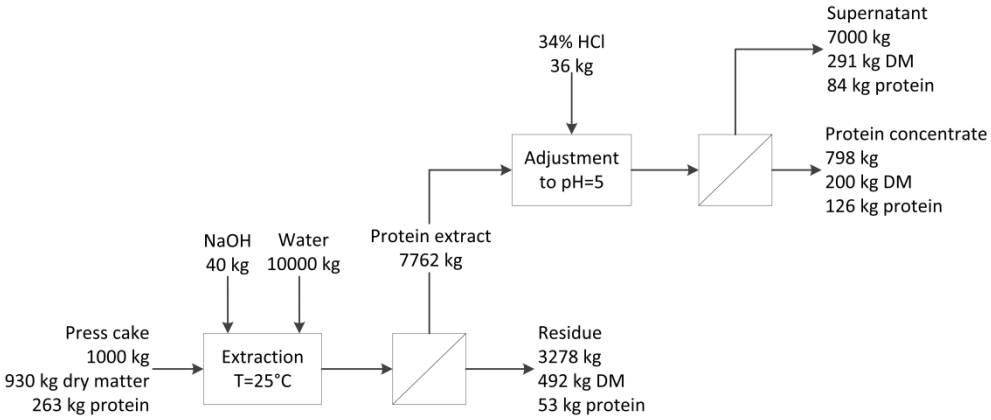


Figure 2.8 Proposed process flow diagram to isolate proteins from rubber seed press cake.

From the proposed process (Figure 2.8), several products can be obtained. Starting with press cake containing 22-28%-dw protein content, a protein concentrate with 48-63 %-dw protein can be obtained from this process. Protein concentrate price could be comparable to the price for soybean meal (44-48%-dw protein) that is USD 400-425/tonne or for cottonseed meal (41%-dw protein) that is USD 350/tonne[72].

Next to protein concentrate, briquettes can be produced by pressing the residue from protein extraction at elevated temperatures. This process is low cost, can be operated by an untrained operator, and is almost without losses in dry weight. The residue can also be burned without oxygen to produce charcoal, however only 25-30% of the original residue

is then converted into product. The market for this product depends on local conditions. The briquette can be used for cooking or as an energy source in rubber production (Figure 2.3). Alternatively, the residue can be used as low-protein ruminant feed.

The supernatant after precipitation, which still holds roughly 30% of the press cake dry weight, can be used as liquid fertiliser for application in the rubber plantations. Fertiliser quality can be improved by selecting the appropriate alkaline and acid combination for the extraction and precipitation. In our experiments, sodium hydroxide (NaOH) was used as the alkali source because it is a strong alkaline, easy to obtain, and widely used in industries. Other alkali sources that can be used are calcium hydroxide, potassium hydroxide, and ammonia; the latter is already used by farmers to prevent latex coagulation in the field. Instead of hydrochloric acid, sulphuric or phosphoric acid can be used for precipitation of protein.

2.4.3 Leaves

Isolation of protein from leafy materials can be done via mechanical pressing or alkaline extraction. The former has been extensively studied and implemented, from pilot to commercial plants[73–75]. The simplest mechanical pressing requires chopping and grinding leaf materials, pulping, and pressing to get protein-rich juice and press cake. Based on visual observation, rubber leaves are considered as soft biomass (unlike grass or alfalfa), therefore screw extrusion might not be suitable due to low friction coefficient[76]. However, leaf protein concentrate has been produced from cassava leaves, which are also soft leaves, both using screw extruder[77] and hydraulic press[78].

Protein-rich (press) juice can be processed into leaf protein concentrate via steam coagulation or isoelectric point precipitation, for use as animal feed or other protein applications. To improve protein quality and, consequently, increase the protein value, press juice can be treated with ultrafiltration or other means of purification. Activated carbon adsorption can remove the chlorophyll from the protein rich juice, results in a RuBisCo-rich fraction that can be used in food and beverage[79].

The other method to isolate protein from the leaves is using alkali. High temperature and alkali amount is required to obtain high extraction yield[80,81]. The advantage of alkaline extraction over mechanical pressing is the possibility to process dried material as well as fresh leaves. Alternatively, alkaline conditions can also be used to extract protein from the press cake that is left after press juice extrusion.

Ammonia pre-treatment, e.g. ammonia fibre explosion (AFEX), may increase extraction yield and allow the use of milder condition for alkaline extraction[82,83]. During AFEX, lignocellulosic material is treated with liquid ammonia under pressure followed by a rapid pressure release that breaks the fibres. AFEX pre-treatment followed by alkaline extraction is especially beneficial when leaf extraction for protein is combined with ethanol production[84].

Rubber leaves contain several anti-nutritional factors, particularly cyanide and tannins, and the influence of processing on these compounds should be taken into account. Alkaline conditions may hinder the formation of gaseous hydrogen cyanide that serves as a cyanide removal mechanism. However, as shown in processing of cassava leaves that also contain cyanide, chopping and drying the leaves before alkaline treatment and two-step drying after alkaline treatment can reduce the amount of cyanide[85]. Tannins and several other toxins and anti-nutritional factors, e.g. phorbol esters, phytate, and glucosinolate, can also be degraded or removed under alkaline conditions[85–87].

Bals and Dale[73] presented several scenarios for both mechanical pressing and alkaline extraction of leaves, in conjunction with the lignocellulosic biorefinery process. They concluded that compared to mechanical pressing, alkaline extraction gives less revenue due to lower protein recovery, but the overall process is less sensitive to changes in process conditions and biorefinery scale. Protein content in the final product is the determining factor in profitability[73], therefore an alkaline extraction process that can achieve up to 95% protein recovery may be a feasible alternative[81]. Combination of mechanical pressing and alkaline extraction to isolate proteins from rubber tree leaves is presented in Figure 2.9. With this process, three protein products can be obtained: protein concentrate from press juice (40%-dw protein), protein concentrate from press cake (52%-dw protein), and a RuBisCo fraction (70%-dw protein). For feed applications, the price for protein concentrates could be comparable to the price for soybean meal (USD 400-425/tonne)[72]. Based on estimated market price for cosmetic-grade proteins (90%-dw protein, USD 1100/tonne)[75], the price of USD 800/tonne for the RuBisCo fraction could be expected.

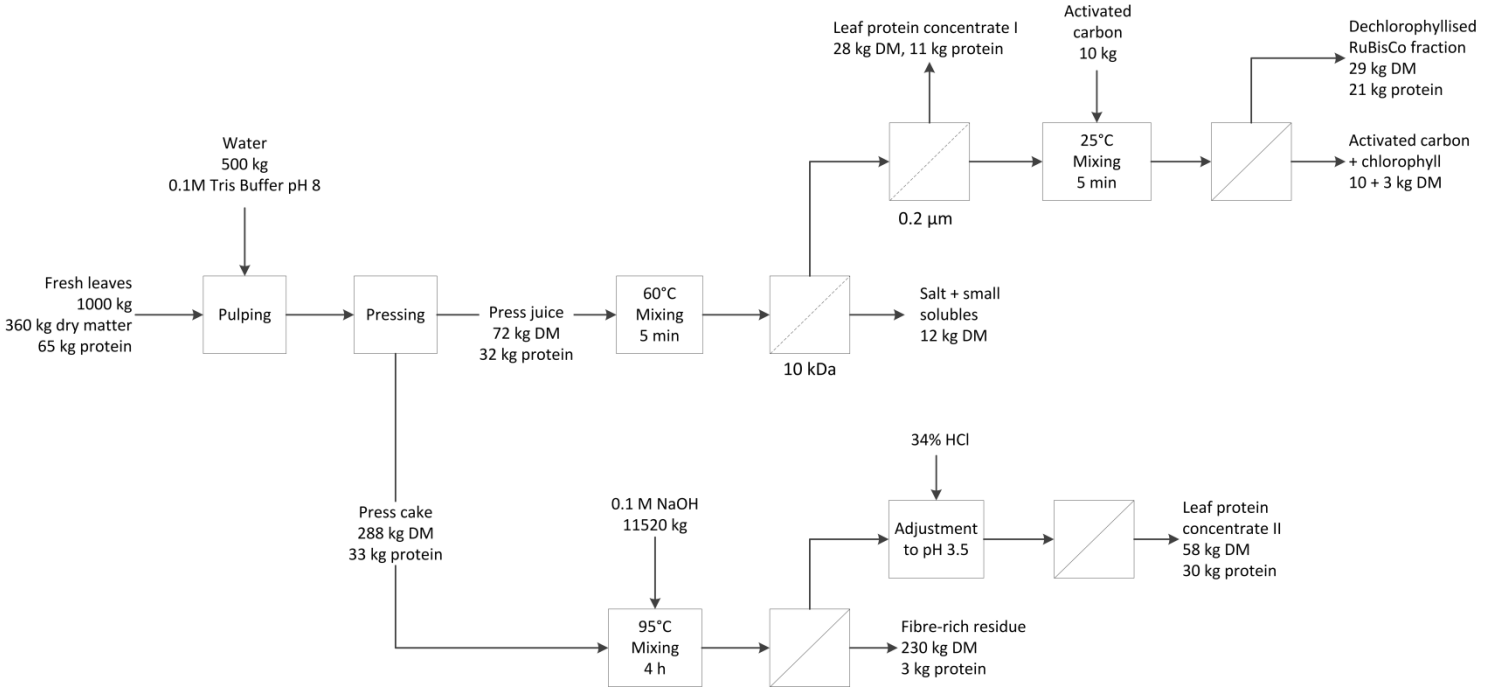


Figure 2.9 Proposed process flow diagram to isolate proteins from rubber tree leaves.
Material balance was calculated from Kamm *et al.*[75], Van de Velde *et al.*[79], and Zhang *et al.*[81].

2.5 Discussions

Utilisation of protein fractions from rubber tree, particularly from rubber seeds and leaves, presents opportunities to increase revenue from rubber plantations. How to realise this opportunity might be a challenge that is not only determined by the availability of technology, but also social aspects and resource availability, which are both location-specific. Most rubber plantations in Indonesia are owned by smallholders who operate the plantation themselves and sometimes employ 1-2 day-workers. Furthermore, as a commodity, rubber price is prone to fluctuation. Additional income from seed or leaves processing will benefit most to farmers whose daily income depend on latex tapping, and might come in handy when rubber price is low. The biorefinery approach might increase the feasibility of the process by utilising all fractions of rubber seeds or leaves, including protein. Furthermore, it is expected that learning process might reduce the biorefinery cost, proving the technology is sustainable[88].

The processes presented in this article (Figure 2.5, 2.8, and 2.9) can be applied either in local (small scale) or in centralised (large scale) biorefinery units. The application of certain equipment or technology is often only feasible at a large scale due to economy of scale. For instance when aiming for a large scale biodiesel production, the optimal result can be achieved via seed collection from several large plantations that allows long-term processing at a centralised site[89]. For leaves processing (Figure 2.9), protein refining to RuBisCo maybe more beneficial at a large scale aiming for industrial markets.

Despite the benefits of large scale processing, local (plantation or village-based) processing may also present some benefits: processing can be adjusted to the farmers' daily activities, within a known community, and with low energy input with local use of the undried products for feed. In general, small scale (pre)processing of biomass is more beneficial for processes with low capital and low energy use[90]. For the case of seed biorefinery (Figure 2.5) and protein extraction from press cake (Figure 2.8), the highest energy consumption is in the drying the starting material and product(s). When starting materials or products are not used directly, *in situ* drying is still preferred to prevent fungal growth and therefore alternatives to reduce energy consumption, e.g. sun drying, should be considered. For local processing, leaves processing (Figure 2.9) can be modified for products that are suitable for local use.

Local processing also enables the recycle of nutrients and minerals to the soil. The seeds and leaves of rubber tree are currently not utilised, and only left on the plantation ground to become humus. Harvesting of the seeds and leaves, therefore, might reduce the soil organic carbons and nutrients in the plantation. One alternative for nutrients recycle is using the liquid fraction from the protein extraction as fertiliser.

2.6 Conclusions

Utilisation of protein fractions from rubber tree might increase the economics of rubber tree plantations. In Indonesia where most rubber plantations are owned by smallholder farmers, this can be a source of additional income for the farmers. Protein extraction from rubber seeds can be incorporated within a biorefinery plant that produces biodiesel as its main product. The protein extraction can be performed with the available knowledge and technology, and the product can be applied for animal feed. Protein extraction from rubber tree leaves can aim for animal feed proteins for local use or more polished products for food and industrial use. Utilisation of protein in the latex is not economically feasible at this moment, but may be feasible when specific use of the latex protein(s) can be identified.

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Chapter 3

Biorefinery Methods for Separation of Protein and Oil Fractions from Rubber Seed Kernel

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Abstract

Biorefinery of rubber seeds can generate additional income for farmers, who already grow rubber trees for latex production. The aim of this study was to find the best method for protein and oil production from rubber seed kernel, with focus on protein recovery. Different pre-treatments and oil separation methods were tested, and alkaline conditions were used to extract protein. Next to processes with subsequent oil and protein recovery, a one-step combined oil and protein extraction was tested. Our study showed that oil separation is not necessary to obtain high protein recovery, however most of the extracted oil is present as an emulsion. The origin of the seeds and their treatment on the plantation before processing were most important for high oil and protein recoveries, and in all cases tested had more influence on recoveries than its subsequent method of processing. Pressing the rubber seed kernel to separate the oil fraction followed by protein extraction from the press cake gives the highest protein recovery with satisfactory recovery for oil.

Keywords: rubber seed, vegetable oil, protein, alkaline extraction, biorefinery

3.1 Introduction

The rubber tree (*Hevea brasiliensis*) is mainly cultivated for its latex, which can be processed to natural rubber and used in various products. Thailand, Indonesia and Malaysia are the largest natural rubber producing countries; their combined harvested area accounts for two-thirds of the world's harvested area[1]. Even in these countries, the seed of the rubber tree is not widely collected for commercial use except for seeding, which accounts for less than 25% of seed with selected breed and quality[2].

The annual production of rubber seed varies from 300 to 2060 kg/ha[3,4]. Plantation conditions often pose difficulties in collecting and rot preventing, therefore the realistic collectable yield without a dedicated collection method might be as low as 150-200 kg/ha per year[5,6]. Seed weight in fresh condition is between 3 to 6 g (fresh weight), and consists of 42-51% hull and 49-58% kernel[5]. On dry weight basis, the kernel of rubber seed contains 40-50% oil[3,5] and 19-23% crude protein[5,7,8]. Based on a conservative estimation of 200 kg/ha with 30% moisture content, 38 kg-oil/ha and 13 kg-protein/ha are available annually.

Oil and protein production from rubber seeds can generate additional revenue to the latex production. The use of rubber seed oil as an alternative feedstock for biodiesel production has already been investigated[6,9]. Other potential applications include corrosion inhibitor[10], metal soap[11], and precursor for resins and polymers[12–14]. Even though these potentials have been identified, studies on optimising oil separation from rubber seed are still limited. Ebewele *et al.*[15] reported that the maximum 0.45 g-oil/g-kernel could be obtained by Soxhlet extraction using n-hexane as the solvent, while only 0.28 g-oil/g-kernel could be obtained using mechanical pressing at optimised condition. Higher results (0.21-0.34 g-oil/g-kernel) were obtained using supercritical carbon dioxide[16,17]. The combination method using mechanical pressing with hexane addition gave as high as 0.49 g-oil/ g-kernel[9].

Early studies on full-fat and de-oiled rubber seed kernel suggested their potential use as food and feed materials because of their protein content[18–20]. Rubber seed kernel proteins contain 33-36% essential amino acid; lysine and methionine are the most limiting[5,7,21]. Heat and pressure treatment, soaking, and oil separation were observed to cause only limited changes in the amino acid composition[21]. Solvent oil extraction and soaking rubber seed kernel in 0.01 M HCl or NaOH decreased the protein quality as observed in experiments with rats[7], possibly due to protein denaturation. Soaking the full-fat kernel with water at 65°C, however, showed slightly improved protein quality compared with the untreated kernel, possibly due to the leaching out of anti-nutritional factors. Other studies on the use of rubber seed kernel still give conflicting results. Biological assays on rats and

chickens fed with diets containing de-oiled rubber seed kernel showed lower weight gain and reduction in food intake and fertility[8,22,23]. On the other hand, de-oiled rubber seed was used to replace 50% of protein in common carp diet without adverse effects[2]. To the authors' knowledge, no work on protein extraction from the rubber seed has been reported yet.

In oil containing biomass, oil is stored in the cell as oil bodies that are covered with proteins[24]. Mechanical oil pressing or solvent extraction is used to separate oil, and oil recovery from these processes is influenced by several factors including temperature, pressure, and moisture content[25,26]. Application of high temperature aids in releasing the oil from the cells by means of breaking the cell structure, lowering oil viscosity, and adjusting moisture content. At high temperature, proteins that cover oil bodies also denature and coagulate, which helps releasing oil from the cell[25]. Higher temperature, however, also influences proteins that are not associated with oil bodies and in general reduces the solubility of these proteins. High efficiency of oil pressing or extraction, therefore, might give reversed effect on protein extraction. Protein extraction from Jatropha seed showed that higher protein recovery was obtained from full-fat kernel instead of de-oiled kernel[27]. Protein extraction from microalgae with protease addition, however, shows that protein recovery from de-oiled microalgae was higher than full-fat microalgae[28].

Combined oil and protein extraction is an alternative method to separate protein and oil fractions, and has been used for oil and protein extraction from peanut, sesame, canola, soybean, and rapeseed[29–33]. The method takes advantage of the insolubility of oil in water to create separate oil and aqueous protein phases. The recoveries of oil and protein are mainly influenced by pH and temperature[34]. The use of protease has been reported to increase both oil and protein recoveries[29,30,32].

The aim of this study was to obtain high protein recovery from rubber seed kernel, without major losses in oil recovery. Different pre-treatments and oil separation methods were tested, and alkaline conditions were used to extract protein. Next to processes with subsequent oil and protein recovery, a one-step combined oil and protein extraction was tested. The influence of several processing parameters was examined, and results could be explained by interpreting differences and taking interactions between oil, protein, and other components into consideration. The envisaged process should have the highest protein recovery and a reasonable oil recovery, taking into accounts both protein and oil qualities. Energy and chemical uses were considered within the context of the intended use of the protein and oil fractions.

3.2 Materials and methods

3.2.1 Materials

Two types of rubber seeds were used in the experiments. The first batch was obtained from Subang, West Java, Indonesia, and the second batch was obtained from Bengkulu, Sumatera, Indonesia. The seeds were stored at room temperature in open containers until use.

The chemicals used for experiments and analysis were of analytical grade, unless otherwise specified. Protease enzyme (Protex 40XL) was obtained from Genencor International BV, the Netherlands. As specified by the manufacturer, the temperature range of the protease was 25-60°C, pH range was 9-12, and activity was 52 MPU/g.

3.2.2 Pre-treatment

Before further treatment, the seeds were de-hulled manually to separate good condition kernels from the ones infected with fungi. The good kernels were cut into four parts to optimize drying. Some kernels were dried at 60°C for three days and others were dried at 105°C for 24 h.

3.2.3 Oil separation

Pre-dried Subang kernels were subjected to hydraulic pressing or hexane extraction.

3.2.3.1 Oil separation by hydraulic pressing

Before pressing, moisture content of the cut and pre-dried kernels was measured. To rule out the influence of different moisture content when measuring the influence of pre-drying temperature, dry kernels were exposed to ambient air to bring the moisture content to an equal value (3%). Pressing was performed using a laboratory scale hydraulic press that could operate from 30 to 120°C with a maximum pressure of 25 MPa. For this experiment, the applied pressure was 25 MPa and temperature was 60°C or 100°C. At 60°C, cell disruption and decrease in oil viscosity were expected, while protein coagulation could be avoided. At 100°C, both effects were expected.

Pressing was performed in ten batches for each condition, using ±7 g of kernel per batch. Pressing time for each batch (including heating) was 30 min. De-oiled residues from the pressing (referred hereafter as press cake) were stored at 4°C until further use.

3.2.3.2 Oil separation by hexane extraction

Pre-dried kernels (at 60 or 105°C) were ground with a commercial coffee grinder, and stored in a desiccator until further use. Extraction was performed using technical grade n-hexane in a Soxhlet (70°C) for 6 h. De-oiled residue from the hexane extraction (referred hereafter as meal) was dried at 60°C to remove residual hexane, and stored at 4°C until further use.

3.2.4 Protein extraction

Before protein extraction, press cakes were ground with a commercial coffee grinder. The meals from hexane extraction were already in powder form; therefore no other pre-treatment was applied.

To 4 g material, 40 g of 0.1 M NaOH (1:10 solid-to-solvent ratio) was added in a 100 ml Erlenmeyer flask. Flasks with the extraction mixture were placed in a water bath at 25°C or 60°C and shaken at 110 rpm for 1 h. The mixtures were subsequently transferred to 50 ml centrifuge tubes and centrifuged at 1520x*g* for 20 min. The extracts and freeze-dried residues were stored at -18°C until analysis. All experiments were performed in duplicate.

3.2.5 Combined extraction

The combined extraction was performed in the similar procedure as protein extraction, only used full-fat kernel as the extracted material. Extraction was performed for 1 or 6 h. For experiments with native or inactivated protease, 5% protease volume per weight protein was added before extraction. Inactivation of protease was performed by incubation at 90°C for 10 min prior to the addition.

After centrifugation (1520x*g* for 20 min), four phases were formed: free oil, emulsion (containing oil and protein), aqueous phase, and solid residue. The free oil, emulsion, and aqueous phase were transferred into a separation funnel following a method developed by Lamsal and Johnson[35]. The transfer caused the phases to mix; therefore the mixture

was left for 1 h at room temperature until the phases were separated again. The free oil phase at the top part consisted of a thin layer and the distinction with the emulsion phase was not very clear. Petroleum ether 40/60 (10 ml) was added carefully to the funnel to extract the free oil, but not the oil in the emulsion. After incubation for 1 h, the three phases were collected in separate containers. Petroleum ether from free oil phase was removed using rotary evaporator, and oil content was determined by weighing. Emulsion and a known amount of aqueous phase sample were freeze-dried; all samples were stored at -18°C until analysis.

3.2.6 Analytical methods

3.2.6.1 Oil content analysis

Oil content of kernels, press cakes, and meals were analysed using Soxhlet at 70°C with n-hexane as the extracting solvent for 4-6 h, followed by hexane evaporation and weighing of the oil. When the oil content was higher than 5% of the material dry weight, extraction was repeated for another 2 h using fresh solvent to ensure all the oil was extracted.

To analyse the oil content in the emulsion and aqueous phase from combined extraction, 20 ml of petroleum ether 40/60 was added to freeze dried samples and mixed by vortex for 30 s. The samples were incubated overnight, followed by centrifugation at 3000x*g* (4°C, 20 min). Two phases, petroleum ether containing extracted oil at the top and remaining solid at the bottom, were formed and separated. Petroleum ether was removed from the collected oil using rotary evaporator. Oil content was determined by weighing. Oil content of the residue was calculated by difference.

3.2.6.2 Protein content analysis

Protein content of materials and extraction products were analysed using Kjeldahl method[36]. Results were calculated with nitrogen-to-protein conversion factor of 5.7, as determined in this article.

3.2.6.3 Degree of hydrolysis

Degree of hydrolysis of the extracts was determined by the modified OPA method[37]. Samples were dissolved in OPA reagent (o-phthaldialdehyde in ethanol and SDS), and the

spectrophotometric absorbance was measured at 340 nm and corrected with absorbance of the unhydrolysed sample. Serine was used as the standard.

3.2.6.4 Amino acid analysis

Amino acid composition of the kernel was determined using Ultra High Performance Liquid Chromatography. Samples were hydrolysed using 6 M HCl containing 1% (w/v) phenol at 110°C for 24 h[38]. For estimation of tryptophan, the samples were hydrolysed using 4.2 M NaOH at 110°C[39]. The hydrolysates were dissolved in methanol and loaded into Ultra-HPLC Dionex RSLC (Dionex Corporation, USA). Detection was done at 263 nm and 338 nm[38].

3.2.6.5 Calculation

Oil and protein contents were both calculated as percentage of dry matter. Oil recovery (%) was calculated as weight of separated oil divided by weight of oil in the kernel. Protein recovery (%) was calculated as weight of separated protein divided by weight of protein in the kernel.

3.2.7 Statistical analysis

Values from at least two measurements are expressed as average ± standard deviation. The significance of differences between values was tested with Student's t-test; $p < 0.05$ was regarded as significant.

3.3 Results and Discussion

3.3.1 Raw material properties

Rubber seeds are usually available in the plantation once a year during a period of one month. Fruiting season of rubber trees starts during the transition between dry season and rainy season. When the fruits are ripe, they will split and the seeds inside will fall to the ground and are available for collection. Because collection was performed during the rainy season, and in a high humidity area like Indonesia, moisture content of the rubber seeds was relatively high and most of it was contained in the kernel (Table 3.1). Seeds

stored at room temperature are prone to fungal contamination; therefore seed moisture content of 7% is advised for storage[15]. We observed that the seeds from Bengkulu, which had lower moisture content, had less fungal contamination compared to the seeds from Subang during prolonged storage (2-10 months).

Table 3.1 Composition of rubber seed, based on wet weight (w) or dry weight (dw)

Parameter	Unit	Subang sample	Bengkulu sample
Whole Seed			
Weight (fresh)	g	4.8 ± 0.7	4.9 ± 0.6
Hull fraction	%-w	40 ± 5	39 ± 4
Kernel fraction	%-w	60 ± 5	61 ± 4
Kernel			
Moisture (fresh)	%-w	50 ± 7	28 ± 1
Oil content	%-dw	48.4 ± 2.5	49.7 ± 0.3
Protein content	%-dw	16.9 ± 0.3	17.2 ± 0.6

Rubber seed properties are varied by seed type and origin, and influenced by the local conditions for seed growing, harvesting, and storage. The amino acid composition of the kernel shows variability both between Subang kernel and Bengkulu kernel, and between our results and literature values (Table 3.2). Compared to literature, our results show higher levels of alanine and valine, and lower levels of histidine, tyrosine, tryptophan, and methionine. Cysteine and methionine were partially converted during acid hydrolysis[40]; therefore the value of these and the total amino acids are underestimated. Compared to other oilseeds[41], rubber seed kernel has lower levels of glutamic acid, isoleucine, and lysine, but higher levels of aspartic acid, arginine, and valine.

Literature values of kernel protein contents vary between 19 and 23%[5,7,8]. The conversion factor of 6.25 used in these studies might overestimate the calculated protein content and a closer examination of these values in literature showed that approximately 10% of the Kjeldahl nitrogen was not protein. Comparison between total amino acid measurements via HPLC and nitrogen content via Kjeldahl of our results gave a nitrogen-to-protein conversion factor of 5.7 for Subang kernel and 5.6 for Bengkulu kernel; similar to literature values with omitted non-protein nitrogen. These values are also comparable to literature values of other oilseeds[41–43]. The value of 5.7 was chosen to calculate the protein content for this study.

Table 3.2 Amino acid composition of rubber seed kernel protein.

Amino acids	Unit	Amount for different kernel type			
		Subang	Bengkulu	Nigeria[a]	Sri Lanka[b]
Aspartic acid + asparagine		12.5 ± 0.1	12.4 ± 0.2	12.4 ± 0.1	11.2
Glutamic acid + glutamine		16.6 ± 1.3	16.5 ± 0.3	17.5 ± 0.2	16.5
Histidine		2.0 ± 0.2	2.4 ± 0.0	2.2 ± 0.0	3.1
Serine		5.7 ± 0.1	5.4 ± 0.0	5.4 ± 0.0	4.7
Arginine		10.2 ± 0.1	10.6 ± 0.0	11.3 ± 0.0	10.2
Glycine		5.1 ± 0.0	4.8 ± 0.1	4.3 ± 0.1	5.9
Threonine	% of	4.2 ± 0.1	3.8 ± 0.1	3.6 ± 0.0	3.8
Tyrosine	total	2.4 ± 0.1	2.0 ± 0.1	3.0 ± 0.0	2.9
Alanine	amino	5.9 ± 0.1	5.6 ± 0.1	4.9 ± 0.0	4.3
Proline	acid	5.0 ± 0.4	6.2 ± 0.1	4.7 ± 0.0	4.3
Tryptophan		0.9 ± 0.1	0.6 ± 0.1	1.5 ± 0.0	1.6
Valine		8.4 ± 0.1	8.3 ± 0.1	6.6 ± 0.1	6.5
Methionine		0.3 ± 0.3[c]	0.4 ± 0.3[c]	1.2 ± 0.1	1.3
Phenylalanine		4.8 ± 0.1	4.7 ± 0.1	5.5 ± 0.1	5.7
Isoleucine		3.9 ± 0.1	3.7 ± 0.1	3.7 ± 0.0	3.7
Leucine		7.8 ± 0.1	7.5 ± 0.1	6.9 ± 0.0	7.7
Cysteine		0.8 ± 0.0[c]	0.7 ± 0.2[c]	1.5 ± 0.0	1.8
Lysine		3.7 ± 0.4	4.4 ± 0.3	3.7 ± 0.0	4.9
Total amino acid	%-dw	16.8 ± 1.6	17.1 ± 2.5	20.4	19.3
Nitrogen-to-protein conversion factor		5.7	5.6	5.7	5.6

[a] Calculated from Fetuga *et al.*[7].

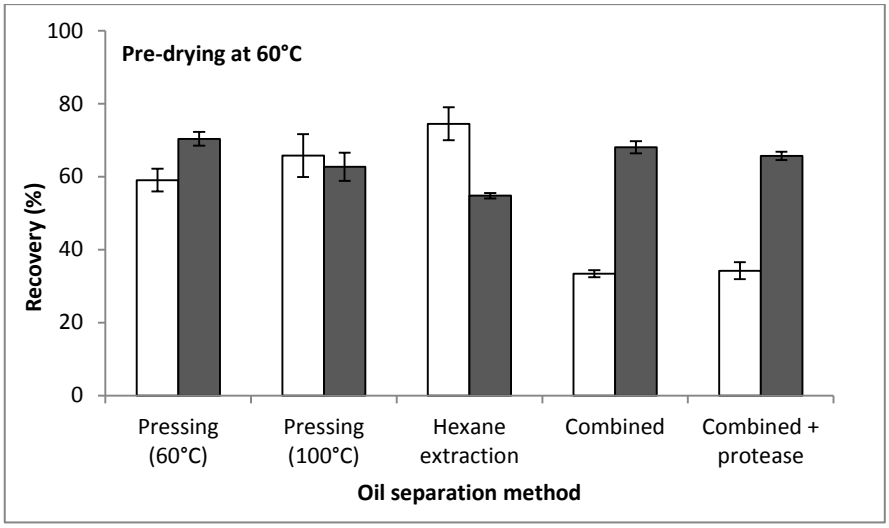
[b] Calculated from Ravindran and Ravindran[5].

[c] Partially destroyed during hydrolysis for HPLC analysis.

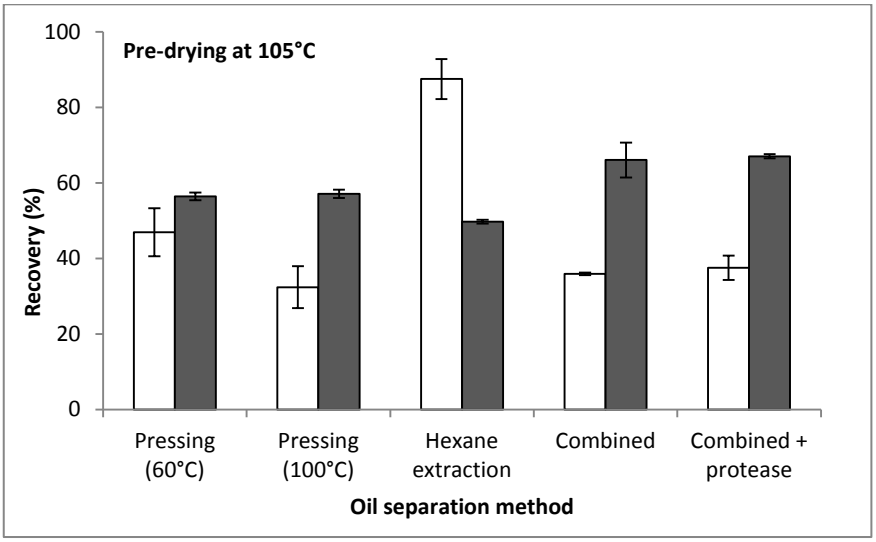
3.3.2 Oil separation method

3.3.2.1 Oil separation method and oil recoveries

As shown in Figure 3.1a, oil recovery from the combined method was less than from pressing or hexane extraction. Oil recoveries from pressing and combined extraction were only almost similar when the kernel was pre-dried at 105°C (Figure 3.1b), and much lower compared to hexane extraction in all cases.



(a)



(b)

Figure 3.1 Oil recovery (□) and protein recovery (■) in extract as a percentage from total original amount present in kernel. (a) Kernel was pre-dried at 60°C for three days or (b) at 105°C for 24 h. Combined and protein extractions were performed at 25°C for 1 h. Oil recovery from the combined extraction was the total amount from free oil, emulsion, and aqueous phase.

In extraction with organic solvent, oil is extracted through several mechanisms: leaching, washing, diffusion, and dialysis[44]. These mechanisms are not entirely applicable in extraction with water, due to the insolubility of oil in water. A proposed model of oil extraction with water suggests the process starts with the release of oil from completely disrupted cells and protein solubilisation, followed by oil coalescence, emulsion formation, and disruption that releases oil[45]. This repeating process results in more oil in the bulk liquid.

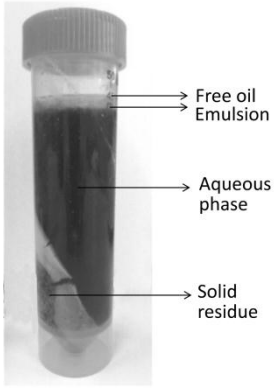


Figure 3.2 Typical results after centrifugation for combined extraction.

Table 3.3 Material balance between free oil, emulsion, aqueous, and residue phases after combined extraction[a].

Phase	Dry weight recovery (%)		Oil recovery (%)		Protein recovery (%)	
	Without protease	With protease	Without protease	With protease	Without protease	With protease
Free oil	1 ± 1	2 ± 0	2 ± 2	3 ± 1	nd	nd
Emulsion	13 ± 2	15 ± 1	24 ± 2	26 ± 2	5 ± 4	3 ± 1
Aqueous	30 ± 2	29 ± 1	7 ± 1	5 ± 2	63 ± 3	63 ± 2
Solid residue	52 ± 0	52 ± 2	67 ± 1	66 ± 2	33 ± 3	32 ± 2

[a] Kernel was pre-dried at 60°C for three days. Extraction was performed at 25°C for 1 h.
nd: not determined.

Table 3.3 shows distribution of dry weight, oil, and protein in all the four phases after combined extraction at 25°C; the result was also typical for other combined extraction conditions (Figure 3.2). From the extracted oil, 46-79% was present in the emulsion phase

and only 3-38% was present as free (non-emulsified) oil. Furthermore, 9-24% of the oil was also present in the aqueous phase. Due to the difference in density, free oil and emulsion can be separated relatively easily from the other phases. The oil in the emulsion can be separated by freezing-thawing, adjusting emulsion pH to protein isoelectric point, or enzymatically using protease or phospholipase[35,46]

When extraction temperature was increased from 25°C to 60°C, free oil recovery increased from 1-8% to 6-14% while oil recovery in the emulsion decreased from 19-31% to 16-23%; therefore the total oil recovery did not change. Higher temperature did not influence the oil recovery in the aqueous phase. At higher temperature, emulsions were less stable and oil viscosity was lower. Therefore more oil was present in the free oil phase.

For biodiesel production, combination of mechanical pressing and solvent extraction is often used[47]. Pressing is applied to feedstock with high (>20%) oil content and gives better oil quality. Solvent extraction is used for feedstock with oil content <20% or as a second extraction step after pressing. Before processing, a refining process is needed to remove unwanted compounds from the oil. Between processes investigated here, combined extraction presents the highest difficulty to be integrated in biodiesel production.

3.3.2.2 Oil separation method and protein recovery

After pressing and hexane extraction, almost all (93-99%) of the protein in the kernel was retained in the press cakes and meals. Consequently, protein content in the press cakes and meals corresponded inversely with the amount of oil separated from the kernel, leading to a higher relative protein content in the press cakes and meals. Overall, 50-71% protein from the total original amount of protein in the kernel could be recovered in the extract (Figure 3.1), comparable to protein recoveries from other materials such as safflower seed[48], rapeseed meal[49], and Jatropha seed kernel and press cake[27].

Protein recovery from the combined process was comparable to press cakes (from pressing) and higher than meal (from hexane extraction) when protein extraction was performed at the same temperature (Figure 3.1). Most of the extracted protein (88-98%) was present in the aqueous phase, and the rest was present in the emulsion phase (Table 3.3). The presence of oil in the material did not seem to influence protein extractability. Indeed, even though the highest oil recovery was obtained by hexane extraction, the meal gave the lowest overall protein recovery compared to the other materials. Lestari *et al.*[50] observed a lower protein recovery from Jatropha press cake with additional hexane extraction, compared to press cake without hexane extraction. The lower recovery was

attributed to the change of press cake particles during hexane extraction, leading to dryer and sturdier particles. Despite this difference, they observed that the extracted protein had similar solubility[50]. In our case, the higher protein recovery from the combined extraction suggests that on average 67% of the protein in the kernel was easily extractable. However, the use of high temperature and organic solvent reduced protein extractability, very possibly via protein denaturation. Next to temperature, also the processing time is of influence, since protein recovery from meal after hexane extraction for 6 h at 70°C was lower than after pressing at 100°C for 30 min.

The presence of oil in the aqueous phase might reduce protein quality when high purity is needed. As animal feed, the oil can serve as energy source and does not give adverse effect on protein digestibility and retention[23]. Only less than 10% of the extracted protein was present in the emulsion.

3.3.3 Pre-drying

3.3.3.1 Pre-drying and oil recovery

Moisture content of the kernel is known to influence oil recoveries from subsequent pressing or hexane extraction[25,26], therefore pre-drying was applied to reduce the moisture content. Oil recovery from hexane extraction increased significantly at higher pre-drying temperature, despite the slight different in moisture contents after pre-drying. This suggests that the application of higher temperature itself, instead of the moisture content, influenced oil recovery. This is in agreement with other studies that showed that drying the kernel at 160°C for 30 min before solvent extraction gave higher oil recovery compared with pre-drying at lower temperature for longer period[21]. Higher drying temperature might facilitate disruption of cell and proteins associated with oil bodies, which allows the oil to flow out of the kernel.

For hydraulic pressing, on the other hand, oil recoveries from kernel pre-dried at 60°C were higher than kernel pre-dried at 105°C despite similar moisture content of both kernels. Furthermore, increasing pressing temperature increased oil recovery for the kernel pre-dried at 60°C, probably by lowering oil viscosity. The reverse influence, the decrease of oil recovery when pressing temperature was increased, was observed for the kernel pre-dried at 105°C. We observed that kernel pre-dried at 105°C was harder than kernel pre-dried at 60°C, even though the moisture content was brought back to 3% by exposing the dry kernel to ambient air. The harder kernel will give more resistance to pressing and the rigid surface might decrease the ease with which the oil flows out of the

kernel, resulting in lower oil recovery. The influence was not observed with the hexane extracted kernels, since these were grinded before oil extraction.

Increasing pre-drying temperature from 60°C to 105°C increased the oil recovery from combined extraction without protease. The influence of pre-drying temperature was larger than the other parameters and, although the oil recovery was considerably lower, the influence was similar to the influence observed in hexane extraction. In general, the increase in pre-drying temperature did not influence oil distribution between free oil and emulsion. The increase of oil recovery when the pre-drying temperature was increased indicates that more cells were disrupted at higher temperature. The main oil recovery comes from the completely disrupted cells. This also explains the lower oil recovery compared to hexane extraction, as in the latter, additional oil recovery can be obtained from diffusion through undisrupted cells.

3.3.3.2 Pre-drying and protein recovery

Increasing pre-drying temperature from 60°C to 105°C did not influence the protein recovery during combined extraction. On the other hand, increasing pre-drying temperature from 60°C to 105°C decreased protein recovery from press cakes and meal in most cases (Figure 3.1). The decrease in protein recovery may be attributed to protein denaturation at higher temperature, resulting in protein coagulation and a decrease in solubility. Similar influence was previously observed when high temperature was applied even during short period; for instance press cake from Chilean hazelnut that received heat treatment (60°C, 5 min) before pressing also gave lower protein recovery compared to press cake that did not receive the treatment[51].

Figure 3.3 shows comparative influence of increasing pre-drying temperature from 60°C to 105°C and from increasing protein extraction temperature from 25°C to 60°C. Protein extraction is often governed by diffusion[34,52], and because increasing extraction temperature increases diffusivity, protein recovery consequently increases. Protein recovery increase from increasing extraction temperature was largest after the most severe oil separation, the hexane extraction, while it was least for the pressing, and even negative for the combined extraction. As shown in Figure 3.3, however, the influence of pre-drying temperature in decreasing protein recovery was more evident. The net influence therefore shows a decrease in protein recovery (shown as negative difference) for all materials. This suggests that at certain degree of denaturation, the formed coagulate inhibits diffusion even at higher extraction temperature.

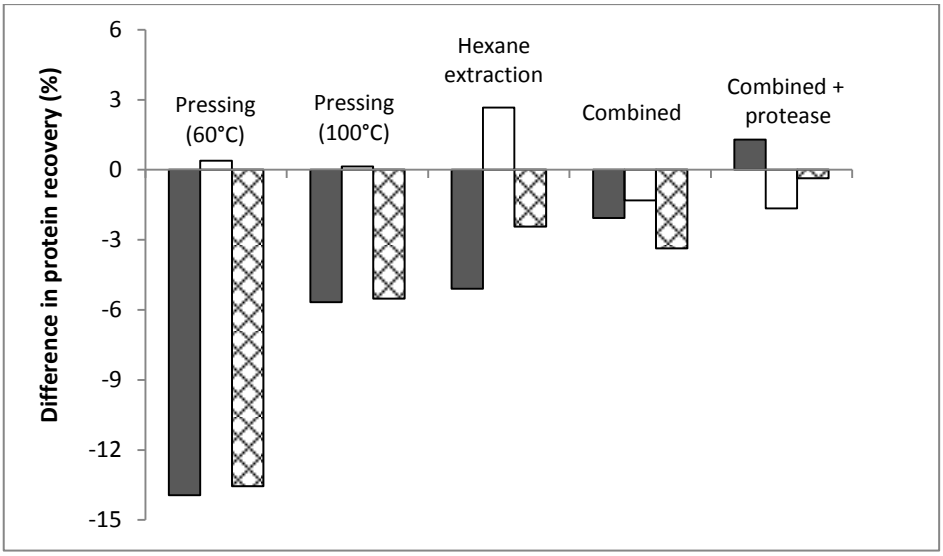


Figure 3.3 Differences in protein recovery from increasing pre-drying temperature from 60°C to 105°C (■) and from increasing protein extraction temperature from 25°C to 60°C (□). Cross-filled bars (⊠) indicate net influence. Results from pre-drying at 60°C and protein extraction at 25°C are used as reference (0).

3.3.4 Protease addition

3.3.4.1 Protease addition and oil recovery

During combined extraction of kernel pre-dried at 60°C, addition of protease and inactivated protease increased oil recovery as free oil and emulsion. We observed that extraction under alkaline conditions without protease resulted in 8% degree of hydrolysis on average (Figure 3.4). Degree of hydrolysis increased when protease was added showing that more hydrolysates were formed.

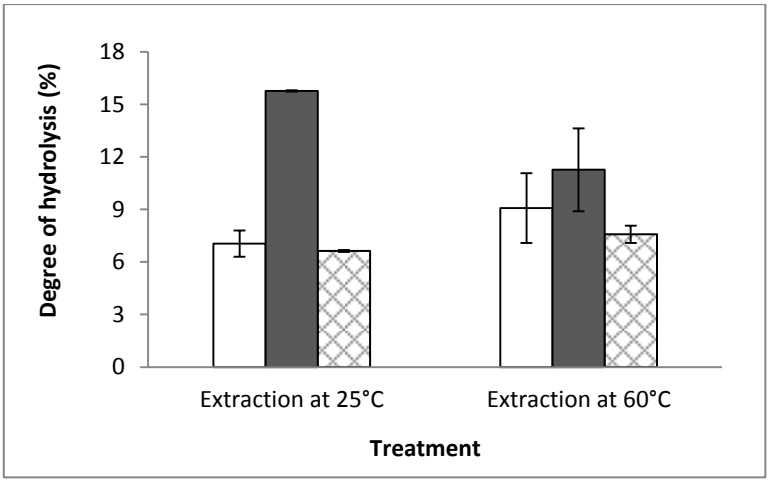


Figure 3.4 Degree of hydrolysis from kernel pre-dried at 60°C and extracted for 1 h without protease (☐), with protease (■), and with inactivated protease (☒).

Previous studies had observed oil recovery increase upon protease addition due to production of protein hydrolysates[29,30]. However, significant increase was often observed within short period of time when the degree of hydrolysis was still relatively low[45]. We observed that addition of inactivated protease also increased free oil and emulsion recoveries, therefore physical interactions due to protein addition are probably more important. Formation of small peptides might expose their hydrophobic side chains and facilitate hydrophobic interaction with apolar tail domains of lipid. Likewise, inactivated protease that was present in unfolded state might follow similar mechanisms[53–55]. Also, hydrolysed and denatured proteins form a weaker interfacial film, which makes it easier to disrupt and coalesce oil droplets[45]. These two mechanisms: hydrophobic interaction and weaker interfacial film, might explain how addition of protease or inactivated protease renders more oil available as free oil or emulsion.

3.3.4.2 Protease addition and protein recovery

During the combined extraction, addition of protease did not aid protein recovery. Influence of physical interactions from native protein (protease) addition on protein extraction was not observed. The application of higher protein extraction temperature decreased the degree of hydrolysis in experiment with added protease. This implies that hydrolysis might first occur on the already soluble proteins, therefore in our case the

protease worked better at 25°C where more protein was dissolved, and in overall no increase in protein recovery was observed from protease addition.

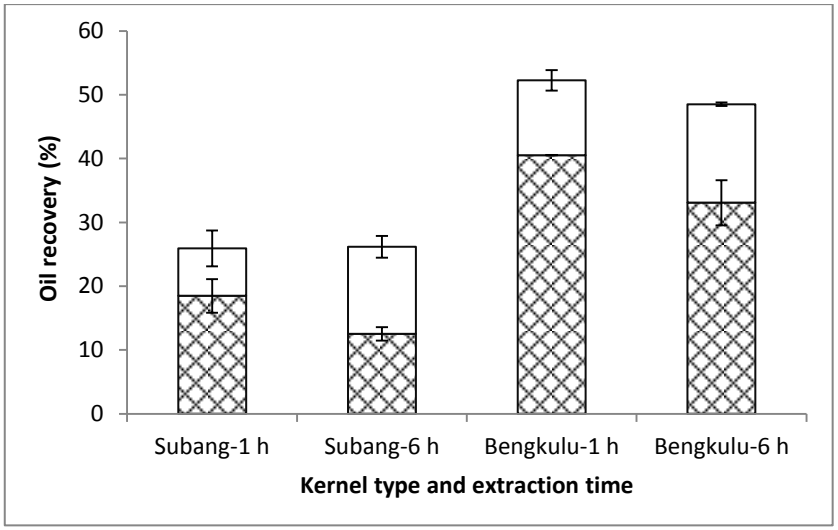
Protein recoveries were decreased by addition of inactivated protease, which might indicate that denatured protein induced protein coagulation. Protein unfolding exposed hydrophobic side chains of the protein, increase surface hydrophobicity and facilitate aggregate formation[56,57].

A study on the use of rubber seed protein as animal protein component suggests that alkaline treatment is causing decrease in solubility and protein quality in general[7]. The study, however, used the insoluble fraction of the rubber seed kernel after soaking with 0.01 M NaOH; the lower protein quality therefore might not be related to protein denaturation, but instead due to the lower solubility of the native proteins in this fraction. Discussions in previous sections suggest that most of the extracted proteins in our study were soluble native proteins, since the denatured proteins were rendered insoluble. The advantage of having native proteins is the relatively easy separation, for instance by isoelectric precipitation. However, further studies are needed to investigate the properties of these proteins.

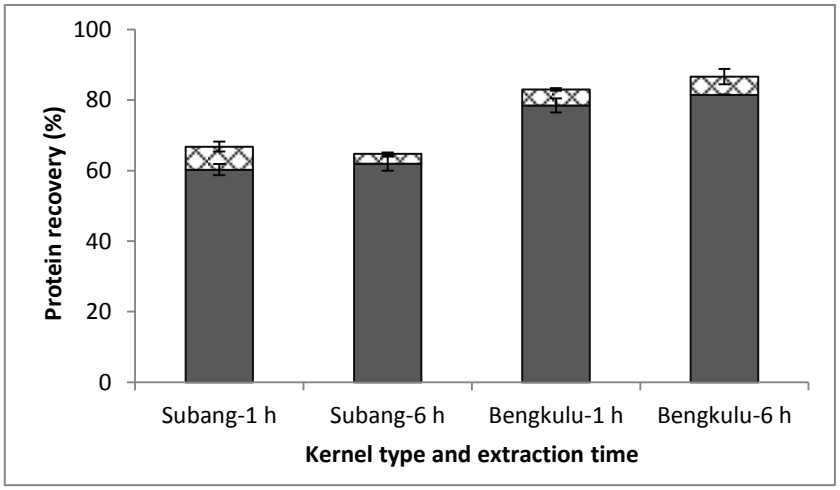
Studies with soybean and rapeseed proteins, among others, suggested some functional properties e.g. emulsifying and foaming can be improved by partial hydrolysis[58,59]. However, separation of hydrolysed proteins is more difficult because isoelectric points of the hydrolysates cover a wider range of pH than the proteins. Membrane filtration was proposed to recover hydrolysates from the aqueous fraction[60,61].

3.3.5 Seed type

The Bengkulu kernels showed considerable higher oil and protein recoveries (Figure 3.5) compared with the Subang kernels that were used in most of the experiments, even though oil and protein contents of these two types of kernel are comparable (Table 3.1). This suggests that seed properties, e.g. arrangement of oil and protein within the cell or composition of the oil or protein, played an important role in oil and protein extraction from rubber seed kernel. Next to seed type, also the local conditions for seed growing, harvesting, and storage will influence kernel properties.



(a)



(b)

Figure 3.5 (a) Oil recovery in the free oil (☐) and emulsion (☒) phases. (b) Protein recovery in emulsion (☒) and aqueous (■) phases. Kernels were pre-dried at 60°C for three days and the extractions were performed at 60°C.

3.3.6 Extraction time

Extending extraction from 1 h to 6 h did not influence the total oil recovery. Prolonged extraction, however, resulted in the increase in free oil recovery and decrease in emulsion recovery (Figure 3.5a). Probably, more oil moved from the emulsion to the free oil phase in time via continuous oil coalescence and emulsion disruptions. Extending extraction from 1 h to 6 h also did not influence the total protein recovery (Figure 3.5b).

3.3.7 Side reactions

The presence of other components like phenolic compounds and sugars might also influence protein extraction. Interactions between these components and protein also depend on extraction temperature, and will lead to changes in the properties of the protein(-complex) e.g. size, shape, or net charge. Rubber seed kernel contains 14% sugar (dry weight)[20], 21% are in the form of reducing sugars that might react with protein via the Maillard reaction. Next to that, rubber seed oil contains over 81% unsaturated fatty acid[8], which usually indicates a high content of phenolic compounds both in the full fat and the de-oiled seeds[62] that may form phenolic-protein complexes[63]. All these reactions are characterised by the formation of dark colours. We observed that the protein extracts had darker brown colour compared to the corresponding starting materials (kernel, press cake, or meal) used for extraction.

Several amino acids with a secondary amine, e.g. lysine, tryptophan, and tyrosine, are known to react with phenolic compounds or sugars[64,65]. We compared the amount of lysine in the materials and extracts and, with the assumption that the amount of the extracted lysine was proportional to the protein recovery, we calculated the amount of theoretical lysine in the extracts. From the theoretical value, similar amount of lysine was only observed in the extracts from press cakes that were pre-dried at 60°C. Less amounts of the theoretical lysine were observed in the extracts from combined extraction (27-59%), press cakes that were pre-dried at 105°C (40-67%), and meal (64-82%). Higher protein extraction temperature did not influence the amount of lysine in the extract. This suggests that the reactions occurred already during pre-drying or oil separation, and oil pressing and hexane extraction conditions influenced the amount of complex formed in the press cakes and meals.

3.3.8 Outlook for application

Based on annual collected seeds of 200 kg/ha, 38 kg-oil/ha and 13 kg-protein/ha are available. A recent study estimates a more optimistic production of 1553 kg/ha per year, corresponding to 259 kg-oil/ha[4]. These values are lower than for other oilseeds e.g. soybean or rapeseed[66], however, using the rubber seeds requires no additional input to the established rubber plantation. Furthermore, the productivity can still be increased when collection yield is improved, or when agricultural practice is also optimised for seed production.

To select the best strategy for optimal oil and protein extractions, other factors such as energy consumption, scaling, and the intended use of oil and protein fractions should also be taken into account. In accordance with the aim of this study, these parameters were considered in the following order: protein recovery, oil recovery, protein and oil quality, energy and chemical use.

Protein recovery was the highest for the combined extraction and for the protein extraction after pre-drying and pressing at 60°C. From these two, pre-drying and pressing at 60°C gave the highest (59%) oil recovery, and is therefore selected as the best method. However, oil recovery is lower than the highest recovery from hexane extraction. Furthermore, oil quality from pressing was also better than combined extraction, as the latter was mostly in the form of an emulsion. Combined extraction required less energy input for the extraction; however the separation of the resulting phases was more complicated. Current methods to separate oil and protein from the emulsion: freezing-thawing, pH adjustment, or enzymatic treatment[35,46], require additional energy or chemical input, or both.

The highest oil recovery was obtained from hexane extraction. This process, however, resulted in the least protein recovery. Both oil and protein qualities might also be influenced by high temperature and contact with solvent.

As edible oil or biodiesel feedstock, oil price is generally higher than protein meal, which is often sold as animal feed. In general, high oil recovery is preferred, and hexane extraction is most efficient and applicable at large scale. On a smaller scale, pressing the seed or kernel is preferred due to low capital investment[67], and local resources can be applied for local products. Local processing also allows recycle of minerals back to the plantation. Here, further optimisation of the process is still possible.

3.4 Conclusions

Our study shows that oil and protein can be extracted simultaneously in one process; oil separation is not necessary for high protein recovery. However, the oil recovery is relatively low. Interactions between oil and protein molecules, including emulsion formation, play important roles during the extraction. The emulsion formation may limit the practical applicability of this method. Protease addition does not increase protein recovery, however formation of hydrolysates might aid in oil extraction.

Seed type and handling before processing were most determining for high oil and protein recoveries. Due to the high moisture content in the kernel, pre-drying is a necessary step to reduce the moisture before oil separation. Lower moisture content also allows longer storage time for the kernel. However, higher pre-drying temperature tends to decrease protein recovery from press cakes and meals, therefore pre-drying at low temperature is preferred.

In general, treatments that result in more oil tend to decrease the protein recovery. Protein recovery from press cake that was pre-dried and pressed at 60°C was comparable to the recovery from combined extraction that gave lower oil recovery, suggesting that seed pressing can give optimised results to obtain both oil and protein from rubber seed kernel.

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Chapter 4

Production of Hydrophobic Amino Acids from Biobased Resources: Wheat Gluten and Rubber Seed Proteins

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Abstract

Protein hydrolysis enables production of peptides and free amino acids that are suitable for usage in food and feed, or can be used as precursors for bulk chemicals. Several essential amino acids for food and feed have hydrophobic side chains; this property may also be exploited for subsequent separation. Here, we present methods for selective production of hydrophobic amino acids from proteins. Selectivity can be achieved by selection of starting material, selection of hydrolysis conditions, and separation of achieved hydrolysate. Several protease combinations were applied for hydrolysis of rubber seed protein concentrate, wheat gluten, and bovine serum albumin (BSA). High degree of hydrolysis (>50%) could be achieved. Hydrophobic selectivity was influenced by the combination of proteases and by the extent of hydrolysis. Combination of Pronase and Peptidase R showed the highest selectivity towards hydrophobic amino acids, roughly doubling the content of hydrophobic amino acids in the products compared to the original substrates. Hydrophobic selectivity of 0.6 mol-hydrophobic/mol-total free amino acids was observed after 6 h hydrolysis of wheat gluten and 24 h hydrolysis of rubber seed proteins and BSA. The results of experiments with rubber seed proteins and wheat gluten suggest that this process can be applied to agro-industrial residues.

Keywords: biorefinery, protein hydrolysis, protease, rubber seed, wheat gluten, hydrophobicity

4.1 Introduction

As the building blocks of proteins, amino acids are important components in food and feed. Alternatively, amino acids can be used for chemicals production to reduce fossil fuel consumption[1]. From the 20 proteinogenic amino acids, isoleucine, leucine, valine, phenylalanine, tryptophan, methionine, threonine, histidine, and lysine are essential amino acids as they cannot be synthesised by humans and most farm animals. This makes them important in the human and animal diet. From these amino acids, the first six have hydrophobic side chains[2]. Amino acid hydrophobicity is often defined by its partitioning between two liquid phases[3], and this property can be important in downstream processing. Producing mixtures rich in hydrophobic amino acids is therefore an interesting process to investigate based on the ease in further processing and their potential application as a group in food and feed. This approach increases the feasibility of a biorefinery route from protein to food/feed and bulk chemicals[4].

The hydrophobicity of amino acids has been extensively studied as hydrophobic interactions play a dominant role in stabilising protein structures[3,5]. Amino acids with hydrophobic side chains tend to reside in the interior of a protein to minimise contact with water. This tendency can be approximated by determining amino acid partition between water and organic phase[6]. The partitioning can also be calculated from amino acid solubility in an organic solvent, and expressed as free energy changes of transfer from organic solvent to water. With this approach, tryptophan shows to be the most hydrophobic[5,6]. Alternatively, the partitioning can be calculated based on phase-partitioning behaviour of molecular fragments that build the amino acid. Phenylalanine is shown as the most hydrophobic amino acid based on this approach[2]. Despite methods differences, there is a good agreement that the following amino acids: phenylalanine, leucine, isoleucine, tyrosine, tryptophan, valine, methionine, and proline can be grouped as hydrophobic.

Amino acids can be produced by chemical synthesis, fermentation, or protein hydrolysis[7]. Protein hydrolysis has a high potential because the proteins can be obtained from several sources including agro-industrial residues, which include residues from first generation bioethanol or biodiesel production, leaves, grass, stover, microalgae, and animal slaughter waste, with varying protein content from 5% to 90%[8]. Dried distillers grains with solubles (DDGS) is an important by-product from bioethanol production. The weight of DDGS is roughly the same as the produced ethanol[9]. Wheat DDGS contains 36-38% protein that is predominated by gluten (80-85% of wheat protein) and has remarkably high (34%) content of glutamic acid/glutamine[8,9]. The other potential agro-industrial residues are rubber seeds. They are available from rubber tree (*Hevea brasiliensis*) plantations, of which the latex is the main product that is used in natural rubber production. Recently

there are growing interests in using rubber seeds for oil and protein production[10,11]. Rubber seed press cake, the residue after oil pressing, contains 22% protein that consists of one-third hydrophobic amino acids[11]. With the increasing production of biofuel, the availability of wheat DDGS, rubber seed press cake, and similar residues are expected to increase in the coming years.

Complete protein hydrolysis can be performed using concentrated acid or alkali at high temperature. This process, however, may result in partial degradation or racemisation of some amino acids, including the essential ones[12,13]. Hydrolysis in subcritical water or using microwave can be performed in shorter duration and less extreme pH, therefore might hinder these problems[14,15]. Albeit liberating less free amino acids, enzymatic hydrolysis can be performed at lower temperature and neutral or slightly alkaline pH, therefore making operation easier and preventing amino acid racemisation. By modifying hydrolysis conditions, it is also possible to control the degree of hydrolysis and the resulting hydrolysate profile. Protein hydrolysates can be used in food or drink supplements (e.g. sports, weight-control, or geriatrics), or in clinical nutrition (e.g. for patients with allergy or liver disease). As native proteins can sometimes induce allergenic reactions, hydrolysis of the proteins can be used to yield short peptides that are less allergenic and have higher digestibility[16]. While proteases have different specificities, it is also possible to selectively hydrolyse specific amino acid bonds or groups of amino acids by selecting different proteases[17].

Another alternative method to modify hydrolysate profiles is using non-aqueous solvents during hydrolysis. Different hydrolysate profiles were observed during casein and β-lactoglobulin hydrolysis in 0–60% ethanol[18,19]. On the other hand, casein hydrolysis in water-immiscible n-hexane, isooctane, and ethyl acetate showed similar hydrolysate profile despite differences in degree of hydrolysis[20]. In non-aqueous solvent, both the substrate and the peptides resulting from cleavage of non-terminal residues have different solubilities compared to solubilities in water. The applied (exo-)protease thus may be exposed to a different part of the protein/peptide, resulting in different free amino acid profiles.

The objective of this research was to selectively produce hydrophobic amino acids from agro-industrial residues. Wheat gluten (as representative of wheat DDGS) and rubber seed protein concentrate were used in the hydrolysis experiments, and the course of hydrolysis was followed in time. Hydrolysis in ethanol was also performed to study the production of free amino acids and the influence of ethanol on selectivity. Experiments with bovine serum albumin (BSA) were used as a reference.

4.2 Materials and methods

4.2.1 Materials

Rubber seed protein concentrate (48% protein) was prepared from rubber seed press cake by alkaline extraction of the press cake using 0.1 M NaOH at solid-to-liquid ratio of 1:10 (w/v), 25°C, for 1 h, followed by precipitation at pH 5 (4°C, 24 h) and freeze drying. Wheat gluten was obtained from Cargill (the Netherlands). BSA and Alcalase 2.4L FG were obtained from Sigma-Aldrich (USA). Validase FP concentrate, Pronase, and Peptidase R were obtained from DSM (the Netherlands), Roche Diagnostics (Germany), and Amano (Japan), respectively. Chemicals used were of analytical grade.

4.2.2 Solubility of rubber seed proteins at different pH

Solubility of rubber seed proteins was determined according to Morr *et al*.[21]. Rubber seed protein concentrate was dispersed in water to get a homogeneous mixture with final concentration of 1% (w/w) protein. The pH was adjusted to the desired pH (1 through 13) using 6 M and 0.1 M HCl or NaOH. The mixture was stirred at 250 rpm, 25°C (2mag magnetic stirrer, Germany) for 1 h, followed by centrifugation at 3000x*g*, 20°C, for 30 min. The supernatant was separated and analysed for protein content.

The experiment was performed in triplicate. Solubility (%) was calculated as the weight of dissolved protein in the supernatant divided by the total protein weight in the mixture.

4.2.3 Solubility of rubber seed proteins at different ethanol concentration

Rubber seed protein concentrate was dispersed in water at the concentration of 3% (w/w) protein, and the pH was adjusted to pH 8.5 using 6 M and 0.1 M NaOH. To this mixture, water and ethanol were subsequently added to get 10–70% (w/w) ethanol concentration and final protein concentration of 1% (w/w). The mixture was stirred at 250 rpm, 25°C (2mag magnetic stirrer, Germany) for 1 h, followed by centrifugation at 3000x*g*, 20°C, for 30 min. The supernatant was separated and analysed for protein content.

The experiment was performed in triplicate. Solubility calculation was similar to solubility at different pH.

Table 4.1 Hydrolysis conditions.

Proteases combination	pH	t = 0 – 1.5 h			t = 1.5 – 24 h		
		Protease	Activity[a]	T (°C)	Protease	Activity[a]	T (°C)
Validase 2x	7	Validase FP concentrate	400,000 HU/g[b]	55	Validase FP concentrate	400,000 HU/g[b]	55
Validase + Peptidase	7	Validase FP concentrate	400,000 HU/g[b]	55	Peptidase R	420 U/g[d]	40
Pronase + Peptidase	7	Pronase	7,000 U/g[c]	55	Peptidase R	420 U/g[d]	40
Alcalase 2x	8.5	Alcalase 2.4L FG	900 U/g[c]	55	Alcalase 2.4L FG	900 U/g[c]	55

[a] The activity as given by the supplier

[b] HU = Hemoglobin Unit

[c] Unit determined by non-specific protease assay, 1 U will hydrolyse casein to produce colour equivalent to 1.0 µmol of tyrosine per minute

[d] Unit determined by L-Leucyl-Glycyl-Glycine method

4.2.4 Enzymatic protein hydrolysis using proteases combinations

To study the hydrolysis of our selected substrates, four combinations of protease mixtures were tested (Table 4.1), based on results of previous experiments with wheat gluten[22]. Validase FP Concentrate and Pronase are mixtures of endo- and exo-proteases with broad specificity. Peptidase R yielded the highest free amino acids compared to other exo-proteases tested. Alcalase 2.4L FG was also selected due to reported specificity towards hydrophobic amino acids[23].

Rubber seed protein concentrate was dispersed in water to get a mixture with concentration of 5% (w-protein/w-solvent). The pH was adjusted to fit the protease optima (Table 4.1) using 6 M and 0.1 M NaOH, and Britton-Robinson buffer was added at 0.01 M. The mixture was stirred at 250 rpm (2mag magnetic stirrer, Germany). The optimal temperature (see Table 4.1) was kept with a circulating-water bath (Julabo). After 30 min, protease at 1% w/w-protein was added and time was set as t = 0. Another 1% protease was added at t = 1.5 h to a total protease concentration of 2%. Samples were taken at t = 0, 1, 3, 6, 9, and 24 h. To inactivate the protease after reaction, the sample tubes were incubated at 90°C for 10 min and stored on ice immediately thereafter, until centrifuged at 7000x*g*, 4°C for 20 min. The supernatant was separated and filtered through a 0.45 µm Minisart filter to remove insoluble matter. BSA was hydrolysed in a similar procedure using a combination of Pronase and Peptidase R. The experiments were performed in triplicates. Identical experiments without protease addition were performed as control.

Wheat gluten was hydrolysed with all protease combinations in Table 4.1. The experiments were carried out in duplicates as described previously[22]; experimental set-ups were similar to experiments with rubber seed protein concentrate except no buffer was added and the experiments with Validase FP Concentrate was performed at pH 6.

4.2.5 Enzymatic protein hydrolysis in ethanol

Rubber seed protein concentrate or BSA was dispersed in water at the concentration of 2.5% (w-protein/w-solvent), and the pH was adjusted to the desired pH using 6 M and 0.1 M NaOH. Water, ethanol, and Pronase dissolved in 0.1 M Britton-Robinson buffer were subsequently added to get the final concentrations of 1% (w/w) protein, 0–50% (w/w) ethanol, and 5% w-protease/w-protein. The mixture was incubated at 55°C for 24 h. To inactivate the protease after the reaction, the sample tubes were incubated at 90°C for 10 min and stored on ice immediately, until centrifuged at 7000x*g*, 4°C for 20 min. The supernatant was separated and filtered through 0.45 µm Minisart filter to remove

insoluble matter. The experiment with rubber seed protein concentrate was performed in triplicate and the experiment with BSA was performed in duplicate.

4.2.6 Analysis

The analysis was performed once for each sample. The analysis was repeated when the standard deviations of replicate treatments were higher than 10% of the mean value.

4.2.6.1 Protein content

Kjeldahl and modified Lowry methods were applied to measure protein content in determination of rubber seed proteins' solubility. Kjeldahl results were calculated with nitrogen-to-protein conversion factor of 5.7[11].

The modified Lowry method[24] was applied to determine protein content in the hydrolysate, as this method only requires samples in small volume, and therefore enables frequent sampling during the experiment. In the presence of free amino acids, the calculation for protein concentration was modified as discussed in subchapter 4.3.2:

Protein concentration = measured soluble protein + free amino acids – tyrosine – tryptophan

All units are in mg-protein/ml.

4.2.6.2 Degree of hydrolysis

Degree of hydrolysis was determined using a modified OPA method[25]. Based on amino acid composition, the total peptide bonds were 7.8 meqv/g for rubber seed proteins, 7.5 meqv/g for wheat gluten, and 8.1 meqv/g for BSA.

4.2.6.3 Amino acid composition

To measure amino acid composition of the substrates, samples were first acid-hydrolysed at 110°C for 24 h using 6 M HCl containing 1% (w/v) phenol[26]. Alkaline hydrolysis (4.2 M NaOH, 110°C, 24 h) was performed specifically for tryptophan determination[27]. The hydrolysates were dissolved in methanol and filtered through 0.2 µm Minisart filter; this

procedure was also applied to the hydrolysates from the experiments to measure free amino acids. The filtered solutions were loaded onto Ultra-HPLC Dionex RSLC (Dionex Corporation, USA) where the amino acids were separated using an Acquity UPLC BEH C18 reversed phase column. Norleucine was used as standard. Detection was performed at 263 nm and 338 nm[26].

4.2.7 Statistical analysis

The values of different treatments were compared using Student's t-test or ANOVA with LSD post-hoc analysis; $p < 0.05$ was regarded as significant.

4.3 Results

4.3.1 Amino acid composition

The three substrates used in our experiments contained comparable amounts of hydrophobic amino acids (Table 4.2).

Valine, proline, and leucine were the hydrophobic amino acids with the highest fraction in rubber seed protein concentrate, wheat gluten, and BSA, respectively (Table 4.2). Hydrophobic amino acids are predominantly present in the interior of the protein[5], as this conformation stabilises the protein in aqueous solution. To enable contact between hydrophobic amino acids and the protease, the protein must be unfolded.

Table 4.2 Amino acid side chain hydrophobicity (Δf) and amino acid composition of rubber seed protein concentrate, BSA, and wheat gluten.

Amino acid[a] (AA)	Abbre-viation	Δf[b] (cal/mol)	Amino acid fraction (mol/mol-total amino acids)		
			Rubber seed protein concentrate	Wheat gluten	BSA
Phenylalanine	Phe	2650	0.04	0.03	0.05
Leucine	Leu	2420	0.08	0.07	0.12
Isoleucine	Ile	2970	0.04	0.04	0.02
Tyrosine	Tyr	2870	0.02	0.02	0.04
Tryptophan	Trp	3220	0.01	0.01[c]	0.00
Valine	Val	1690	0.11	0.04	0.07
Methionine	Met	1300	0.01	0.02	0.01
Proline	Pro	2600	0.06	0.15	0.05
Cystine/cysteine	Cys	1000[d]	0.00	0.00	0.00
Alanine	Ala	500	0.08	0.04	0.09
Glycine	Gly	0	0.08	0.06	0.03
Threonine	Thr	400	0.04	0.03	0.06
Serine	Ser	-300	0.07	0.06	0.05
Lysine	Lys	1500[e]	0.02	0.04	0.10
Histidine	His	450	0.02	0.01	0.03
Glutamic acid/glutamine	Glx	550[f]	0.13	0.33	0.14
Aspartic acid/asparagine	Asx	540[g]	0.12	0.03	0.10
Arginine	Arg	730	0.09	0.02	0.04
Total hydrophobic amino acids[h]			0.35	0.37	0.35

[a] The amino acids are listed from the most hydrophobic (phenylalanine) to the least hydrophobic (arginine) as calculated with phase-partitioning constants of molecular fragments[2].

[b] Δf (hydrophobicity) = free energy change for transfer from ethanol to water at 25°C[5,6]. Values for ethanol were selected instead of average values of organic solvents due to the relevance with our experiment.

[c] Calculated from Woychik *et al.*[28].

[d] Data from Bigelow[29].

[e] The high hydrophobicity of lysine is due to the presence of norleucine side chain that is very hydrophobic (Δf = 2700 cal/mol). However, as lysine is positively charged, it is not grouped as hydrophobic.

[f] Value for glutamic acid

[g] Value for aspartic acid

[h] Phenylalanine, leucine, isoleucine, tyrosine, tryptophan, valine, methionine, proline[2].

4.3.2 Protein solubility

Solubility of rubber seed proteins at different pHs was measured to indicate the available protein fraction in the solution at the start of hydrolysis. At pH 7, where some of the experiments were conducted (Table 4.1), only 16% of protein was soluble. Protein concentrate was prepared using alkaline extraction, therefore it consisted mostly of alkaline-soluble fractions. As expected, most of the proteins were soluble at pHs up and above 8.5 (Figure 4.1). The lowest solubility in water occurred between pH 4 and 5, which indicates its isoelectric point. BSA is fairly soluble at pH 7[30], with isoelectric point at pH 5[31]. Wheat gluten solubility is less than 5% at pH 7, which is estimated as its isoelectric point[32].

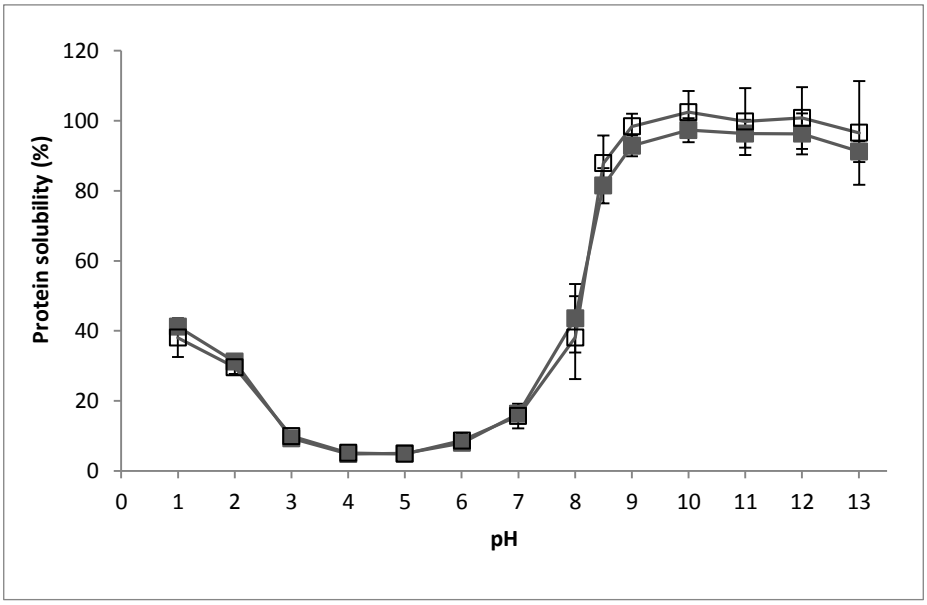


Figure 4.1 Solubility of rubber seed protein at 25°C as a function of pH, as determined by modified Lowry (□) and Kjeldahl (■).

Protein denaturation reduces protein solubility, however, the conformational change may expose the interior amino acids to the proteases. There was no significant difference ($p > 0.05$) of rubber seed proteins solubility between 0 and 10% w/w ethanol (Figure 4.2), but solubility decreased at higher ethanol concentrations, indicating the protein was denatured. BSA was completely soluble in water up to 0.56 g-BSA/g-solution, and the solubility did not change in up to 30% w/w ethanol. At 37% w/w ethanol, complete

solubility of 0.05 g-BSA/g-solution was still observed[30]. The use of 50-65% v/v ethanol is reported to even increase wheat gluten solubility from 2 to 37 g-gluten/l-solvent[33].

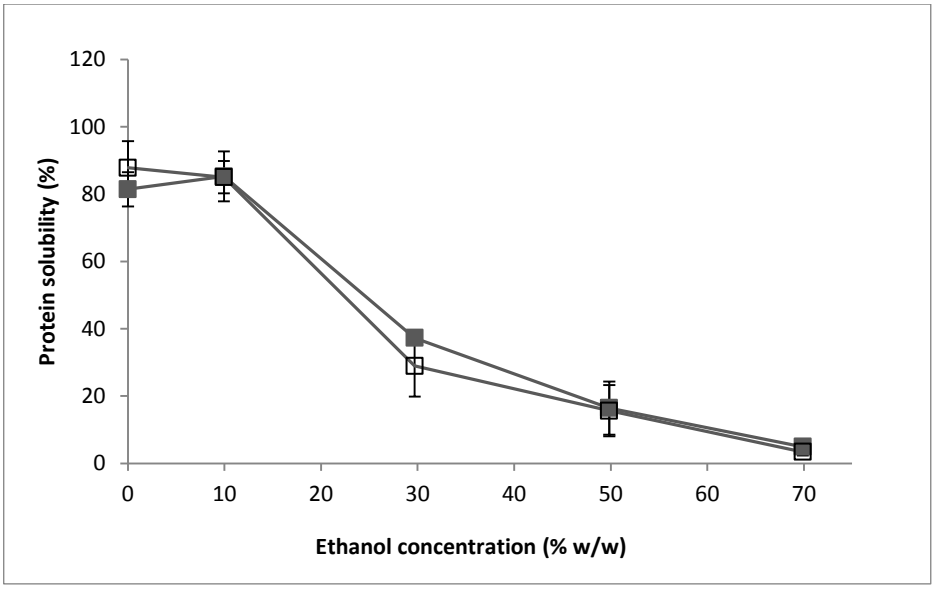
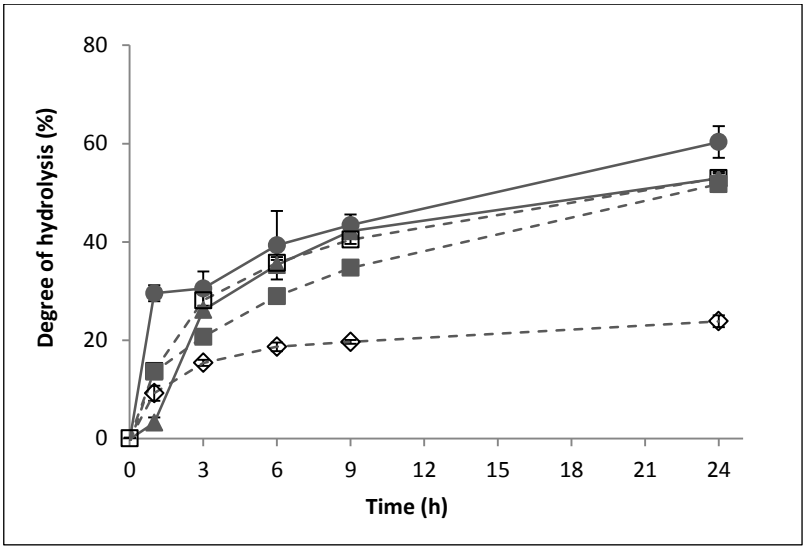


Figure 4.2 Solubility at different ethanol concentrations for rubber seed proteins at pH 8.5, 25°C, as determined by modified Lowry (□) and Kjeldahl (■).

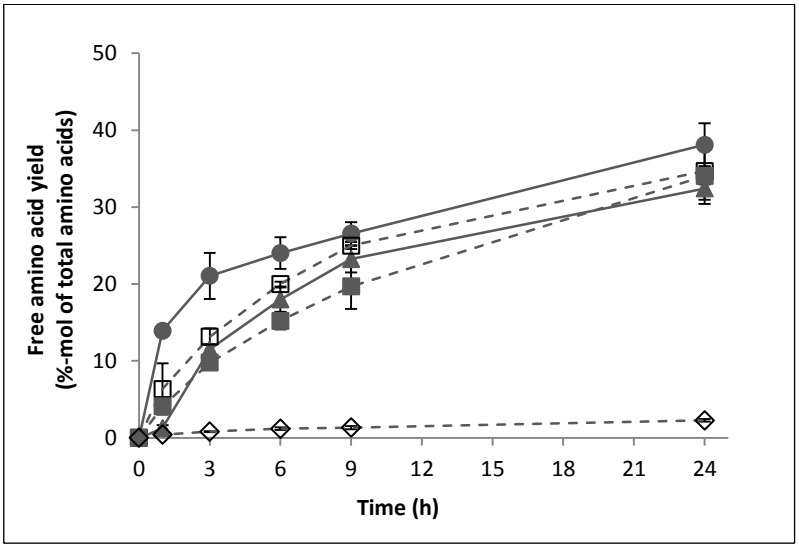
Due to the low detection limit (up to 0.4 mg/ml), protein determinations by modified Lowry had higher standard deviations at high protein concentrations. However, comparison between this method and Kjeldahl for rubber seed proteins shows good correlation based on linear regression (R^2 = 0.986):

Kjeldahl solubility (%) = 0.93 Lowry solubility (%) + 2.43 %

Free amino acids other than tyrosine and tryptophan may not be detected with Lowry[34]. However, this method requires only small sample volume that enables frequent sampling during the experiment. Based on these results, we used the modified Lowry method[24] corrected with free amino acids concentrations from HPLC measurements to determine protein contents of the hydrolysates.



(a)



(b)

Figure 4.3 Degree of hydrolysis (a) and free amino acid yield (b) during 24 h hydrolysis of BSA with Pronase + Peptidase (—●—) and hydrolysis of rubber seed proteins with Validase 2x (- -□- -), Validase + Peptidase (- -■- -), Pronase + Peptidase (—▲—), and Alcalase 2x (- -◇- -).

4.3.3 Hydrolysis with protease combinations

4.3.3.1 Influence of protease

Figure 4.3a and 4.3b show, respectively, the degree of hydrolysis and the yield of liberated free amino acids relative to the total available amino acids in the experiment. After 24 h hydrolysis of rubber seed proteins, comparable degree of hydrolysis and free amino acid yield were observed for the three protease combinations at pH 7: Validase 2x, Validase + Peptidase, and Pronase + Peptidase. With increasing degree of hydrolysis, the amount of solubilised protein for these experiments also increased (Figure 4.4). We previously observed this in experiments with wheat gluten[22]. Up to t = 3 h, the increase in protein solubility was mainly attributed to the formation of peptides. Material balance between fractions and the high degree of hydrolysis in all experiments suggests that the peptides were very short-chained, and probably mainly present as di- or tri-peptides. After 3 h, the increase in protein solubility was the result of free amino acids liberation.

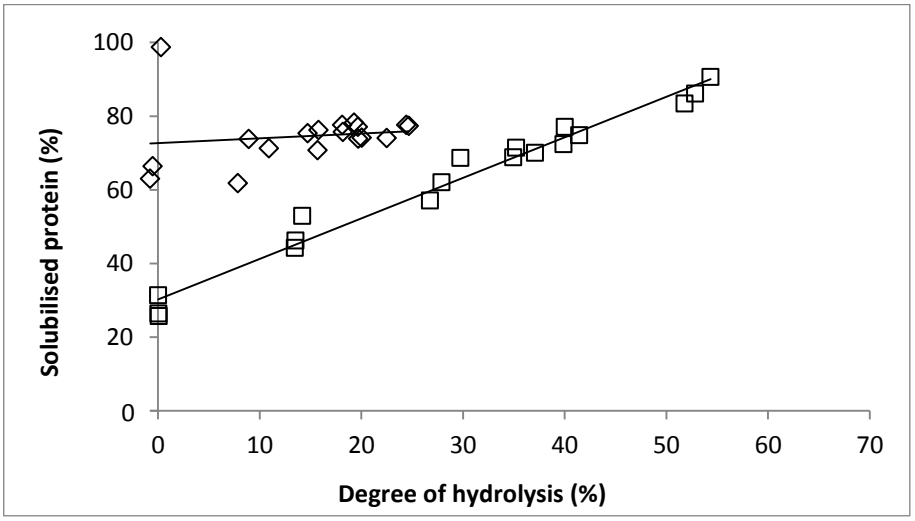


Figure 4.4 Protein solubility as a function of degree of hydrolysis during 24 h hydrolysis of rubber seed proteins with Validase 2x (□) and Alcalase 2x (◊). The lines have different starting points because of the different pH's of the mixtures (7 versus 8.5).

Despite the higher solubility of rubber seed proteins at pH 8.5 (Figure 4.1), the experiment with Alcalase 2x gave the lowest degree of hydrolysis (Figure 4.3a). Alcalase 2.4L FG is an

endo-protease from *Bacillus licheniformis* that has lower activity compared to the other proteases (Table 4.1), therefore the amount of liberated free amino acids was lower than the other experiments (Figure 4.3b). Furthermore, low exo-protease activity and inhibition of proteases from *B. licheniformis* by short peptides have been reported[23]. This is consistent with our results that the hydrolysate entailed mostly peptides and less free amino acids. As free amino acids were partially accountable for the increase in protein solubility, the amount of solubilised protein for the Alcalase 2x experiment also did not change even though the degree of hydrolysis increased during the 24 h (Figure 4.4).

4.3.3.2 Influence of substrate composition

After 24 h hydrolysis with Pronase + Peptidase, the free amino acid yield from wheat gluten was 52 ± 13% of total amino acids, which was higher than both rubber seed proteins (32 ± 2%) and BSA (38 ± 3%). Figure 4.5 shows the yield of individual amino acids based on the total amino acids available in the substrates. For all amino acids except lysine and proline, different yields between substrates were observed ($p < 0.05$), indicating that substrate composition influenced the liberation of amino acids during hydrolysis.

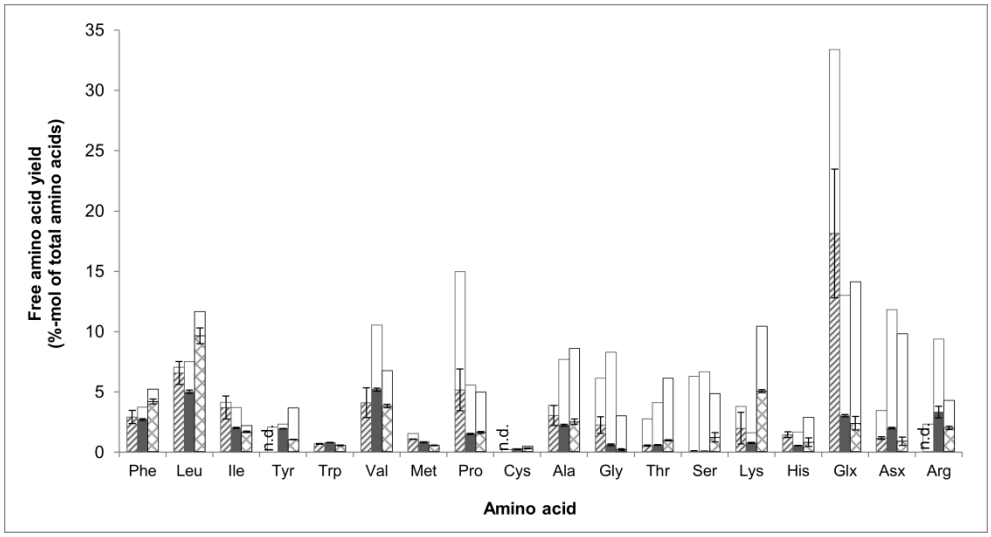


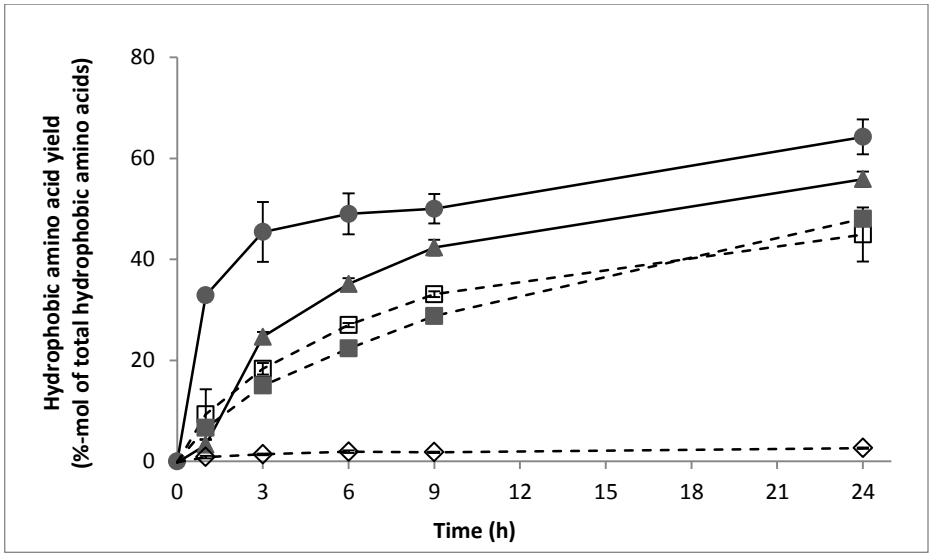
Figure 4.5 Free amino acid yield after 24 h hydrolysis of wheat gluten (▨), rubber seed proteins (■), and BSA (☒) with Pronase + Peptidase; unfilled bars (☐) indicate the available amino acid in the substrate.
n.d = value below detection level

Previous studies have shown that combination of endo- and exo-proteases leads to higher degree of hydrolysis and yields more free amino acids[22,35]. This was further illustrated when in our experiments the amount of free amino acids from wheat gluten in the experiment with Validase + Peptidase combination was higher than in the Validase 2x combination[22]. For rubber seed proteins, on the other hand, the amount of free amino acids was similar or even higher (t = 3 h and 6 h) for the Validase 2x combination than the Validase + Peptidase combination (Figure 4.3b). Peptidase R has a high proline-specific aminopeptidase activity[36], which suggests that the difference might be attributed to the amount of proline in wheat gluten (0.15 mol/mol-total amino acid) that was almost three-times higher than that in rubber seed proteins (Table 4.2; Figure 4.5). This might also explain the higher free amino acid yield of wheat gluten compared with BSA, as the latter also has low proline content.

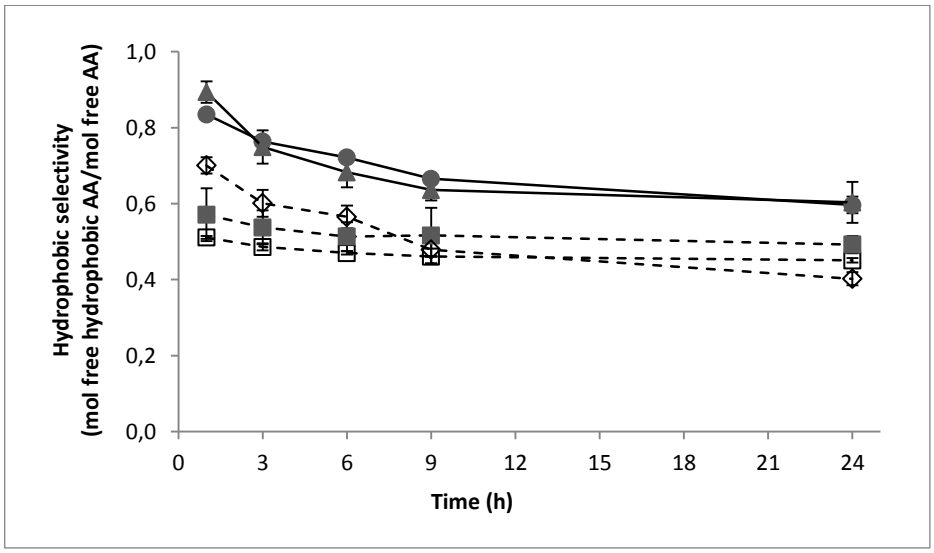
4.3.3.3 Hydrophobic amino acids yield

Figure 4.5 shows that not all amino acids were liberated to the same degree. During hydrolysis of rubber seed protein concentrate, each protease combination resulted in different hydrophobic amino acid yield and selectivity. After 24 h of hydrolysis, 45-56% of the total hydrophobic amino acids in the substrate could be recovered in the hydrolysate (Figure 4.6a), higher than the overall free amino acid yield compared to the total amino acids (Figure 4.3b).

Hydrophobic selectivity is defined as the amount of free hydrophobic amino acids: phenylalanine, leucine, isoleucine, tyrosine, tryptophan, valine, methionine, and proline[2], relative to the total liberated free amino acids on molar-base. Selectivity for each combination was highest at t = 1 h and decreased over time, except for the Validase + Peptidase combination (Figure 4.6b). There was no significant difference ($p > 0.05$) of hydrophobic selectivity between Validase FP Concentrate with and without Peptidase R , except for t = 24 h (Figure 4.6b). Furthermore, the higher selectivity of Pronase compared to Validase FP Concentrate was already observed at t = 1 h when only Validase FP Concentrate or Pronase was added and no second protease mixture. Pronase is a non-specific protease mixture. The hydrophobic selectivity might be attributed to the presence of leucine aminopeptidase[37]. This is consistent with our results showing that free leucine, phenylalanine, and valine were the amino acids that contributed most to the selectivity.



(a)



(b)

Figure 4.6 Hydrophobic amino acid yield **(a)** and selectivity **(b)** during 24 h hydrolysis of BSA with Pronase + Peptidase (—●—) and hydrolysis of rubber seed proteins with Validase 2x (- -□- -), Validase + Peptidase (- -■- -), Pronase + Peptidase (—▲—), and Alcalase 2x (- -◇- -).

Table 4.3 Hydrophobic amino acid selectivity (mol free hydrophobic amino acid/mol total free amino acid).

Substrate	Hydrophobic amino acid fraction in the substrate	Protease					
		Validase + Peptidase		Pronase + Peptidase		Pronase	Pronase, 10% ethanol
		6 h	24 h	6 h	24 h	24 h	24 h
Rubber seed proteins	0.35	0.51 ± 0.04	0.49 ± 0.02	0.68 ± 0.04	0.60 ± 0.05	0.55 ± 0.05	0.56 ± 0.06
Wheat gluten	0.37	0.42[a]	0.47 ± 0.03	0.56[a]	0.46 ± 0.02	n.a.	n.a.
BSA	0.35	n.a.	n.a.	0.72 ± 0.00	0.60 ± 0.02	0.40 ± 0.00	0.45 ± 0.00

[a] Value from one measurement.

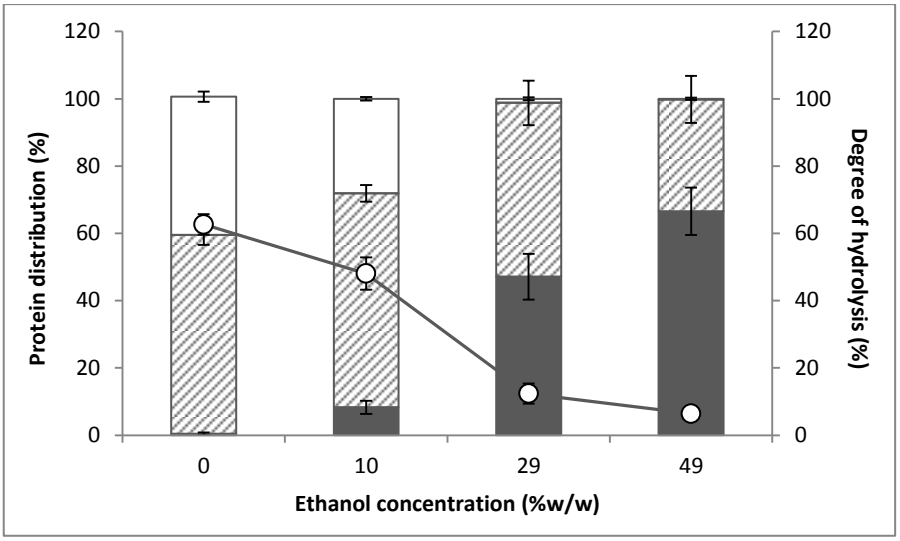
n.a. = data not available

Alcalase 2.4L FG is considered to have specificity towards hydrophobic amino acids[23], and its selectivity increases as the peptide size decreases[38]. In our experiments, however, the selectivity of Alcalase 2.4 L FG after 24 h was lower than the other protease combinations (Figure 4.6b). This might be because even though Alcalase cleaved peptide bonds next to hydrophobic amino acids, it did not always liberate free amino acids due to the lack of exo-protease activities.

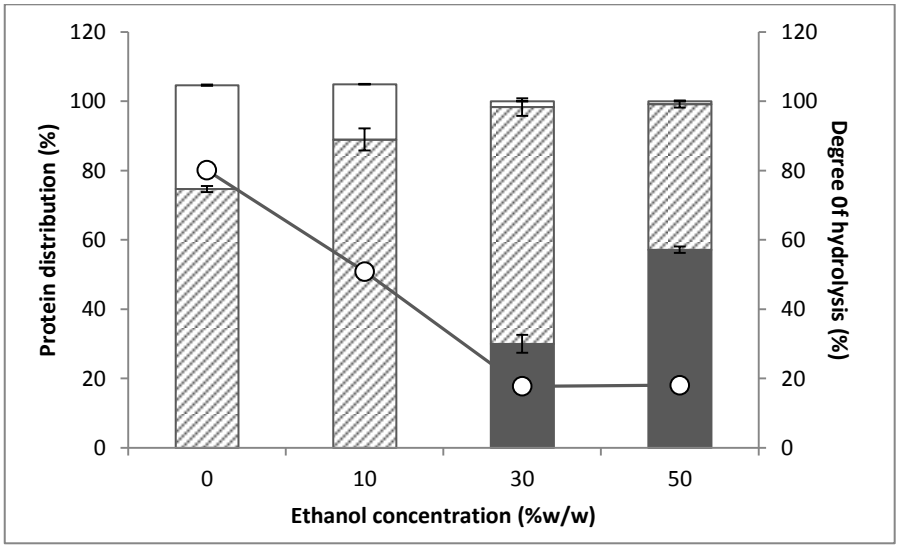
For the Pronase + Peptidase combination, comparison between the hydrophobic amino acid fraction in the substrate and selectivity in the hydrolysate at t = 6 h (Table 4.3) shows two-fold increase of selectivity for rubber seed proteins and BSA hydrolysates. For wheat gluten, a slightly less 1.5 times increase was observed. For the same protease combination, at t = 24 h, hydrophobic selectivity for rubber seed proteins and BSA hydrolysates were both still high at 0.60 mol/mol, while wheat gluten hydrolysate was only 0.46 mol/mol. The difference might be attributed to the high amount of liberated glutamic acid/glutamine from wheat gluten. The glutamic acid/glutamine fraction in wheat gluten was 0.33 mol/mol-total amino acid (Table 4.2) and the liberated glutamic acid/glutamine at t = 6 h and t = 24 h were 0.20 and 0.35 mol/mol-total free amino acid, respectively, which significantly dominated the hydrolysate profile. Similar influence of glutamic acid/glutamine on wheat gluten hydrolysis was also observed for the Validase + Peptidase combination (Table 4.3; Sari *et al.*[22]).

4.3.4 Hydrolysis in ethanol

Hydrolysis in ethanol was performed to establish ethanol influence on amino acids yield and selectivity. Figure 4.7a and 4.7b show that at 10% ethanol, around 50% degree of hydrolysis could still be obtained. As much as 28% and 16% of the original protein from rubber seed proteins and BSA, respectively, were liberated to free amino acids. This shows that the Pronase was still active at 10% ethanol, albeit at lower activity. At 30% ethanol, however, not only did the free amino acid yield decrease compared to the experiments at 0 and 10% ethanol, but also the protein solubility was similar (for rubber seed proteins) or lower (for BSA) than in the experiments without protease. Here, the protease itself can be denatured, and may have formed an insoluble complex with the peptides[11].



(a)



(b)

Figure 4.7 Degree of hydrolysis (○) and protein molar distribution between insoluble (■), peptide (▨), and free amino acid (□) fractions after 24 h hydrolysis of rubber seed proteins (a) and BSA (b) using Pronase at different ethanol concentration.

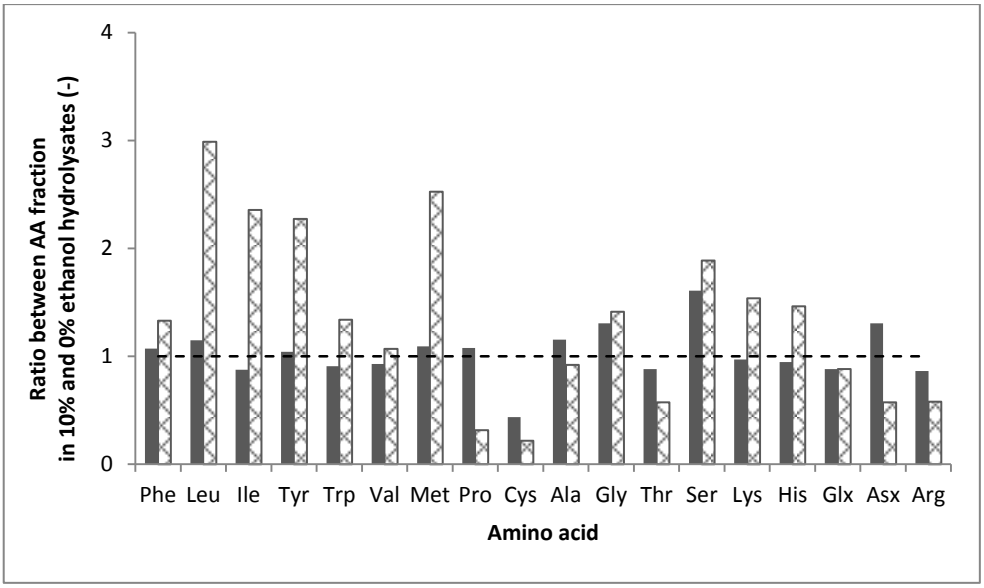


Figure 4.8 Ratio of free amino acid fraction in 10% ethanol hydrolysate to the one in 0% ethanol hydrolysate after 24 h hydrolysis of rubber seed proteins (■) and BSA (⊠) with Pronase. The dashed line indicates a ratio of 1, when the amino acid fraction in 10% ethanol hydrolysate was equal to the one in the 0% ethanol hydrolysate.

It was expected that at higher ethanol concentrations, hydrophobic selectivity could be higher, even when the total free amino acid yield was lower. The selectivity increase, however, was only observed for BSA between 0 and 10% ethanol (Table 4.3). Comparison between free amino acid fractions in 10% ethanol hydrolysate and 0% ethanol hydrolysate (Figure 4.8) shows no clear pattern of ethanol influence on free amino acid composition in the hydrolysate. Protein conformational change due to ethanol may expose other parts in different proteins and in a different fashion compared to when ethanol was not present. Also, the protease we used was a mixture of several enzymes that each may respond differently to ethanol presence. At higher ethanol concentrations both selectivity and yield decreased, which shows that ethanol addition could not be used to increase selectivity for protein hydrolysis into free hydrophobic amino acids.

Degree of hydrolysis of BSA at 0% ethanol was 80% (Figure 4.7b), suggesting that most of the proteins were completely hydrolysed. However, only 30% protein was liberated to free amino acids. This either suggests that the amount of free amino acids was underestimated, or some secondary hydrolysate products were formed e.g. pyroglutamic acid or diketopiperazine[39,40]. In the presence of both leucine aminopeptidase and

carboxypeptidase, terminal proline can form diketopiperazine instead of being liberated as free proline[39,41]. Indeed, we observed that the amount of free proline in the hydrolysate was very low. The presence of protease with proline-aminopeptidase activity, e.g. Peptidase R, may surmount diketopiperazine formation. This is consistent with the results for experiments with a combination of Pronase + Peptidase.

4.4 Discussion

There are three points where hydrophobic selectivity can be achieved, namely selection of starting material with high hydrophobic amino acids, selection of hydrolysis conditions, and separation of the final hydrolysate. The amounts of hydrophobic amino acids for the three substrates used in our experiments were 0.35, 0.37, and 0.35 mol/mol-total amino acid for rubber seed protein concentrate, wheat gluten, and BSA, respectively. These values are higher than e.g. soybean, sunflower, and Jatropha seed press cake/meal and protein isolate, which have 0.30-0.33 mol-hydrophobic/mol-total amino acid, but close to rapeseed meal with 0.34 mol-hydrophobic/mol-total amino acid[42–46]. On the other hand, the hydrophobic fraction of wheat gluten is still lower than corn gluten meal that has 0.43 mol-hydrophobic/mol-total amino acid[47].

Our results show that the amount of free hydrophobic amino acids in hydrolysate relative to the total free amino acids was influenced by the extent of hydrolysis and protease selection. Prolonged incubation increased the overall free amino acid yield, but decreased the selectivity towards hydrophobic amino acids. In our experiments, the highest selectivity towards hydrophobic amino acids was obtained by combining Pronase and Peptidase R; selectivity of 0.6 mol/mol-total free amino acid was observed after 6 h hydrolysis of wheat gluten and 24 h hydrolysis of rubber seed proteins and BSA. Pronase has both endo- and exo-protease activity, and it also showed high hydrophobic selectivity without the presence of Peptidase R, an exo-protease. On the other hand, hydrolysis of potato pulp using combinations of Alcalase or Novo Pro-D as endo-protease and Flavourzyme or Corolase LAP as exo-protease showed higher hydrophobic selectivity of Corolase, regardless of the endo-protease[35]. Experiments with Pronase without Peptidase R addition also showed the possibility of secondary products formation. Therefore in order to achieve high hydrophobic selectivity, selection of the appropriate exo-protease is crucial. Based on our results and on potato pulp hydrolysis results from literature[35], we conclude that combination of Pronase and Corolase LAP may yield hydrolysates with high hydrophobic selectivity.

Both rubber seed proteins and BSA were still soluble at 10% ethanol; this property was hypothesised to be important during hydrolysis. Indeed, around 50% degree of hydrolysis could still be obtained. On the other hand, results of β-casein and β-lactoglobulin hydrolysis suggest that protein structure is more important as proteins with different structures follow different denaturation patterns[19]. Both β-casein and β-lactoglobulin are fairly soluble in 0-30% (v/v) ethanol. However, while β-casein was readily hydrolysed by pepsin at 0-10% ethanol and less hydrolysis was observed at 20% ethanol or higher, β-lactoglobulin hydrolysis by pepsin only occurred at ethanol concentration of 20% or higher. Pepsin has specificity towards aromatic and hydrophobic amino acids, and it was proposed that these amino acids were located in the interior of β-lactoglobulin and were only exposed to pepsin in the presence of ethanol. In contrast, β-casein has an unordered structure and potential cleavage sites were already exposed without denaturation. To optimise hydrolysis and increase selectivity, investigation of the denaturation pattern in the presence of protease, ethanol, and/or other denaturing agents can be of importance.

The use of ethanol did not influence hydrophobic selectivity, except for BSA at 10% ethanol. A decrease of Pronase activity was observed at 10% ethanol and higher. Still, based on the degree of hydrolysis we saw that peptides were formed. Their profile might be influenced by ethanol addition, however we did not identify the peptides and therefore no conclusion can be drawn. Higher hydrophobic selectivity might be achieved by using proteases that can maintain their activity in the presence of ethanol. Trypsin, α-chymotrypsin, subtilisin DY[18], and papain[48] still exhibit some hydrolytic activity in the ethanol concentration up to 70%. Our own preliminary experiments with papain (data not shown), however, showed that the degree of hydrolysis decreased with increasing ethanol concentration and the free amino acid yield was much lower than the yields achieved from proteases used in this experiment.

The use of protease for hydrolysis enables mild processing, thereby avoiding formation of unwanted compounds or even racemisation of amino acids, making the hydrolysates more suitable for food or feed application compared to chemical hydrolysates. We have shown that 50% degree of hydrolysis from our substrates could be obtained within 24 h, indicating the hydrolysate comprised of short-chained peptides and free amino acids. Hydrolysate with high fraction of hydrophobic amino acids may taste bitter; valine, leucine, isoleucine, phenylalanine, tyrosine are some amino acids that are considered have bitter taste[49]. However, bitterness is also influenced by peptide length; free amino acids and di- and tri-peptides are less bitter than peptides with longer chain[50,51]. The final hydrolysate profile can be modified by adjusting hydrolysis time.

Rubber seed protein concentrate and wheat gluten had protein contents of 48% and 74%, respectively. As representative of agro-industrial residues, the results from these

substrates were comparable to BSA that was used in its purified form. This illustrates that protease can be applied for hydrolysis of proteins from (impure) agro-industrial residues to obtain free amino acids. Within a biorefinery framework, the next step after hydrolysis by protease would be the separation of the peptides and free amino acids from the hydrolysate mixture. The peptides and essential amino acids can be used for food or feed applications, while the non-essential amino acids can be used for bulk chemicals production. At this separation stage, hydrophobic selectivity can also be achieved, and this will be the topic for a follow-up article.

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Chapter 5

Precipitation of amino acids from agro-industrial residues

* This chapter was submitted as: Widyarani; Bowden, N. A.; Kolfschoten, R. C.; Sanders, J. P. M.; Bruins, M. E. Precipitation of amino acids from agro-industrial residues.

Abstract

Amino acids are important in human and animal diet, as well as being potential feedstocks for chemical production. Amino acids can be obtained from protein after hydrolysis. In addition, several agro-industrial residues already contain a mixture of free amino acids. The objective of this study was to develop a method for amino acids separation, starting from mixtures containing amino acids, and using anti-solvent precipitation with ethanol. Protamylasse™, rubber seed protein hydrolysates, and grass juice were used in the experiments, representing existing and potential agro-industrial residues. Our results show that in a water-ethanol system, some amino acids had lower solubility in mixtures than as a single component, thereby facilitating precipitation. A sufficiently high total amino acid concentration in the mixture is needed to achieve precipitation, therefore a concentration step is sometimes required. Ethanol precipitation can be applied as a pre-treatment to separate mixtures into groups of amino acids or a polishing step to increase purity.

Keywords: *Amino acid, hydrophobic side chains, Protamylasse™, rubber seed protein hydrolysate, grass juice, ethanol, anti-solvent*

5.1 Introduction

Value-added products can be obtained from proteinaceous fractions in a biorefinery framework. Native proteins, functional peptides, and essential amino acids can be used for food or feed applications. Their presence is often limiting and therefore determines the quality and price. Amino acids also have functionalities that already resemble traditional chemical products, therefore they are interesting as intermediate building blocks for nitrogen-containing chemicals[1].

Amino acids can be produced by hydrolysis of proteins from biofuel residues, e.g. oil seed press cake or dried distiller grains with solubles[2,3]. This assures feedstock availability as biofuel production is also increasing[4]. In addition, several agro-industrial residues already contain a mixture of free amino acids. Examples of these agro-industrial residues include vinasse from sugar cane and sugar beet refinery[5], and Protamylasse™ that is the concentrated potato juice from potato-starch production[6]. However, since the amino acids from hydrolysis or in agro-industrial residues are present as a mixture containing multiple amino acids, a separation process is required.

Separation of single amino acids from a mixture can be performed by crystallisation[7,8], reactive extraction[9], chromatography[10,11], and electrodialysis[12]. Combination of two or more of these methods is often needed to get pure compounds. These methods are mostly applied on fermentation broth that has a high concentration of one or two amino acids[13,14]. Amino acid crystallisation is one of the most applied processes in industrial amino acid production through fermentation. One of the notable amino acid fermentation products is monosodium glutamate[8]. Chromatography has been applied for separation of phenylalanine from impurities, most notably tyrosine[10,11]. Enzymatic reaction or thermo-chemical treatment may alter specific amino acid properties to aid its separation from a mixture[15].

Crystallisation can be performed by water removal, whether or not combined with pH shifting or the addition of an anti-solvent. The solubility of individual amino acids is the most important parameter in crystallisation[16]. When using hydrolysates as amino acid source, one of the challenges is the aqueous system the amino acids are present in. These aqueous systems are often at a much lower concentration than the maximum solubility of the amino acids.

Table 5.1 Changes of amino acid solubility in a mixture containing two amino acids in water[a].

I = Amino acid that was added first at varying concentrations below its saturation point.

II = Amino acid that was added to the solution containing amino acid (I) at amounts exceeding its saturation point.

+ Solubility of amino acid (II) increased when concentration of amino acid (I) in the initial solution increased.

- Solubility of amino acid (II) decreased when concentration of amino acid (I) in the initial solution increased.

= Solubility of amino acid (II) did not change when concentration of amino acid (I) in the initial solution increased.

	I	Polar				Hydrophobic				Special		
II		Asn	Ser	Asp	Glu	Leu	Val	Phe	Tyr	Ala	Gly	Cys2[b]
Polar	Asn									+	+	
	Ser			+	+					+		
	Asp		+		+						+	
	Glu		+	+							+	
Hydrophobic	Leu						=/-[c]		=	-	-	=
	Val					+/=[c]				-		
	Phe										+-[d]	
	Tyr					=					+	+
Special	Ala		+			-	-					
	Gly			+	+	=		+	=			=
	Cys2[b]					+	=		+	+	-/+[e]	

[a] Data compiled from Carta[17], Cohn *et al.*[18], Grosse Daldrup *et al.*[19], Jin and Chao[20], Kuramochi *et al.*[21], Kurosawa *et al.*[22], Soto *et al.*[23] for l- isomers at 25°C, except for Ala/Val, Ala/Ser, Asp/Gly, Phe/Gly systems (dl- isomers), Ala/Asn, Ala/Cys2, Val/Cys systems (dl-Ala, dl-Val), Ala/Leu system (30°C), and Glu(I)/Gly(II) system (60°C). For amino acids abbreviations see Table 5.2.

[b] L-cystine; [c] Result at 25°C/result at 30°C; [d] Increase then decrease; [e] Result from Carta[17]/result from Cohn *et al.*[18]

Amino acid solubility also changes in the presence of mixtures of other amino acids, and the mechanisms are still not fully understood[17,22]. The results from several studies of interactions between two amino acids in solution are presented in Table 5.1. In only water, the presence of a polar amino acid seems to increase the solubility of the other polar amino acids, alanine, glycine, and cystine. On the other hand, the influence of hydrophobic amino acid is not as clear. The presence of hydrophobic amino acid does not seem to influence other hydrophobic amino acid solubility, except for the increase of valine solubility in the presence of leucine[22]. The presence of tyrosine and leucine also does not influence glycine solubility[17], while the presence of phenylalanine increases glycine solubility[23]. Alanine solubility decreases in the presence of leucine or valine and vice versa[19,21].

The other alternative for crystallisation is using ethanol as an anti-solvent. In water-ethanol systems, the solubility of individual amino acids decreases at different selectivity[24–26]. These differences can be used to separate groups of amino acids. For instance when soybean or fish protein hydrolysates were mixed with aqueous ethanol followed by centrifugation, the relative amount of hydrophobic amino acids in the solution increased with increasing ethanol concentration[27]. The simultaneous influence of ethanol addition and interactions between amino acids, therefore, will determine the final solubility in the mixture.

The objective of this study was to develop an energy-efficient method for amino acid separation from aqueous system containing amino acid mixtures. The effectiveness of ethanol as an anti-solvent was investigated. Experiments were performed using Protamylasse™, hydrolysate of rubber seed protein, and grass juice. Protamylasse™ contains 150-180 g of proteins, peptides, and free amino acids per litre. Asparagine is notably abundant[6,28]. Interest is growing for using rubber seeds for oil and protein production[29,30]. Rubber seed proteins (RSP) contain high amounts of aspartic acid, glutamic acid, arginine, valine, and leucine. Hydrolysis of these proteins with proteases, however, results in mixtures with different free amino acid composition that can be steered to produce mixtures that are more rich in hydrophobic amino acids[31]. Grass juice is the liquid product after grass pressing and protein precipitation. The liquid still contains a mixture of amino acids that are interesting to use for further valorisation.

5.2 Materials and Methods

5.2.1 Materials

Protamylasse™ is a residue from potato starch production. Potato juice is obtained after separation of the starch and fibre from the potatoes. Part of the proteins from the potato juice is separated via steam coagulation. The remaining liquid fraction is concentrated via water evaporation to obtain Protamylasse™. Protamylasse™ (51% dry matter) for the experiments was obtained from AVEBE, the Netherlands.

Rubber seed protein (RSP) hydrolysates were obtained after hydrolysis of RSP concentrate using protease and dilute acid[3,31]. Protein concentrate was immersed in water at 5% w-protein/w-water and 0.01 M Britton-Robinson buffer was used to keep the pH at 7. Pronase and Peptidase R (1% w-protease/w-protein each) were added at t = 0.5 h and t = 2 h, respectively, and total incubation time was 24.5 h. To stop protease activity, the mixture was incubated at 90°C for 10 min. Part of the mixture was centrifuged at 3000x*g*, 4°C, 20 min, the supernatant was removed and filtered through Schleicher and Schuell filter No. 604 to get RSP enzymatic hydrolysate. Another part of the mixture that had not been centrifuged was mixed with 6 M HCl at a ratio of 5 : 1 (w-mixture/w-acid) and incubated at 95°C for 48 h to further hydrolyse the mixture. After the incubation, this mixture was let cool until room temperature and the pH was neutralised using 6 M and 0.1 M NaOH; the final pH was 7.6. The mixture was filtered through Schleicher and Schuell filter No. 604 to get RSP combined hydrolysate. The latter shows a broader and extended amino acid pattern compared to the RSP enzymatic hydrolysate, and the glutamine and asparagine have been converted to glutamic acid and aspartic acid due to the high acid concentrations.

Grass juice is the residue from protein production from grass. Grass is ground, pulped, and extruded to separate the protein-rich liquid fraction from the fibre-rich solid fraction. Protein in the liquid fraction is separated via steam coagulation or isoelectric precipitation. The amino acids are not separated during this process and remain in the liquid (grass juice). Grass juice for the experiments was obtained from Grassa BV, the Netherlands.

Chemicals used in the experiments and analyses were of analytical grade. Pronase was obtained from Roche Diagnostics (Germany). Peptidase R was obtained from Amano (Japan).

5.2.2 Precipitation with fixed starting material concentration and varying ethanol concentration

Prior to the experiment, RSP enzymatic hydrolysate was concentrated in a rotary evaporator to a concentration of 410 µmol/g free amino acids. Protamylasse™ or concentrated hydrolysate was added into empty tubes at a fixed amount equivalent to 5% w/w in the starting mixture before precipitation. Milli-Q water was added at decreasing amounts to each tube. Subsequently, ethanol was added at increasing amounts to obtain mixtures with 0-95% w/w ethanol concentration. The tubes were mixed by vortex for 10 s, then immediately centrifuged at 7000x*g*, 20°C for 5 min. The supernatant was transferred into empty containers using graduated pipettes, and stored at -18°C until analysed.

5.2.3 Precipitation with fixed ethanol concentration and varying starting material concentration

Protamylasse™ was added into empty tubes at amounts equivalent to 5-40% w/w in the starting mixture before precipitation. Milli-Q water was added at decreasing amounts to make the total water fraction of 40% w/w. Subsequently, ethanol was added to obtain mixtures with 60% w/w ethanol concentration. The tubes were mixed by vortex for 10 s, then immediately centrifuged at 7000x*g*, 20°C for 5 min. The supernatant was transferred into empty containers using graduated pipettes, and stored at -18°C until analysed.

5.2.4 Precipitation with both ethanol and starting material concentrations varied

Prior to experiment, Protamylasse™ was diluted with Milli-Q water to a concentration of 213 µmol/g free amino acids. Grass juice was concentrated with rotary evaporator to a concentration of 225 µmol/g free amino acid. RSP hydrolysates were used without any pre-treatment.

Diluted Protamylasse™, RSP hydrolysate, or concentrated grass juice was added into empty tubes at decreasing amounts. Subsequently, ethanol was added at increasing amounts to each tube to obtain starting mixtures with 10-95% w/w ethanol concentration. The tubes were centrifuged at 7000x*g*, 20°C for 5 min. The supernatant was transferred into empty containers using graduated pipettes, and stored at -18°C until analysed.

5.2.5 Amino acid analysis

Prior to measurement, frozen samples were thawed at room temperature and mixed. To measure free amino acid contents, the starting materials and supernatants from the experiments were dissolved in methanol and filtered through 0.2 μm Minisart filter. Norleucine (0.04 mM) was used as the internal standard. The filtered solutions were loaded onto Ultra-HPLC Dionex RSLC (Dionex Corporation, USA), and detections were performed at 263 nm and 338 nm[32].

An amino acid was considered to be precipitated when the concentration in the supernatant was lower than in the starting mixture at $p < 0.05$.

5.3 Results and Discussions

5.3.1 Starting material properties

Free amino acid composition of the starting materials is presented in Table 5.2.

Protamylasse™ contained 142 g of sugars, 226 g of ash[28], 113 g of proteins/peptides, and 174 g of organic acids per litre (own measurement). Citric acid (94 g/l) was the most abundant organic acid. Malic acid, lactic acid, and acetic acid concentrations were 24 g/l, 29 g/l, and 27 g/l, respectively. Protamylasse™ also contained 8 g of γ-aminobutyric acid (GABA) per litre.

Next to free amino acids, RSP enzymatic and combined hydrolysates also contained 10 g and 29 g of proteins/peptides per litre, respectively. GABA concentrations were less than 0.1 g/l. Sugars and lipid contents were not determined in this study, but it was assumed that they were present in (partially) hydrolysed forms.

Grass juice contained 3 g of sugars, 12 g of ash[34], and 45 g of organic acids per litre (own measurement). Malic acid (19 g/l) and lactic acid (25 g/l) were the most abundant organic acids. Grass juice also contains 0.9 g/l of GABA.

Table 5.2 Free amino acid composition of the starting materials.

Amino acid	Abbre-viations	pI[a]	Concentration in starting material (µmol/g)			
			Protamy-lasse™ (pH = 5.8)	RSP enzymatic hydrolysate (pH = 5.7)	RSP combined hydrolysate (pH = 7.6)	Grass juice (pH = 6)
Polar uncharged:						
Asparagine	Asn	5.4	135 ± 3	3 ± 0	0 ± 0	0.9 ± 0.1
Glutamine	Gln	5.7	0 ± 0	6 ± 0	0 ± 0	0.4 ± 0.0
Serine	Ser	5.7	13 ± 0	4 ± 0	11 ± 0	2.4 ± 0.0
Threonine	Thr	5.6	7 ± 3	3 ± 0	6 ± 0	1.6 ± 0.1
Negative:						
Aspartic acid	Asp	2.8	69 ± 2	3 ± 0	29 ± 0	5.0 ± 0.0
Glutamic acid	Glu	3.2	31 ± 1	6 ± 0	28 ± 0	2.5 ± 0.0
Positive:						
Arginine	Arg	10.8	21 ± 0	1 ± 0	5 ± 0	1.1 ± 0.0
Histidine	His	7.6	3 ± 0	2 ± 0	3 ± 0	0.2 ± 0.0
Lysine	Lys	9.7	10 ± 1	3 ± 0	4 ± 0	1.3 ± 0.1
Hydrophobic:						
Isoleucine	Ile	6.0	6 ± 0	7 ± 0	6 ± 0	1.3 ± 0.0
Leucine	Leu	6.0	5 ± 0	17 ± 0	15 ± 0	2.4 ± 0.0
Valine	Val	6.0	17 ± 0	21 ± 0	18 ± 0	2.3 ± 0.1
Phenylalanine	Phe	5.5	6 ± 0	7 ± 0	7 ± 0	1.2 ± 0.1
Tryptophan	Trp	5.9	0 ± 0	2 ± 0	0 ± 0	0.2 ± 0.0
Tyrosine	Tyr	5.7	5 ± 0	0 ± 0	1 ± 0	0.7 ± 0.0
Methionine	Met	5.7	2 ± 0	2 ± 0	3 ± 0	0.5 ± 0.0
Proline	Pro	6.3	5 ± 1	5 ± 0	9 ± 0	1.1 ± 0.0
Special:						
Alanine	Ala	6.0	30 ± 1	9 ± 0	16 ± 0	5.4 ± 0.1
Glycine	Gly	6.0	4 ± 0	3 ± 0	18 ± 0	1.5 ± 0.1
Cysteine	Cys	5.1	0 ± 0	0 ± 0	2 ± 0	0.0 ± 0.0
Total (µmol/g)			368 ± 8	104 ± 0	181 ± 0	32 ± 1
Total (g/l)			48 ± 1	13 ± 0	23 ± 0	4 ± 0

[a] Isoelectric point at 25°C[33]

5.3.2 Amino acid precipitation at fixed starting material concentration: Replacing water with ethanol

For experiments at fixed starting material concentration, the starting mixture contained 5% w/w starting material and water-ethanol at various concentrations. Figure 5.1 shows that overall, RSP enzymatic hydrolysate and Protamylasse™ were completely soluble up to 50% and 60% ethanol, respectively. However, different amino acids precipitated at different ethanol concentrations. The amino acid fractionation between the supernatant and the precipitate were also different. The concentration of an amino acid in each supernatant was related to both solubility and initial concentration in the mixture. In complex mixtures such as Protamylasse™ and RSP hydrolysate, the maximum solubility of individual amino acids was different from their solubility in mixtures that only contain one amino acid.

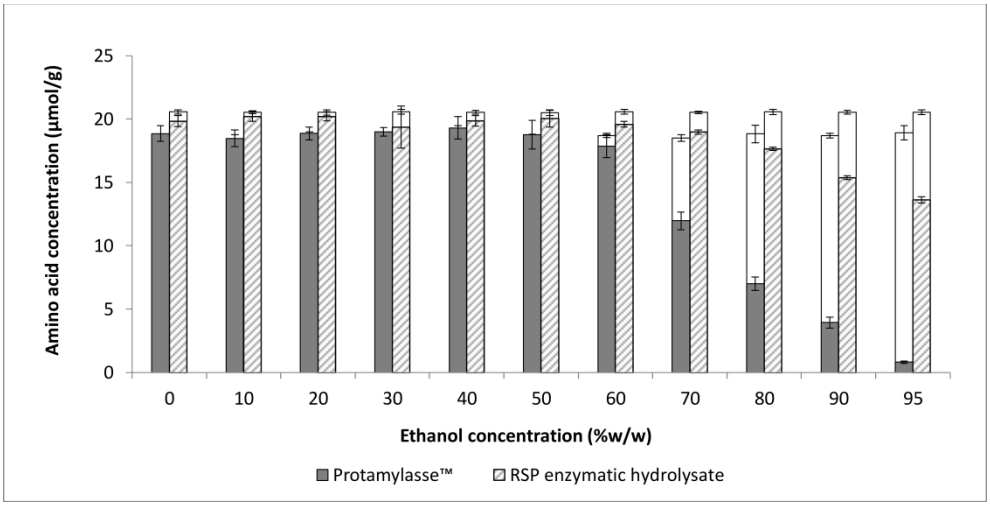

Figure 5.1 Total amino acid concentrations in the starting mixtures (unfilled bars) and supernatants from Protamylasse™ and RSP enzymatic hydrolysate at different ethanol concentrations, from experiments with fixed starting material concentration and varying ethanol concentration.

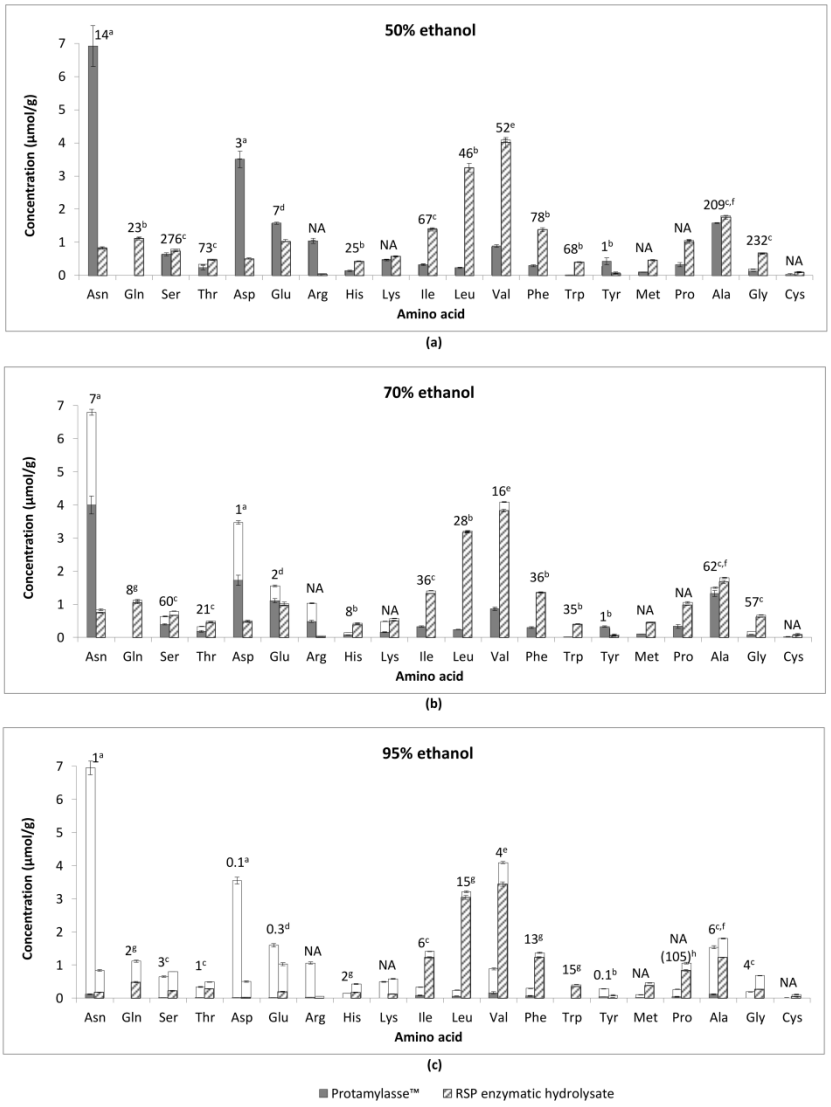

Figure 5.2 Amino acid concentrations in the starting mixtures (unfilled bars) and supernatants of Protamylasse™ and RSP enzymatic hydrolysate at 50% (a), 70% (b), and 95% (c) ethanol, from experiments with fixed starting material concentration and varying ethanol concentration. Numbers above bars indicate maximum solubility of l-isomer at 25°C, unless otherwise stated.
NA = data not available.
[a] Interpolated from McMeekin *et al.*[35]; [b] Interpolated from Nozaki and Tanford[26]; [c] Interpolated from Ferreira *et al.*[24,25]; [d] Interpolated from Dunn and Ross[36]; [e] Interpolated from Zhang *et al.*[37,38], maximum solubility at 20°C; [f] Data for dl-alanine; [g] Extrapolated from Nozaki and Tanford[26]; [h] Data not available. Number between brackets show maximum solubility in 100% ethanol at 19°C[39].

Figure 5.2a, 5.2b, and 5.2c show the amino acid concentrations in the starting mixture and supernatant at 50%, 70%, and 95% ethanol, respectively. Amino acid concentrations at other ethanol concentrations are presented in the Appendix. The concentrations were compared to individual amino acid's solubility from literature, as shown in Figure 5.2. As biobased sources, amino acids in Protamylasse™ and RSP enzymatic hydrolysate were most likely present as l-isomers. Consistent data on maximum solubility in aqueous ethanol is scarce because most studies only focused on a few amino acids. Therefore, the data has been compiled from several sources. Data for methionine, arginine, lysine, and cysteine are not available.

Almost all amino acids in our experiments precipitated at concentrations below their maximum solubility as single amino acids (Figure 5.2; Table A.1-A.4). The exception was aspartic acid from Protamylasse™ that at 50% ethanol already exceeded its maximum solubility based on data for the single amino acid (Figure 5.2a), but only precipitated at 70% ethanol and higher (Figure 5.2b). Table 5.1 indicates that the solubility of some amino acids increases in each other's presence, particularly for polar amino acids. The decrease we observed, therefore, might be due to different interaction patterns between amino acids in the presence of ethanol or due to the presence of non-amino acid components.

At 70% ethanol, all polar amino acids from Protamylasse™ started to precipitate while the hydrophobic ones stayed in the solution (Figure 5.2b). The combined aspartic acid/ asparagine fraction increased from 56% (mol/mol) in the starting material to 69% in the precipitate. The combined aspartic acid/asparagine fraction in the precipitate decreased at higher ethanol concentration as more amino acids precipitated, and was as low as 57% again at 95% ethanol where practically all amino acids precipitated (Figure 5.2c).

At 70% ethanol, all amino acids from RSP enzymatic hydrolysate precipitated except aspartic acid, glutamic acid, tyrosine, tryptophan, phenylalanine, and leucine (Figure 5.2b). The precipitated amino acids were 6-18% of the amount in the starting material except for arginine (58%) and cysteine (32%); both were present at low concentrations and therefore uncertainty in measurements was high. At 95% ethanol, however, 79-95% hydrophobic amino acids were still present in the supernatant (Figure 5.2c), except tyrosine that has lower solubility than the other amino acids.

The relative abundance of hydrophobic amino acids in the supernatant of RSP enzymatic hydrolysate suggests that at high ethanol concentrations, interactions between hydrophobic amino acids resulted in the increase of overall solubility of hydrophobic amino acids, which is similar to the influence of polar amino acids interactions in water-only solution (Table 5.1). However, this was not observed in Protamylasse™ at 95%. RSP enzymatic hydrolysate had 59% (mol/mol) hydrophobic amino acids in the starting

material, much higher than Protamylasse™ that only had 13%. This suggests that the increase of hydrophobic amino acids solubility in high ethanol concentration may only occur in starting materials with a sufficiently high fraction of hydrophobic amino acids.

5.3.3 Influence of starting material concentration

When maximum solubility is the sole factor that determines the amount of amino acids that remain in the supernatant, it is expected that the amino acids concentration in the supernatant is constant regardless of the concentration in the starting mixture. Therefore, we performed an experiment with different starting material concentrations in a fixed ethanol concentration at which changes would be most measurable. At 50% ethanol, all amino acids from Protamylasse™ were still present in the supernatant (Figure 5.2a), while at 70% ethanol, some amino acids already precipitated but some were still present in the supernatant (Figure 5.2b). This shows that the 60% ethanol concentration was the most sensitive to changes.

When precipitation occurred at 60% ethanol, the total amino acid concentrations from Protamylasse™ in supernatants were always around 27 ± 2 µmol/g as expected. The change was relatively small compared to the increase in Protamylasse™ concentration (Figure 5.3a). For individual amino acids, this was not always the case. At the start not all amino acids were at maximum solubility yet. Also after precipitation, the relative composition changed and this influenced the solubility of individual amino acids in the water-ethanol mixture.

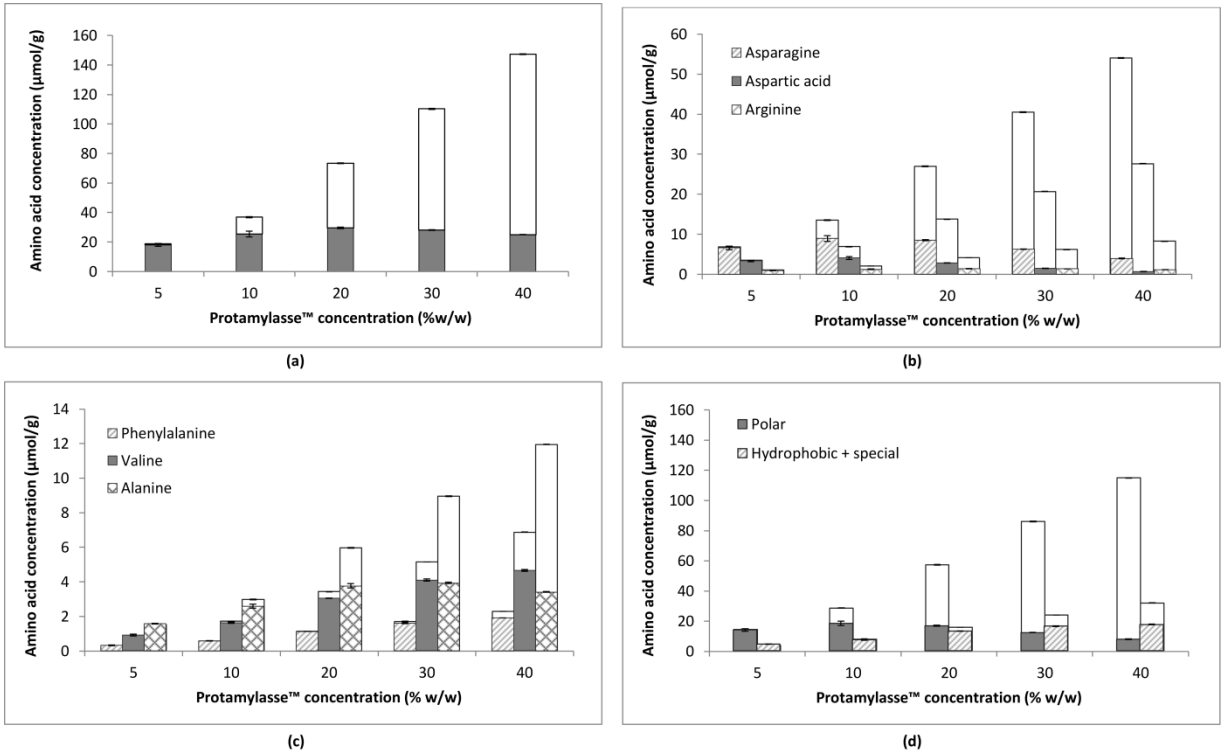

Figure 5.3 Total amino acid (a), selected polar amino acid (b), selected hydrophobic and special amino acid (c), and combined polar amino acids and hydrophobic and special amino acids (d) concentrations in the starting mixture (unfilled bars) and supernatant at 60% ethanol and different Protamylasse™ concentration.

Data for other amino acids are presented in Table A.5 and A.6 in the Appendix

5.3.3.1 Polar amino acids

Asparagine's maximum solubility when present as a single amino acid at 60% ethanol is 10 µmol/g[35]. At 5% Protamylasse™ concentration, asparagine was completely soluble in 60% ethanol (Figure 5.3b). Precipitation was observed at 10% Protamylasse™; the asparagine concentration in the supernatant was 9 ± 1 µmol/g, similar to the maximum solubility. At higher Protamylasse™ concentrations, however, the amino acid concentrations in the supernatant decreased despite more amino acids were present in the starting mixture.

Aspartic acid concentration in the supernatant at 60% ethanol and 10% Protamylasse™ was 4 µmol/g, higher than the maximum solubility as single amino acid (2 µmol/g[35]). At higher Protamylasse™ concentrations, the amino acid concentrations in the supernatant also decreased and were even lower than its maximum solubility at 30% and 40% Protamylasse™ (Figure 5.3b).

Other polar amino acids: arginine, lysine, histidine, glutamic acid, and serine also started to precipitate at 10% Protamylasse™. Precipitation occurred despite the maximum solubility was higher than the starting mixture concentration for some amino acids, e.g. glutamic acid and serine. Glycine, which is considered to be a non-polar amino acid, also showed similar pattern.

5.3.3.2 Hydrophobic amino acids

Maximum solubilities of phenylalanine and valine as single amino acid in 60% ethanol are 65 µmol/g and 84 µmol/g, respectively[26,37]. Figure 5.3c shows that phenylalanine and valine concentrations were lower than this maximum solubility. Precipitation occurred at 20% Protamylasse™ for valine and 30% Protamylasse™ for phenylalanine, higher than the required Protamylasse™ concentration for precipitation of polar amino acids. Even then, the supernatant concentration still increased at higher Protamylasse™ concentrations. Similar patterns were observed for other hydrophobic amino acids: proline, tyrosine, methionine, tryptophan, isoleucine, and leucine.

5.3.3.3 Alanine

Dl-alanine maximum solubility as a single amino acid in 60% ethanol is 105 µmol/g[25]; the solubility of l-alanine may be lower but the difference should not be more than one order of magnitude. Even though alanine concentrations in the starting materials were lower than their maximum solubility, alanine started to precipitate at 10% Protamylasse™

concentration (Figure 5.3c). With the increase of Protamylasse™ concentration, the concentration in the supernatant first increased and then decreased again when Protamylasse™ concentration increased further. The pattern was between that observed for the polar and hydrophobic amino acids, suggesting what would happen to hydrophobic amino acids if higher Protamylasse™ concentration can be applied.

5.3.3.4 Changes in composition as a result of higher starting material concentrations

With increasing Protamylasse™ concentration, the concentration of polar amino acids in the supernatant decreased, while the concentration of hydrophobic amino acids increased (Figure 5.3b-d). Consequently, amino acid composition in the supernatant changed. At 5% Protamylasse™, the supernatant consisted of 76% polar amino acids and 24% hydrophobic and special amino acids while at 40% Protamylasse™, the supernatant consisted of 30% polar amino acids and 70% hydrophobic and special amino acids. This again suggests the increase of overall solubility of hydrophobic amino acids in water-ethanol solution due to interactions between hydrophobic amino acids. However, as the influence of single amino acids was not measured independently, the solubility might also be influenced by the other components that also increased when more Protamylasse™ was present. This effect will be discussed in subchapter 5.3.5.

5.3.4 Precipitation by variation of ethanol and starting material concentrations: Ethanol addition

A more practical approach to anti-solvent separation is the simple addition of ethanol to the starting material. This will enable precipitation at lower ethanol concentration than the previous experiments since no water is added to the mixture. Figure 5.4a-d show that precipitation occurred for all starting materials, but precipitation started at different ethanol concentrations. The amino acid fractionation between the supernatant and the precipitate were also different.

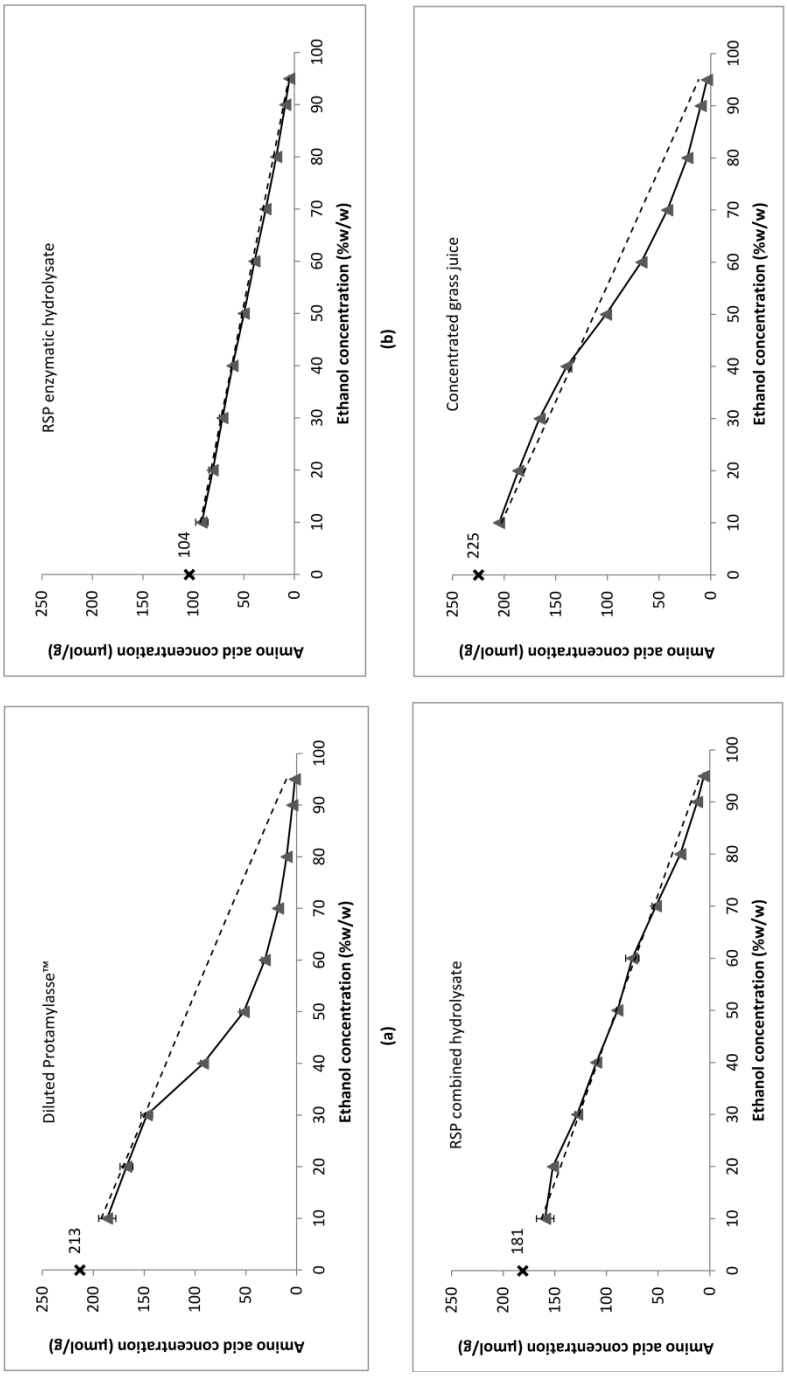

Figure 5.4 Amino acid concentration in the supernatant from diluted Protamylasse™ (a), RSP enzymatic hydrolysate (b), RSP combined hydrolysate (c), and concentrated grass juice (d). Dashed lines indicate amino acid concentrations in the starting mixtures.
x = Total amino acid concentration in the starting material before ethanol addition.

Protamylasse™ started to precipitate at 40% ethanol, and at this concentration already 28% of total amino acids in the starting mixture precipitated (Figure 5.4a). RSP enzymatic hydrolysate, on the other hand, only started to precipitate at 80% ethanol and only 19% of total amino acids in the starting mixture precipitated (Figure 5.4b). This is probably due to the low amino acid concentrations in the mixture. Protamylasse™ and grass juice had the highest and comparable total amino acid concentration; they had more precipitation compared to RSP hydrolysates (Figure 5.4a-d). This may suggest that there was a minimum total amino acid concentration that was required to achieve precipitation, and below this value, precipitation did not occur or occurred only at high ethanol concentration. This was the case with Protamylasse™, which is industrially concentrated potato juice that is more than ten times thicker than the original material. This was also illustrated in RSP enzymatic hydrolysate precipitation, which could precipitate at lower ethanol concentration (60%) when concentrated starting material was used (Figure 5.1). The minimum total amino acid requirement, however, may be different for different starting materials.

The total amino acid concentration of RSP combined hydrolysate was roughly 20% lower than Protamylasse™ and grass juice (Figure 5.4a, 5.4c, 5.4d). Unlike these two materials, RSP combined hydrolysate started to precipitate at much higher ethanol concentration (80%), similar to RSP enzymatic hydrolysate (Figure 5.4b, 5.4c). The large difference between precipitations of RSP combined hydrolysate and Protamylasse™ or grass juice suggests that there were influences of other factors, particularly starting material composition.

With increasing ethanol concentrations, the composition of the amino acid groups in the supernatant shifted from polar to hydrophobic amino acids. The shift was most apparent in Protamylasse™ (Figure 5.5a), which had the least hydrophobic amino acids in the starting material. Also the supernatant from grass juice showed an apparent shift and had even higher hydrophobic amino acids content at 90% and 95% ethanol compared to Protamylasse™ (Figure 5.5d). RSP enzymatic hydrolysate had relatively the most hydrophobic amino acids in the starting material, but the low mixture concentration resulted in only small changes in supernatant composition at higher ethanol concentration (Figure 5.5b). The low mixture concentration also influenced RSP combined hydrolysate (Figure 5.5c), where precipitation mostly occurred on aspartic and glutamic acid.

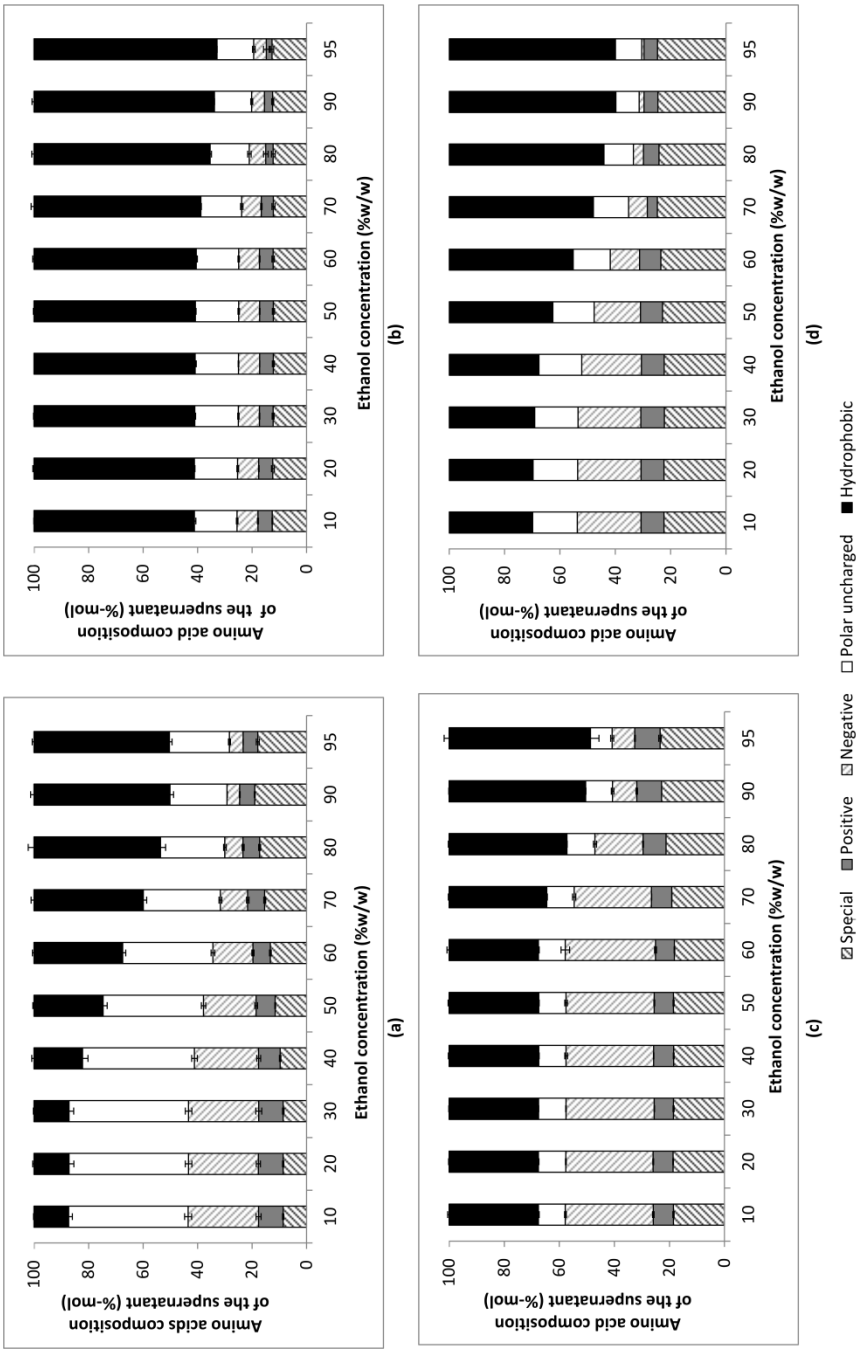

Figure 5.5 Distribution of special, positive, negative, polar uncharged, and hydrophobic amino acid groups in the supernatant after precipitation of diluted Protamylasse™ (a), RSP enzymatic hydrolysate (b), RSP combined hydrolysate (c), and concentrated grass juice (d) at different ethanol concentrations.

On a mole-fraction basis, grass juice contained more hydrophobic amino acids than Protamylasse™ (Table 5.2). This might be the reason the precipitation started at higher ethanol concentration, even though the starting mixture concentration was higher (Figure 5.4a, 5.4d). On the other hand, the absolute concentrations of hydrophobic amino acids were almost similar in the two RSP hydrolysates (Table 5.2), despite the different relative compositions due to the higher concentrations of aspartic and glutamic acid in the RSP combined hydrolysate (Figure 5.5b, 5.5c). The similarity of hydrophobic amino acids content might therefore have resulted in similar precipitation behaviour. Next to amino acids, the presence of non-amino acids component might also influence precipitation from these materials.

5.3.5 Influence of non-amino acid components in the mixture

Next to amino acids, the starting materials used in our experiments also contained other components including proteins/peptides, sugars, salts, organic acids, and lipids. Due to the setup of our experiments, the influence of non-amino acid components could not be measured directly. However, their possible influences were taken into consideration.

RSP hydrolysates contained proteins or peptides at roughly the same amount as free amino acids, while Protamylasse™ contained proteins/peptides at twice the amount of free amino acids. Ethanol may change the conformation of some proteins that results in the decrease of their solubility[40]. Proteins and peptides also can make insoluble complexes with phenolic compounds[29]. Since proteins/peptides were present at considerable amounts in our experiment, they might co-precipitate with amino acids[41], and amino acid precipitation thus occurred at lower concentration compared to the amino acid maximum solubility as single amino acid.

Compared to their total proteinogenic amino acid concentrations, Protamylasse™ and grass juice contain high concentrations of GABA. GABA is a non-proteinogenic amino acid that can be formed from conversion of glutamic acid. Unlike glutamic acid, GABA is positively charged and has a higher isoelectric point (7.2) and solubility in water[33], therefore should have a different precipitation behaviour compared to glutamic acid. However, we observed that GABA precipitated with similar pattern as glutamic acid and other polar amino acids.

Protamylasse™ and grass juice contain considerable amounts of organic acids. In experiments with varying ethanol and starting material concentrations where the concentrations of amino acids were both 28 g/l, the concentrations of organic acids were 110 g/l in Protamylasse™ and 350 g/l in grass juice. Organic acids are highly soluble in

water and ethanol[42–44]. They can be applied as the acid source for isoelectric precipitation of proteins[45]. The concentrations in our mixture, however, were not enough to achieve the pH where negatively-charged amino acids precipitate.

The high solubility of GABA and organic acids might respectively increase the concentrations of positive and negative ions in the mixtures. Both Protamylasse™ and grass juice also have high concentrations of potassium[28,34]. RSP concentrate was prepared by alkaline extraction followed by isoelectric precipitation; consequently salts were present at considerable amounts. Furthermore, the amino acids themselves contribute to the ionic strength of the mixtures. The presence of ions can change amino acids solubility, which is influenced by ionic type and strength. In water, salting-in effect was observed for neutral and hydrophobic amino acids, while salting-out effect was observed for positively-charged amino acids[46–48]. Ethanol is less polar than water, therefore the influence of ions might not be as evident. However, salting-in and salting-out with respect to ionic strength had been observed e.g. in n-octanol-water solutions[49].

Table 5.2 shows that all amino acids except polar charged amino acids have isoelectric points between 5.0 and 6.3[33], which were close to the pH of all starting materials except RSP combined hydrolysate that had a slightly higher pH (7.6). At pH 5, metal salts can form insoluble complex with amino acids, but the bonds are likely broken at pH 7[45]. Our experiments showed that polar uncharged and hydrophobic amino acids showed different precipitation patterns. Furthermore, both positively-charged amino acids (pI 7.5-10.8) and negatively-charged amino acids (pI 2.8-3.2) precipitated with similar pattern as polar uncharged amino acids. This suggests that for dilute mixtures at pH close to 7, hydrophobicity of the amino acid side chains, ethanol concentration, and starting material concentration had more influence than pH.

5.3.6 Applications

The results show that ethanol can be best applied in amino acid separation as either a pre-treatment to separate amino acid groups or a polishing step to increase purity. The parameters that need to be considered are the minimum amino acid concentration in the mixture, the ethanol concentration, and the mixture composition, especially the ratio between hydrophobic and polar amino acids. Furthermore, the presence of non-amino acid components also may influence the separation.

In our cases, a mixture with total amino acid concentration of 200 µmol/g or higher is needed to achieve precipitation at 50% ethanol. For mixtures with lower concentrations, a pre-treatment step to concentrate is necessary. This might however result in a viscous

mixture. For instance while the original potato juice only has 4% dry matter, Protamylasse™ in our experiment had 368 µmol/g amino acids and 51% dry matter. Based on visual observation, the material was very viscous. On the other hand, concentrated RSP enzymatic hydrolysate had 410 µmol/g amino acids and was still sufficiently fluid with only 23% dry matter. The high viscosity of Protamylasse™, therefore, was likely due to the abundant presence of other components like sugars. Viscosity reduction can be achieved by ethanol addition, which also increases diffusion rates. In practice, the mixture of the starting material and ethanol should have 15% dry matter or less to enable easy processing.

Amino acid composition determines the required ethanol concentrations for precipitation, and the window of operation may vary between starting materials. Two possible processing scenarios are proposed:

- For mixtures that are rich in polar amino acids, 50-70% ethanol can be applied to obtain a **precipitate** that is rich in polar amino acids. For the case of Protamylasse™, the combined fraction of aspartic acid and asparagine increased from 56% (mol/mol) in the starting material to 69% in the precipitate. Ethanol concentrations of 90% or higher should be avoided, as at these concentrations, hydrophobic amino acids may also precipitate.
- For mixture rich in hydrophobic amino acids, 90% ethanol or higher is required to increase the fraction of hydrophobic amino acid in the **liquid**. In these concentrations, most polar amino acids will precipitate. For the case of RSP enzymatic hydrolysate with prior concentrating step, hydrophobic amino acid fraction increased from 59% (mol/mol) in the starting material to 76% in the supernatant.

Alternatively, a two-step precipitation can be applied: Step 1 to precipitate most (>90%) polar amino acids, while a small amount of hydrophobic ones also precipitate. Step 2 can be used to further purify the precipitated polar amino acids, by re-solubilising the hydrophobic amino acids.

5.4 Conclusions

Ethanol can be applied in the fractionation of amino acids from protein hydrolysate and agro-industrial residues. In such complex mixtures, interactions between amino acids influence the solubility. Our results show that in a water-ethanol system, some amino acids have lower solubility in a mixture than as a single component, facilitating precipitation. Precipitation of polar amino acids mostly occurs at lower ethanol concentration compared to the hydrophobic ones. Meanwhile, interactions between

hydrophobic amino acids in ethanol presence may lead to the increase of overall solubility of hydrophobic amino acids.

Ethanol precipitation can be applied as a pre-treatment to separate mixtures into groups of amino acids or as a polishing step to increase purity. For dilute mixture at a pH close to 7, precipitation is determined by hydrophobicity of the amino acid side chains, ethanol concentration, and starting material composition. In the case of very dilute streams, a concentration step is required to get a sufficiently high mixture concentration to achieve precipitation.

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5.5 References

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Chapter 6

General Discussion

6.1 Introduction

The objective of this research was to design a process for the recovery of proteinaceous fractions from rubber tree for applications that are suitable for local use. In the introduction of this thesis, five research questions were formulated:

1. Which stream(s) can be used to obtain proteins from the rubber tree?
2. How to efficiently isolate proteins from rubber seed and its press cake?
3. Which method is most suitable in a biorefinery framework to obtain amino acids from the proteins?
4. Is it possible to separate amino acids in the protein hydrolysate?
5. What are the possible applications of the proteinaceous fractions for rural and industrial conditions? In particular, which application has the highest value in rural economies for the Indonesian case?

Research questions number 1-4 were discussed separately in previous chapters, and the conclusions are discussed further in subchapter 6.2. The last research question will be discussed in subchapter 6.3 and 6.4, in relation with the findings from chapters 2-5.

6.2 Thesis overview

6.2.1 Biomass selection

Rubber trees are mainly grown for their latex that can be processed into various rubber products. The latex processing, as well as the waste- and side-streams, is already well-defined. In chapter 2, streams with promising protein content were identified based on field visits, interviews, and literature. Protein isolation from latex stream poses two difficulties: dilute streams and attachment to rubber particles. Utilisation of protein in the latex is not economically feasible at this moment, but may be feasible when specific use of the latex protein(s) with high value can be identified.

Next to latex, the seeds and leaves have promising protein contents. It was estimated that annually, 21-144 kg-protein/ha can be obtained from seeds and 380 kg-protein/ha can be obtained from leaves (Figure 2.1). Commercial processes to obtain proteins from these parts are not yet available. Proposed processes to isolate proteins from the seeds and the leaves are presented in Figure 2.8 and Figure 2.9, respectively. In the following discussions, rubber seeds were selected as the model biomass since the protein extraction can be incorporated within a biorefinery concept that produces biodiesel as its main product (Figure 2.5).

6.2.2 Protein extraction

Rubber seed kernel contains 48-50% oil and 17% protein (nitrogen-to-protein conversion factor = 5.7). Prior to protein extraction, the oil can be separated either by pressing that yields press cakes (20-23% protein) as residue or solvent extraction that yields meals (24-29% protein) as residue.

Influence of three process parameters: pre-treatment, oil separation method, and protein extraction temperature on protein recovery were investigated in chapter 3. Using alkaline extraction, up to 80% protein from the total original amount of protein in the kernel could be recovered in the extract, comparable to protein recoveries from other oilseeds and oilseed cakes. Seed type and pre-treatment have the highest influence over protein recovery. Due to the high moisture content in the kernel, pre-drying is a necessary step before oil separation. Increasing pre-drying temperature from 60°C to 105°C tends to decrease protein recovery from press cakes and meals. This decrease in protein recovery may be attributed to protein denaturation at high temperature, resulting in protein coagulation and a decrease in solubility. Solubility decrease was also indicated by low protein recovery from meals, due to the use of high temperature and solvent for long period.

Increasing extraction temperature from 25°C to 60°C slightly increased protein recovery from the press cake (Figure 3.3). On the other hand, extending the extraction time from 1 h to 6 h did not have any influence. The influence of alkali source and concentration were not investigated. An optimisation process is still required for these parameters, particularly extraction temperature and alkali concentration. The extracted protein can be separated from the liquid via isoelectric precipitation; the optimum pH was between 4 and 5.

Oil and protein also can be extracted simultaneously during alkaline extraction of the full-fat kernel. Protein recovery from the kernel was comparable to protein recovery from the press cakes and higher than from the meal (Figure 3.1). The presence of oil in the material did not seem to hinder protein extraction. This is consistent with results from Jatropha kernel and press cake[1], but in contrast with the results from soybean and microalgae where extraction of full-fat materials had 15-44% lower yields compared to the de-oiled materials[2]. This suggests that not only biomass composition but also type of biomass influences protein recovery.

Simultaneous (combined) extraction can be an alternative method to obtain oil and protein from rubber seeds. However, the maximum oil recovery is only 50%, lower than solvent extraction and even pressing. Furthermore, around 80% of the oil is in the form of

creamy emulsion phase that prevent its application for e.g. biodiesel production. Even though the extraction itself may require less energy than separate processes, current methods to separate oil and protein from the emulsion[3,4]: freezing-thawing, pH adjustment, or enzymatic treatment requires additional energy or chemical input, or both. Alternatively, the emulsion can be used for other application without prior separation. Rubber seed oil contains 19% omega-3 fatty acids, and preparation of rubber seed oil emulsion for food supplement has been investigated[5]. Further investigation is needed to study the properties of emulsion from alkaline extraction for this application. The most important concerns are the emulsion stability and whether anti-nutritional factors, e.g. cyanide, are present in the emulsion.

6.2.3 Hydrolysis with protease

Enzymatic protein hydrolysis is a method that can be used to obtain amino acids from protein-rich materials. Amino acids can be applied in food and feed, or used in production of nitrogen-containing chemicals. For these applications, not only degree of hydrolysis, but also hydrolysis selectivity is important. Selectivity can be achieved by selection of starting material, selection of hydrolysis conditions, and separation of hydrolysate. In chapter 4, hydrolysis selectivity towards hydrophobic amino acids was investigated.

Experiments with rubber seed protein concentrate (48% protein) were performed using different combinations of proteases: Alcalase 2.4L FG was used at pH 8.5 and Validase FP Concentrate, Validase FP Concentrate + Peptidase R, and Pronase + Peptidase R at pH 7. After 24 h hydrolysis of rubber seed protein, comparable degrees of hydrolysis were observed for the three protease mixtures at pH 7 (Figure 4.3a), and 32-35% protein was recovered as free amino acids (Figure 4.3b). On the other hand, despite the higher solubility of rubber seed protein at pH 8.5, Alcalase 2.4L FG gave the lowest degree of hydrolysis.

Hydrophobic selectivity was defined as the amount (on molar-base) of free hydrophobic amino acids: phenylalanine, leucine, isoleucine, tyrosine, tryptophan, valine, methionine, and proline, relative to the total liberated free amino acids. The highest hydrophobic amino acid selectivity was obtained after hydrolysis with Pronase + Peptidase R (Figure 4.6b; Table 4.3). Selectivity increased from 0.35 mol-hydrophobic/mol-total amino acids in the starting material to 0.6 mol/mol in the hydrolysate after 24 h. Leucine, phenylalanine, and valine were the amino acids that contributed most to this selectivity.

Hydrolysis in ethanol was performed to establish ethanol influence on amino acids recovery and selectivity. Free amino acids recovery decreased from 41% in experiment

without ethanol to 28% in experiment at 10% (w/w) ethanol, indicating a decrease of protease activity. Furthermore, the use of ethanol did not influence hydrophobic selectivity during hydrolysis. Compared to experiment without ethanol, however, the use of ethanol yields a different hydrolysate profile that may be interesting to investigate further.

The course of hydrolysis in ethanol is determined by substrate type and protease selection. In the presence of ethanol, protein may undergo structural changes, exposing the interior amino acids to the proteases. This can be beneficial if amino acids that are matched with protease specificity are located inside the protein. This can also be combined with hydrolysis in water in two steps hydrolysis. The first step is protein dispersion and limited hydrolysis in ethanol to unfold the protein, and the second step is hydrolysis to free amino acids in water. Alternatively, the first step can be hydrolysis in water using endoproteases to form smaller peptides. In the second step, ethanol is added and the hydrolysis is continued with exoproteases to selectively yield specific free amino acids.

The results of experiments with rubber seed proteins were comparable to wheat gluten and BSA, suggesting this process can be applied in general for agricultural residues. Alternatively, protease can be applied for simultaneous protein extraction and hydrolysis of unprocessed residues, e.g. oilseeds press cakes instead of protein isolate/concentrate. This option presents some advantages e.g. fewer process steps and avoiding salt formation (from isoelectric precipitation). However, longer extraction time may be required and amino acid concentration in the final hydrolysate is lower than if starting material with higher protein concentration is used.

6.2.4 Amino acids separation

Protein hydrolysis results in a mixture containing multiple amino acids. A separation process is required to obtain pure amino acids, e.g. for bulk chemicals production. In chapter 5, ethanol was used as an anti-solvent for selective precipitation of amino acids. In a water-ethanol system, some amino acids in mixtures had lower solubility than as a single component, thereby facilitating precipitation. Ethanol (90% or higher) was able to selectively increase the hydrophobic amino acids content in rubber seed protein hydrolysate from 59% (mol/mol) in the starting material to 76% in the supernatant. Leucine and valine contributed most to this increase.

Ethanol application adds to the available toolbox to separate amino acids from a mixture. Separation of single amino acids from a mixture can be performed by using the following

methods: crystallisation, reactive extraction, chromatography, and electrodialysis. Combination of two or more of these methods is often needed to get pure compounds. In addition, integration between amino acids separation and preceding processes may increase efficiency of the overall process.

In complex mixtures such as protein hydrolysate or agricultural residues, other soluble compounds like sugars and salts are also present. Influence of these compounds should be taken into account. Salt presence in the mixture can be minimised by avoiding extraction/hydrolysis at highly acidic or highly alkaline pH. In the case of very dilute stream, a concentration step can be applied to get a sufficiently high mixture concentration to achieve precipitation.

Specific conversion of amino acids, for instance via enzymatic reaction or thermo-chemical treatment, may aid their separation from a mixture[6]. Part of this conversion can be integrated to, even aimed at during amino acids liberation from protein-containing biomass. For instance during enzymatic hydrolysis of wheat gluten, 10% glutamic acid can be recovered in the form of pyroglutamic acid that is highly soluble in water compared to glutamic acid or aspartic acid, the other negatively charged amino acid[7]. The difference in solubility can be exploited for separation of pyroglutamic acid in acidic pH, after which pyroglutamic acid can be hydrolysed with strong acid at high temperature into glutamic acid[8].

6.3 Techno-economic assessment of protein production from rubber seeds

Based on results from previous chapters, the following proteinaceous fractions can be obtained from the rubber seeds: (native) protein concentrate, protein hydrolysate, and amino acid-rich mixtures. This techno-economic assessment will focus on biorefinery of seed into oil and protein concentrate as this will be the most suitable for local processing and applications. The oil fraction can be directly used locally or further processed into biodiesel[9], while the protein fraction can be applied in animal feed. The aim of this discussion is to have a general idea of the feasibility of the process, therefore some calculations were simplified.

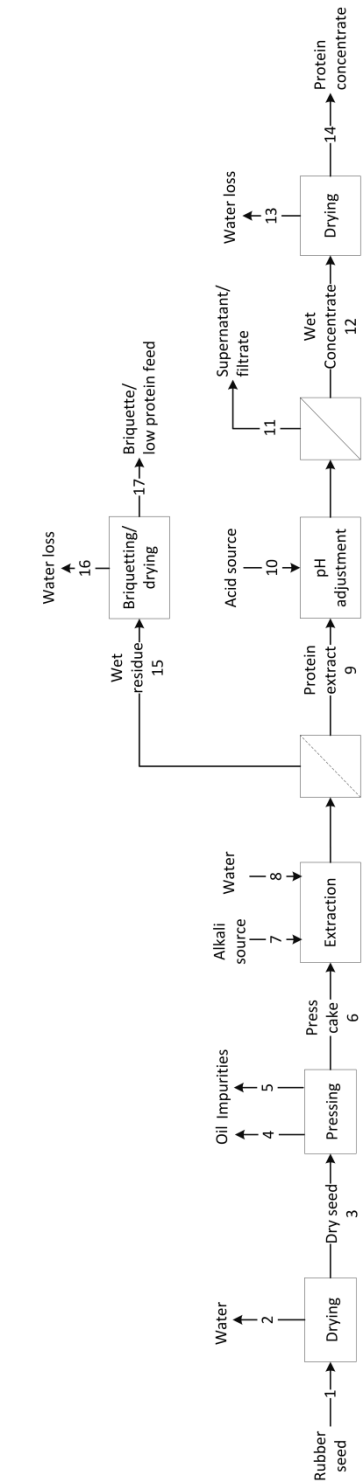

Figure 6.1 Proposed process flow diagram for rubber seed biorefinery. Main route: pressing of the whole seed followed by protein extraction.

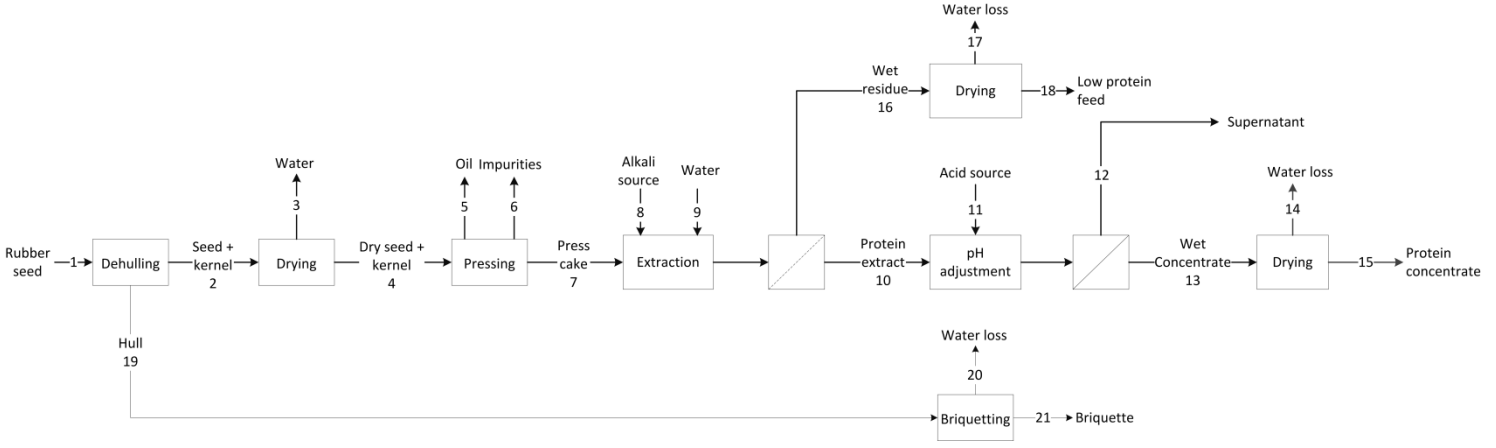

Figure 6.2 Proposed process flow diagram for rubber seed biorefinery. Alternative route-1: pressing of the dehulled seed followed by protein extraction.

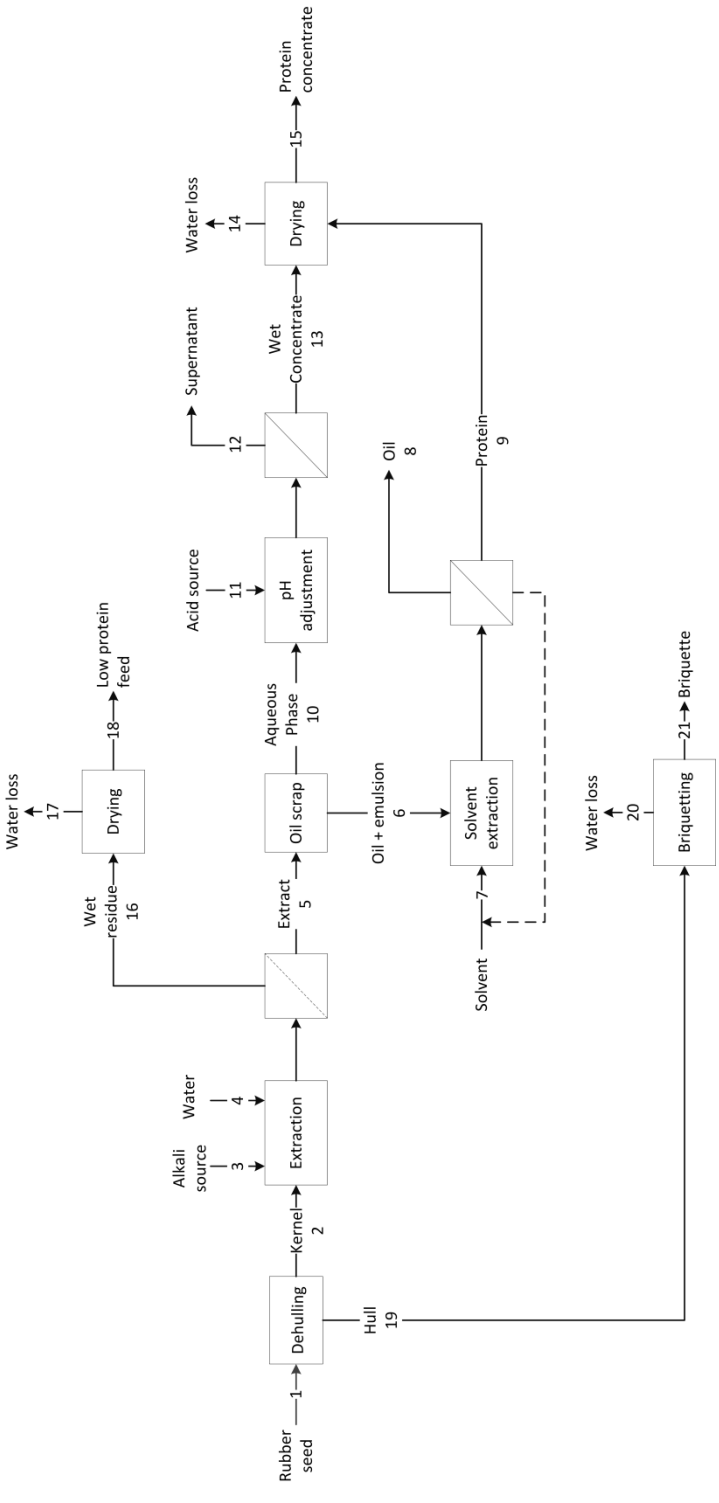

Figure 6.3 Proposed process flow diagram for rubber seed biorefinery. Alternative route-2: simultaneous oil and protein extraction

6.3.1 Material balance

The proposed biorefinery concept of rubber seed is presented in Figure 2.5 and the main processing route is presented in Figure 6.1. The whole (non-dehulled) seed can be pressed to get 203 kg-oil/tonne-seed and 3 kg-press cake/kg-oil with 15%-dw crude protein. Crude protein (nitrogen-to-protein conversion factor of 6.25) was used instead of actual protein (conversion factor of 5.7) to simplify comparison with other protein sources. To obtain the protein, the press cake can be extracted with alkaline solution. Belt or rotary drum filter can be used to separate the protein-containing extract from the residue. Isoelectric precipitation can be applied by adding acid to the extract, followed by centrifugation to separate the concentrate from the supernatant. NaOH and HCl can be used as alkali and acid sources, respectively.

As an alternative to separate the protein fraction by isoelectric precipitation and centrifugation, microfiltration can be applied. In this case, the required acid for pH adjustment may be less than with isoelectric precipitation. Most proteins can be retained with a 0.2 µm membrane, while water, salt, and small solubles can pass through. However, other compounds might be retained as well, lowering the overall purity.

As an alternative route (Figure 6.2), the seeds can be dehulled before pressing. Dehulling can result in a higher oil quality as fewer impurities are present in the oil. Separation of hulls increases the digestibility of the resulting press cake when used as animal feed[10,11]. Furthermore, the fibre-rich hulls can be used for other valuable product(s) or as additional energy source in the process. As friction is required to expel the oil from the seed matrices, a minimum presence of 8% hull is usually applied[11]. With mixture of 85% dehulled seed (kernel) and 15% whole seed, 11% hull is still present and the same oil recovery as from the whole seed can be expected. The amount of press cake was estimated to be 1.6 kg/kg-oil with 28% crude protein.

As previously discussed in Chapter 3, oil and protein can be extracted simultaneously from rubber seed kernel. The oil from this process is in the form of free oil and emulsion[12]. Solvent extraction can be applied after the aqueous extraction to separate the oil while the additional protein can be added to the protein concentrate (Figure 6.3).

To reduce processing cost, current alkali and acid sources can be substituted with cheaper ones, e.g. lime and sulphuric acid. These chemicals, however, have not been tested experimentally and their applicability needs further investigation.

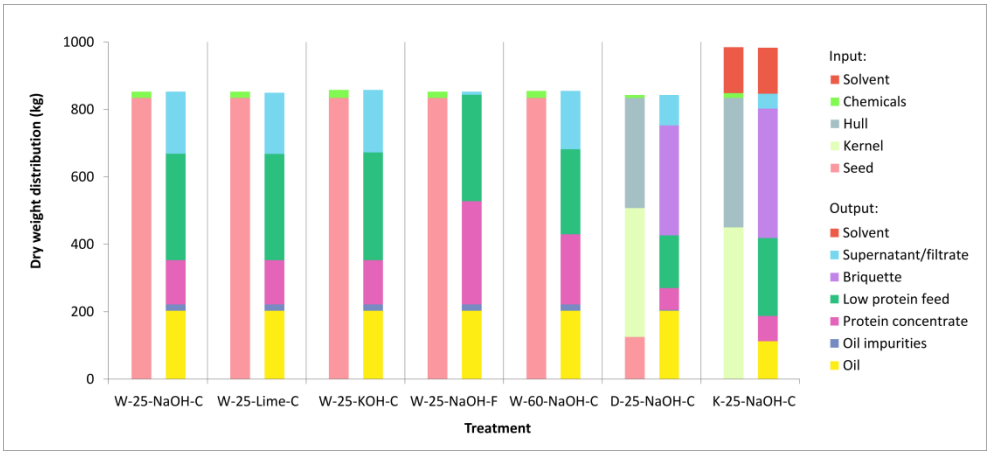

Figure 6.4 Distribution of dry weight in input (left side) and output (right side) from processing 1 tonne (wet) seed that corresponds to 834 kg dry material.
W = whole seed pressing; D = dehulled seed pressing; K = dehulled seed (kernel) extraction
(Pre-drying and dehulling are regarded as pre-treatment. For W and P, seed and kernel refer to input to the oil press. For K, kernel refers to input to extraction process. Hull refers to input to briquetting.)
25, 60 = protein extraction temperature (°C); NaOH, Lime, KOH = alkali source
C = Centrifugation; F = Microfiltration

Figure 6.4 shows the mass balance for different processing options based on the results of experiments with NaOH and HCl, assuming extraction is only influenced by the amount, and not the type, of the added alkali and acid. The more detailed mass balances for these processes are presented in Table B.1-B.5 in the Appendix.

As shown in Figure 6.4, chemicals substitution was not expected to change the output composition. On the other hand, with microfiltration more dry weight can be recovered in the protein concentrate compared to centrifugation. More dry weight in protein concentrate can also be achieved by increasing extraction temperature from 25°C to 60°C.

Protein content of the protein concentrate is determined by both the amount of protein recovered in the concentrate and the presence of non-protein component. All proteins can be recovered by using microfiltration, but most non-protein components are recovered as well. On the other hand by using acid precipitation, even though less protein can be recovered, less non-protein components in the product lead to a higher protein content compared to microfiltration (Table 6.1).

Table 6.1 Comparison of protein concentrates from different processes.

Route	Extraction temperature (°C)	Protein separation	Protein concentrate		
			Quantity[a] (kg/tonne-raw material)	Crude protein (%-dw)	Estimated price[b] (USD/tonne-product)
Pressing of the whole seed followed by protein extraction	25	Centrifugation	138	38	434
	25	Microfiltration	322	25	319
	60	Centrifugation	219	30	363
	60	Microfiltration	386	21	281
Pressing of dehulled seed followed by protein extraction	25	Centrifugation	68	69	716
	25	Microfiltration	159	45	505
	60	Centrifugation	108	54	585
	60	Microfiltration	191	38	436
Simultaneous oil and protein extraction	25	Centrifugation	78	62	655
	25	Microfiltration	118	62	655
	60	Centrifugation	105	46	515
	60	Microfiltration	167	44	490

[a] Protein concentrate with 95% dry weight.

[b] Price was estimated based on protein content[13].

When the seeds are dehulled, the resulting hulls can be processed into briquettes, an additional product. The quantity of protein concentrate is lower than without dehulling (Figure 6.4), however the protein contents are much higher (Table 6.1), because the fibre-rich hulls are separated.

6.3.2 Energy consumption

Figure 6.5 shows the energy consumption for processing 1 tonne raw material. Detailed calculations are presented in Table B.6-B.7 in the Appendix. Drying requires the highest energy consumption. Removing the hull reduces energy consumption significantly, as less material has to be processed in the subsequent steps. Increasing extraction temperature from 25°C to 60°C requires additional energy, but only slightly increases the overall energy requirement.

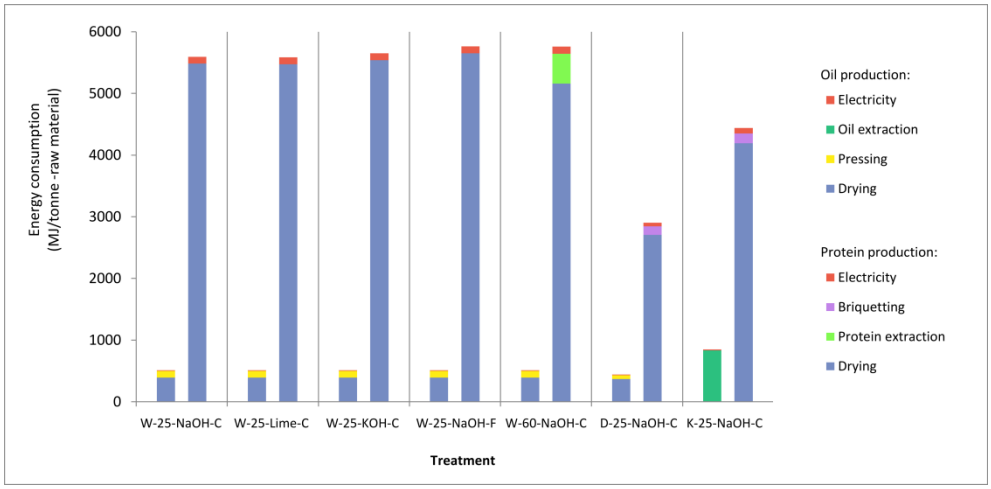

Figure 6.5 Energy consumption for processing 1 tonne seed for oil production (left side) and protein production (right side).
W = whole seed pressing; D = dehulled seed pressing; K = dehulled seed (kernel) extraction
25, 60 = protein extraction temperature (°C); NaOH, Lime, KOH = alkali source
C = Centrifugation; F = Microfiltration

The energy required to produce oil via pressing was estimated to be 507 MJ for whole-seeds and 440 MJ for dehulled seeds. The heating value of rubber seed oil is 37.5 GJ/tonne[14], therefore the produced energy in 203 kg pressed-oil was estimated to be

7594 MJ that equals to energy output/input ratio of 15-17. On the other hand, oil extraction requires 849 MJ, while only producing 113 kg oil (4219 MJ). This only equals to an energy output/input ratio of 5.

The energy required for protein extraction was estimated to be 9-14 MJ/kg-product; 89-98% was for drying. The energy requirement is equivalent to 59-83 MJ/kg-protein for pressed whole seed, 32-45 MJ/kg-protein pressed dehulled seed, and 45-70 MJ/kg-protein for simultaneous oil and protein extraction, indicating protein production from pressed dehulled seed (Figure 6.2) is relatively more energy-efficient. In order to increase energy efficiency, drying energy must be reduced significantly.

6.3.3 Preliminary comparison of different processing options based on economics

To select the process that has the highest feasibility for application, the different processing options were compared based on economics. In this preliminary comparison, only capital cost and processing cost were taken into account. Capital cost was estimated based on the most important equipment cost. When required, the exchange rate of USD 1 = EUR 0.92 was used. The calculation for the economics of different processing options are presented in Table B.8-B.10 in the Appendix, and summarised in Figure 6.6.

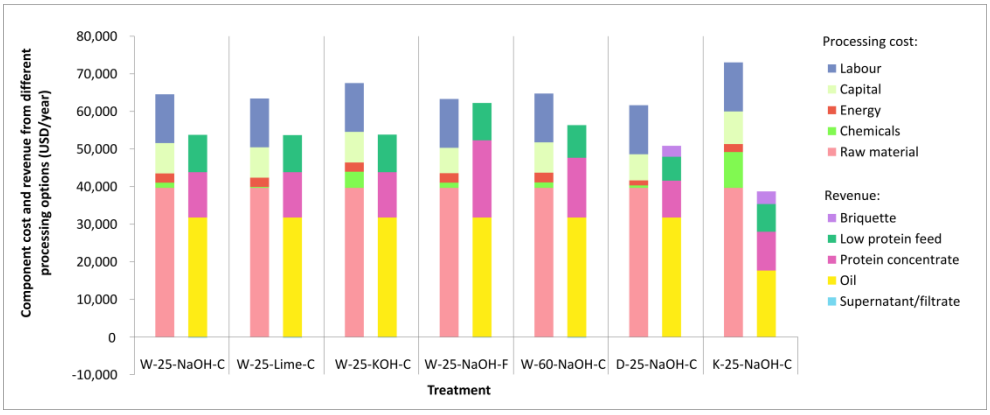

Figure 6.6 Component costs (left side) and revenue (right side) from different processing options.
W = whole seed pressing; D = dehulled seed pressing; K = dehulled seed (kernel) extraction
25, 60 = protein extraction temperature (°C); NaOH, Lime, KOH = alkali source
C = Centrifugation; F = Microfiltration

The cost for the raw material is the highest and constitutes 55-66% of the total processing cost. The highest revenue comes from the oil (40-63%), while the protein concentrate constitutes to 19-37% of the revenue. Oil market price was estimated at USD 786/tonne based on the price of rapeseed oil[15], one of the main feedstocks for biodiesel production. When pressing is used to separate the oil, revenue from oil was estimated at USD 159/tonne-raw material (USD 31833/year), which only constitutes 80% of raw material cost (Figure 6.6). It shows that processing the seeds only for the oil is not economically feasible and additional revenue from protein fraction is needed to improve the economics of oil pressing.

Labour cost constitutes 18-22% of total processing cost. In this calculation, the labour cost was normalised to raw material quantity, therefore the cost is similar for different processes.

Overall, capital cost constitutes 9-13% of total processing cost. Chemicals cost, except for dehulled seed (kernel) extraction, constitutes 0.3-6% of the total processing cost. The capital and chemicals costs for kernel extraction are higher because solvent extraction is used to separate the oil from the emulsion. Dehulling the seed before pressing requires lower capital cost, reduces chemicals and energy consumptions, and yields additional revenue from briquette. On the other hand, revenues from protein fractions were estimated to be lower than process without dehulling because less quantity (in kg/tonne-raw material) can be produced. Increasing extraction temperature and using microfiltration instead of centrifugation can increase revenue without major changes in processing cost. These options (alone or combined) are the most promising for optimisation.

The revenue from simultaneous oil and protein extraction from kernel was estimated to be lower than the other processes. The expected oil recovery by aqueous extraction is only 50% in the form of free oil and emulsion. Solvent extraction is proposed to recover this oil from the oil/emulsion mixture (Figure 6.3). Other methods e.g. freezing-thawing, pH adjustment, or enzymatic treatment can be used as well[12], possibly at a lower cost. However, since the oil constitutes the highest revenue, the lower recovery contributes significantly to the lower revenue compared to pressing, and renders this route less feasible compared to the other options.

6.3.4 Evaluation of processing parameters

The preliminary economic comparison (Figure 6.6) serves to indicate the influence of processing parameters on the cost and revenue. These parameters and possible

modifications for improvement are discussed next. Simultaneous oil and protein extraction from kernel requires the highest processing cost and yields the lowest revenue and therefore is not discussed further.

1. Raw material

The seeds constitute the highest cost component, therefore any changes in seed price will have significant influence on processing cost. In this calculation, rubber seed price was estimated at USD 198/tonne based on its protein content[13]. This approach may not reflect the actual seed price because the oil, instead of protein, is the highest value component in rubber seeds.

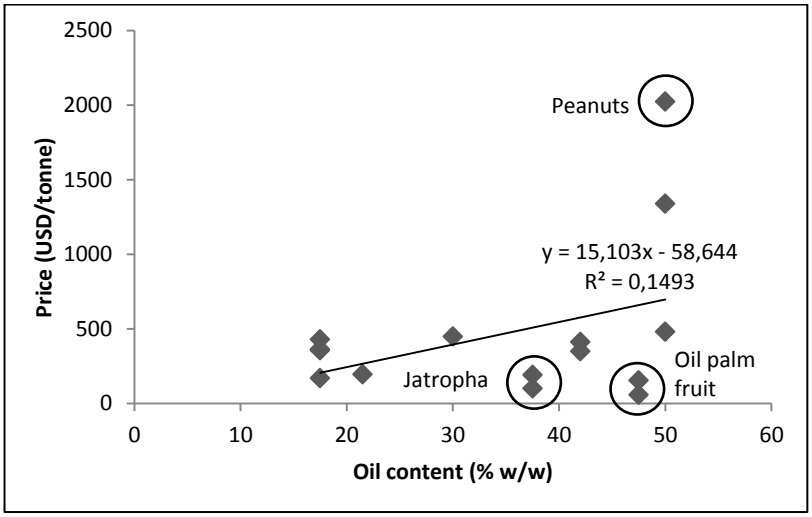

Figure 6.7 Price of plant oil feedstocks as function of oil content.
Dataset for this graph is presented in Table B.11 in the Appendix.

Figure 6.7 shows that the price of oilseeds and other plant oil feedstocks is not directly correlated to its oil content. Oil application as food, biodiesel, or oleochemicals may be the determining factor. Application as food has the highest price as indicated by the high price of peanuts. Soybean, sunflower seed, and rapeseed (Figure 6.8), which oils can be used for food application but also for biodiesel and oleochemicals, have lower price than peanut but higher price than seeds that produce inedible oil such as Jatropha (Figure 6.7). The price is also related

to location, for instance soybean from Argentina has lower price than soybean from the United States[15,16]. For oil palm, the price reflects the low cost production and high yield[17].

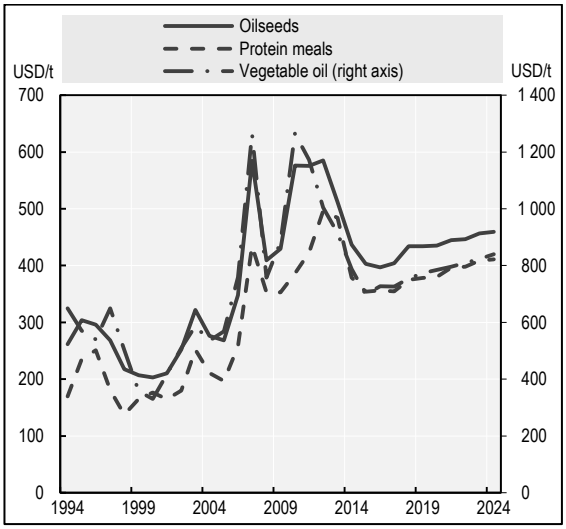

Figure 6.8 Oil seeds, protein meals, and vegetable oil prices change over time[a,18].
[a] Vegetable oil price is based on production weighted average price for palm, soybean, rapeseed, and sunflower oils at European port. Oilseeds and protein meal prices are based on production weighted average prices for soybeans, rapeseed, and sunflower seeds and meals at European port.

Rubber seed is currently not used by most farmers, and may even be considered as waste. A review on rubber seed utilisation estimated that the price of rubber seed is in the range of USD 350/tonne to USD 1000/tonne[19]. However, this may account for seeds for propagation purpose that only comprise small quantities of selected breed and quality. For the case of biodiesel production from rubber seed in Indonesia, the price was estimated at USD 115/tonne[9].

Instead of comparison of rubber seed with plant oil feedstock in general (Figure 6.7 and 6.8), comparison to Jatropha may be closer to rubber seed. Jatropha seed has 35-40% oil content that is inedible[20]. Jatropha seed price was used to be estimated at high price, between USD 140-440/tonne[21], due to high expectations in yield and productivity. Current, more realistic price is estimated between USD 100-190/tonne[16,22]. The price of rubber seed for Indonesian case falls within this range[9]. If this price is applied, the processing cost will be 24-27% lower.

A higher seed quality can be obtained by lowering the moisture content, and this can be applied as a standard requirement for the seeds that also determine the price. Freshly collected seeds can have 30-40% moisture. Sun drying that can be done in the plantation area can reduce the moisture to 15-25%. Dry seeds are preferred in processing because it will reduce the energy cost for pre-drying and prevent losses due to fungal contamination.

2. *Energy cost*

Assuming that 50% of the heat can be recycled, energy constitutes 2-4% of the total processing cost or USD 7-12/tonne-raw material. Without recycle, the energy costs were estimated to be USD 12-22/tonne-raw material. The main energy use in the process is for drying. Sun drying can reduce moisture content in the product to approximately 30% moisture. If only a final drying step is applied, energy requirements were estimated to be 1179 MJ/tonne-raw material (4 MJ/kg-protein) for pressed whole seed and 768 MJ/tonne-raw material (2 MJ/kg-protein) for pressed dehulled seed, reducing energy cost to USD 3-6/tonne-raw material. Alternatively, the protein concentrate can be sold as wet feed, no further drying then is required. The price may be lower than dry feed, but energy cost can be reduced. This is more feasible if the product is used locally, otherwise transportation cost will be high due to the additional weight from water and the product may also deteriorate faster.

In this calculation, coal (USD 2.6/GJ) was used because of its low cost. However, coal use has some drawbacks, particularly high CO_2 and particulate emissions. To counter this problem, an air pollution control system is required and will add to capital cost. The cleaner option is using natural gas at the estimated price of USD 7.0/GJ[23]. If natural gas and only a final drying step are applied, the energy costs were estimated to be USD 5-9/tonne-raw material.

For a process with dehulling before pressing (Figure 6.2), the hull can be applied as an alternative energy source. The hull was estimated to contain 15 GJ/tonne and only 4% moisture. If only a final drying step is applied, 26 kg-hull/tonne-raw material is required to generate heat for the process; this is 8% of the hull produced per tonne raw material. The ash from the burning can be applied to soil as fertiliser.

3. Labour and capital cost

The capital cost for the process with pressed whole seed (Figure 6.1) was estimated based on equipment cost at USD 8100/year, assuming 10% annual depreciation. The price for microfiltration is lower than for a centrifuge with similar capacity, therefore the capital cost may be around 17% lower. Dehulling and briquetting machines are required in the alternative route using pressed dehulled seed (Figure 6.2). The added capital cost for this equipment may be compensated by smaller equipment for subsequent processes. If solar drying is applied for the products, additional costs are required to build the solar dryer unit, but this is compensated by the lower required capacity of the final dryer unit.

In the preliminary calculation, the capital cost was assumed to be scalable to the amount of processed raw material. In practice this is unlikely, because the economy of scale dictates that the smaller the scale, the capital cost per unit (processed material or product) usually increases[24]. However, it has previously been indicated that for the screw extruder that is used in this scenario to expel the oil, processing of smaller amounts of raw material lowers the cost per tonne of material compared to processing larger amounts[25].

The labour costs, as normalised to raw material quantity, were estimated at USD 65/tonne-raw material or USD 13000/year. Assuming the processing plant requires four workers and operates whole year round, the worker's monthly wage was estimated at USD 270. The wage is within the lower end of the average wage for European countries[26], therefore a lower wage may not be possible if the processing is performed in this region. In the three largest rubber producing countries: Thailand, Indonesia, and Vietnam[27], the average monthly wages in 2013 were USD 391, 183, and 197, respectively[28]. Processing in these countries may therefore be cheaper.

4. Oil price

For the preliminary calculation, oil market price was estimated at USD 786/tonne based on the price of rapeseed oil[15], one of the main feedstocks for biodiesel production. The price may be overestimated, as Figure 6.8 shows that the average price of palm oil, soybean oil, sunflower oil and rapeseed oil decreased from USD 1265/tonne in 2010 to USD 700/tonne in 2015[18]. With petroleum price decreasing[23], there is an increased pressure to lower the price of vegetable oil for biodiesel application[29].

5. Protein concentrate

The revenue (turnover) from the protein concentrate is determined by the price and product quantity. Figure 6.9 shows that the revenues from processes with microfiltration are higher than centrifugation because the former can yield more product quantity (Table 6.1).

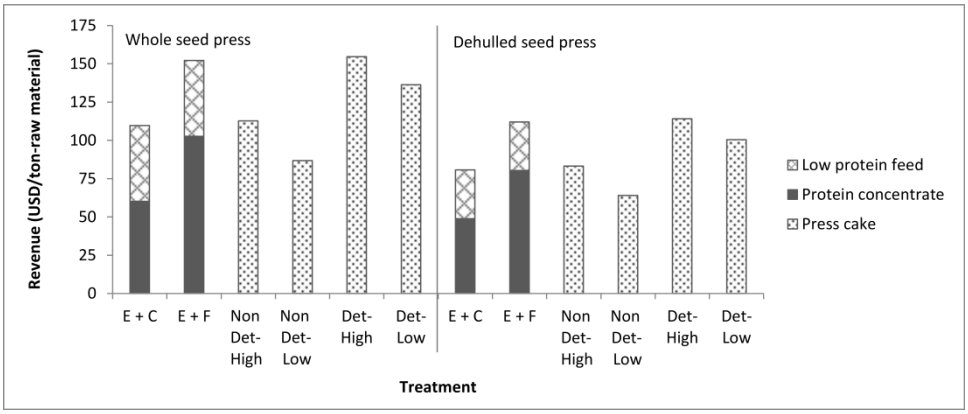

Figure 6.9 Revenue from press cake with different treatment options.
E + C = Extraction at 25°C, lime + H_2SO_4, Centrifugation
E + F = Extraction at 25°C, lime + H_2SO_4, Microfiltration
Non Det = No detoxification treatment
Det = Detoxification according to Sharma *et al.*[30]
High, low = Estimated revenue

The alkaline conditions used in the extraction may influence protein properties. Racemisation or lysinoalanine formation may occur during extraction, particularly at high temperature and pH[31]. On the other hand, digestibility may increase because alkaline treatment increases protein solubility[32,33].

Instead of processing for protein extraction, the press cake can also be sold directly. However, the price may be 27-44% lower than the estimated price based on protein content if anti-nutritional factors are present[13]. Fresh rubber seed kernels contain cyanide equivalent to 1640 mg-HCN/kg-dry kernel[34]. Screw pressing can reduce 61-89% of the initial cyanide in the seed[35], however the press cake still retains part of the cyanide. Figure 6.9 shows the revenue from non-detoxified press cake (Non Det). High price and low price indicate, respectively, 27% and 44% lower prices than the price estimated based on protein content. With estimated high price, the revenue can be

higher than the revenue gained from protein extraction with centrifugation, but still lower than extraction with microfiltration (Figure 6.9)

Alternatively, the press cake can be detoxified. Under acidic conditions or in the presence of β-glucosidase, gaseous hydrogen cyanide (boiling point 25.7°C) is formed. β-Glucosidase is present in rubber seeds[36]. The enzyme has optimum pH of 6 and temperature of 60°C, therefore treatment at 45-65°C and neutral conditions aids hydrogen cyanide formation, which is further removed by evaporation[37,38]. Hydrogen cyanide formation may be inhibited under alkaline conditions, but the inhibition is reversible. Cyanide removal was observed when alkaline extraction was followed by treatment at high temperature[33,38]. Other anti-nutritional factors, e.g. tannins and trypsin inhibitor, also can be removed or are decreased after alkaline treatment[33,38,39].

The following detoxification method for the seeds has been proposed[30]: four months storage at room temperature, soaking in water for 24 h, boiling for 30 min, after which the seeds were dehulled and the kernels were dried for 72 h. The detoxified kernels were incorporated in carp fingerling feed up to 20% without adverse effect. Even though the method has not been tested for press cake, soaking, boiling, and drying may also be applied for press cake detoxification.

Figure 6.9 shows the revenue from detoxified press cake (Det-High) can be similar to protein extraction with microfiltration if no losses occur during the process. In practice, however, some protein and dry matter may be lost. Assuming 10% dry matter and 10% protein losses, selling detoxified press cake (Det-Low) can generate more revenue than selling non-detoxified press cake, but still less than revenue gained from extraction with microfiltration (Figure 6.9).

A drawback for selling the whole seed press cake is the low protein content of only 15%, which makes it less competitive as there are other alternatives for protein sources with similar protein content. Whole seed press cake also contains 48% fibre, which makes the digestibility poor. The presence of small hull particles (55% w/w) in the press cake was reported to be harmful for tissues in digestive organs of pigs, but should be safe for chicken that often swallow dirt or small stones to aid digestion[40]. Dehulled seed press cake, on the other hand, contains 28% protein, which is comparable to e.g. non-dehulled sunflower seed meal. The fibre content is 23% and the hull presence is 17% w/w, which should therefore be more digestible than whole seed press cake. Considering that the detoxification process is simpler than alkaline protein extraction and requires no chemicals, selling detoxified press cake can be an interesting alternative.

Table 6.2 Comparison of residue use as fuel or low protein feed.

Oil separation	Protein extraction temperature (°C)	Residue quantity (kg/tonne-raw material)	As feed		As fuel	
			Crude protein (%-dw)	Estimated price[a] (USD/tonne-product)	Energy[b] (GJ/tonne)	Estimated price[c] (USD/tonne-product)
Whole seed	25	332	6	149	17	44
pressing	60	265	8	163	17	44
Dehulled seed	25	164	11	194	18	48
pressing	60	131	14	220	19	49

[a] Estimated from protein content[13].

[b] Calculated with Atwater constants[41].

[c] Estimated from coal price, USD 2.6/GJ[23].

6. Other by- products

The residue after protein extraction can be used as fuel (e.g. briquette) or as low protein feed. The comparison between these options is presented in Table 6.2.

Based on Table 6.2, more revenue can be obtained by selling the residue as low protein feed instead of as fuel. Residue from whole-seed and dehulled-seed press cake contains 78% and 62% fibre, respectively, which may reduce their digestibility. On the other hand, alkaline treatment may have increased digestibility because the proteins and other components become more soluble, while also removing components like tannins[32,39].

For a process with dehulling before pressing (Figure 6.2), 340 kg-hull/tonne-raw material (4% moisture content) can be obtained. The hull consists mainly (69%) of fibre[34]. It was estimated to contain 15 GJ/tonne and can be sold as fuel for USD 39/tonne. The hull has been studied for activated carbon production, and it shows potential application as adsorbent for wastewater treatment[42]. The price for activated carbon was estimated at USD 2/kg[43].

The other alternative for the residue and the hull is to use it as fibreboard component[44]. Using the hull is more practical because of its low moisture content, while the residue after extraction still contains up to 85% moisture. For this application, the price was estimated at the price of wood chips being USD 100/dry-tonne[45].

7. Utilisation of the water fraction

The water fraction—supernatant or filtrate—is currently regarded as wastewater with a treatment cost of USD 1 per cubic metre[46]. Alternatively, they can be recycled back to the process or applied to the soil as liquid fertiliser. Using the water fraction for the soil is preferred whenever possible to recycle the nutrients back to the plantation; however, several restrictions apply. The optimum soil pH for rubber plantation is 4.5-6[47], therefore the water pH should not be lower than 4.5 for application in rubber plantation. Furthermore, the chemicals used should not have negative influence on rubber tree growth and latex production.

The pH of the supernatant is approximately 5, therefore no adjustment is necessary. The use of lime and H_2SO_4 in the process is preferred to NaOH and HCl because calcium and sulphur are regarded as secondary macronutrients. However, excessive

calcium in the tree may disrupt the latex vessel and cause early coagulation on the excised bark and reducing latex flow[47]. The best combination for liquid fertiliser is potassium hydroxide and phosphoric acid because potassium and phosphorus are primary macronutrients.

Table 6.3 Estimation of liquid fertiliser price.

Chemicals	Cost[a] (USD/tonne-raw material)	Quantity (kg-dry weight/tonne-raw material)				Potential revenue[b] (USD/tonne-raw material)
		Supernatant	N	P	K	
NaOH + HCl	7.0	184	4.9	-	-	3.1
Lime + H_2SO_4	1.4	181	4.9	-	-	3.1
KOH + H_3PO_4	21.6	185	4.9	1.2	4.7	6.0
KOH + HCl	20.6	186	4.9	-	9.1[c]	6.6
Lime + H_3PO_4	3.0	183	4.9	1.2	-	3.4

[a] Chemicals cost for extraction of whole seed press cake.

[b] Elemental price was estimated based on the price of urea, phosphate rock, and potassium chloride[48], normalised to 100%: USD 635/tonne-N, USD 252/tonne-P, and USD 548/tonne-K.

[c] Calculated as potassium chloride[48].

Table 6.3 shows that in general, the nitrogen and salt concentration in the supernatant are too low to generate significant revenue from liquid fertiliser. By using lime with sulphuric or phosphoric acid, potential revenue from liquid fertiliser is higher than chemicals cost. Calcium concentration in the supernatant is less than 5 mg/l, which is still within water quality standard for irrigation[49]. On the other hand, even though the use of potassium hydroxide may generate USD 6-7/tonne-raw material, the chemicals cost can be as high as USD 22/tonne-raw material, making this alternative unfavourable.

The filtrate has a neutral pH, however dry matter content is very low. Micronutrients may be present in the filtrate as ions, therefore recycling the filtrate to the plantation may still present benefit. After polishing, e.g. with reverse osmosis, the filtrate also can be reused in the extraction.

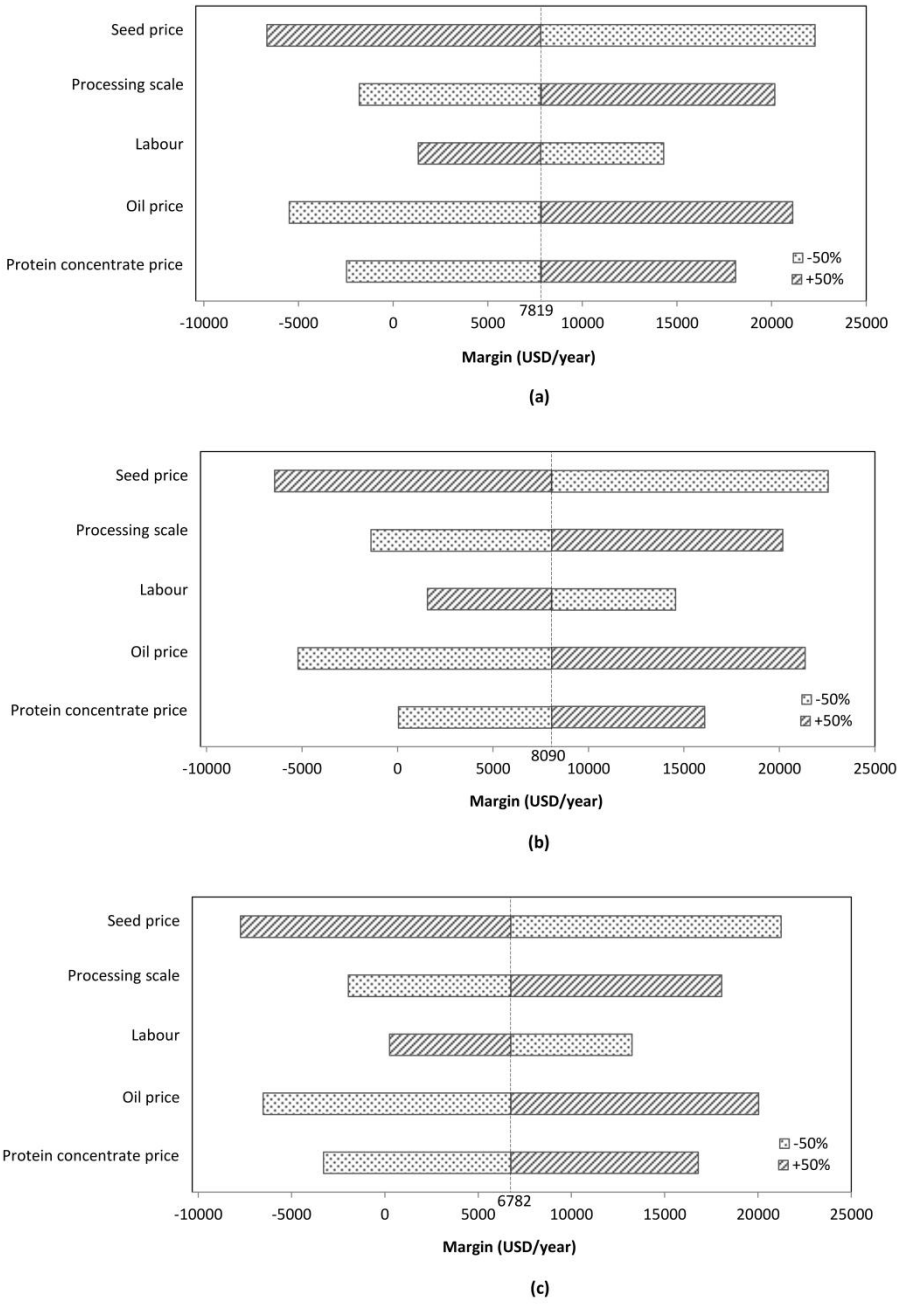

Figure 6.10 Sensitivity analysis for the optimised case: (a) whole seed pressing + extraction, (b) dehulled seed pressing + extraction, and (c) dehulled seed pressing + detoxification.

8. Sensitivity analysis

The processes were optimised based on the evaluation of processing parameters. The assumptions and the detailed processing costs and revenues are respectively presented in Table B.13 and Table B.14 in the Appendix. With the revised parameters, the annual profits were estimated at USD 7819 from pressing the whole seed followed by protein extraction, USD 8090 from pressing the dehulled seed followed by protein extraction, and USD 6782 from pressing the dehulled seed followed by press cake detoxification. A sensitivity analysis was performed on the optimised processes and the results are presented in Figure 6.10a-6.10c.

Figure 6.10a-6.10c show that all processes are highly sensitive to changes in seed price and oil price. Protein product price also has significant influence. Price changes of protein concentrate from whole seed press cake and detoxified press cake have more influence on margin compared to protein concentrate from dehulled seed press cake, showing additional advantage of having product with higher protein content. The influence of processing scale is almost similar to product price. Labour cost has smaller influence compared to the other parameters.

6.3.5 Indonesian case

Indonesian livestock production is growing by 5-8% each year[50]. The sector is dominated by chicken; chicken meat production is higher than the others combined (Figure 6.11). Feed is the highest cost component in livestock production, accounting for 58%, 84%, and 65% for cow, layer chicken, and broiler chicken production costs, respectively[51]. Compound feed production in Indonesia was 15 million tonnes in 2014. Unsurprisingly, 83% of total feed in Indonesian market was used for poultry production. The rest was consumed by aquaculture (11%) and cow and pig (6%)[52].

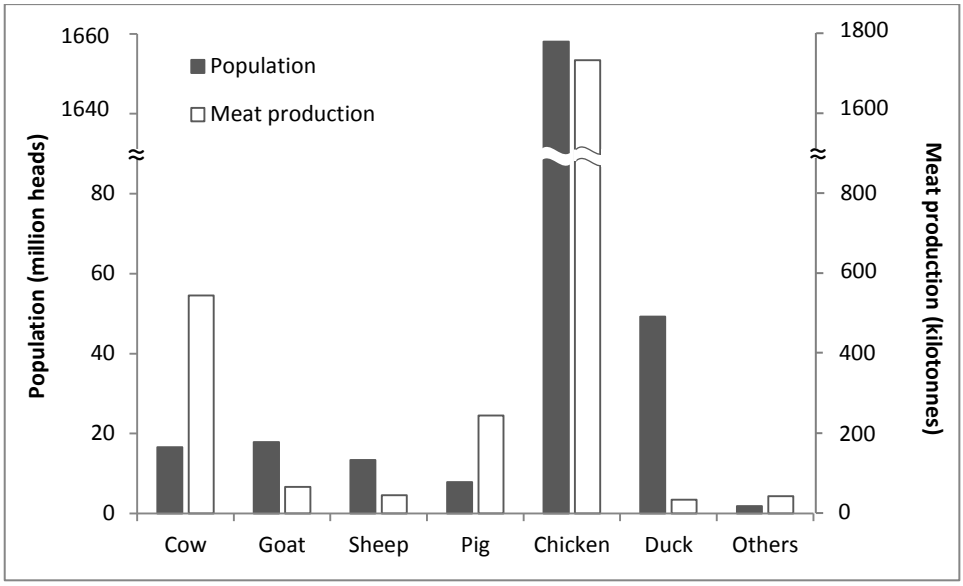

Figure 6.11 Animal population and meat production in Indonesia, 2012[53].

Animal feed industry in Indonesia consists of around eighty companies. Next to that, traditional breeders also often produce feed for own consumptions and local markets. However, more than 65% market is controlled only by four large companies[54].

Table 6.4 Main protein sources for feed ingredients in Indonesia.

Feed ingredient	Crude protein[15,56] (%)	Demand[a] (million tonnes)	Sources (%)	
			Local	Imported
Corn	9-11	7.5	90-95	5-10
Corn gluten meal	60-67	0.5	0	100
Fish meal	64-65	0.8	5-10	90-95
Soybean meal	44-48	3.6[55]	0	100
Rice bran	13-14	2.3	100	0
Wheat pollard	15-18	1.2	0	100
Palm kernel meal	17-22	0.5[55]	100	0

[a] Estimated from Wright and Meylinah[52].

The feed ingredient with the highest demand is corn, accounting for 50% of all feed ingredients (Table 6.4). However, combination with other feed ingredient e.g. soybean meal is required to increase the overall protein content and quality. Most feed ingredients with high protein content have to be imported. Surprisingly, even though Indonesia is the leading palm kernel meal producer, most of the production is exported mainly to the European Union and New Zealand[15]. Only 0.5 million tonnes are used locally as cattle feed; application as non-ruminant feed requires processing due to high fibre content[32]. The limited use is mainly because processing the meal and transporting it to cattle producing areas is considered more costly than the potential revenue[55].

Table 6.5 Essential amino acid content of rubber seed protein concentrate, soybean meal, and palm kernel meal (% dry matter).

Amino acid (AA)	Rubber seed protein concentrate	Soybean meal[a]	Palm kernel meal[b]	AA requirements in feed[c]
Histidine	1.0 ± 0.2	1.5	0.4	0.27
Isoleucine	1.8 ± 0.1	2.5	0.6	0.62
Leucine	3.7 ± 0.3	4.1	1.2	0.93
Lysine	0.9 ± 0.4	3.3	0.7	0.85
Methionine	0.5 ± 0.2	0.8	0.3	0.32
Methionine + cysteine	0.6 ± 0.2	1.6	0.6	0.60
Phenylalanine	2.3 ± 0.2	2.7	0.7	0.56
Phenylalanine + tyrosine	3.9 ± 0.3	4.6	1.3	1.04
Threonine	1.8 ± 0.2	2.1	0.7	0.68
Tryptophan	0.5 ± 0.0	0.7	0.2	0.16
Valine	4.6 ± 0.4	2.6	1.0	0.70
Arginine	6.1 ± 0.8	4.0	2.8	1.00
Glycine + serine	5.0 ± 0.6	5.0	1.9	0.97
Essential AAs[d]	17.2 ± 1.5	20.3	5.9	
Essential + semi essential AAs[e]	30.0 ± 2.5	28.0	11.4	
Crude protein	53.3 ± 0.6	53.5	18.7	18.0

[a] Calculated from average value for soybean meal of USA origin[57].

[b] Calculated from Fetuga *et al.*[58] for untreated palm kernel meal.

[c] Amino acid requirements for 6-8 weeks broiler[59].

[d] Essential amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.

[e] Semi essential amino acids that are included in this table are those included in the requirement in the last column: cysteine, tyrosine, arginine, glycine, and serine.

Crude protein content of rubber seed protein concentrate is similar to soybean meal (Table 6.5). However, even though the total essential amino acid contents are comparable, the contents of lysine, methionine, and cysteine in rubber seed proteins are much lower than soybean meal. In fulfilling the amino acid requirements for poultry, the contents of these amino acids may be limiting. This is often also the case for other protein sources, for instance palm kernel meal (Table 6.5). In practice, these amino acids are usually added as supplements. Rubber seed protein concentrate with added lysine and methionine thus can be used to substitute soybean meal in animal feed.

Abduh *et al*.[9] calculated the techno-economical feasibility of a small scale biorefinery unit (55 tonnes rubber seed oil/year) located in Palangka Raya, Indonesia. Total production cost of this unit was estimated at USD 55852/year, yielding USD 27987 annual revenue from oil and USD 27897 annual revenue from untreated press cake. Feasibility of the process is mainly determined by production scale. However seed price, labour cost, and revenue from press cake also have significant influence[9].

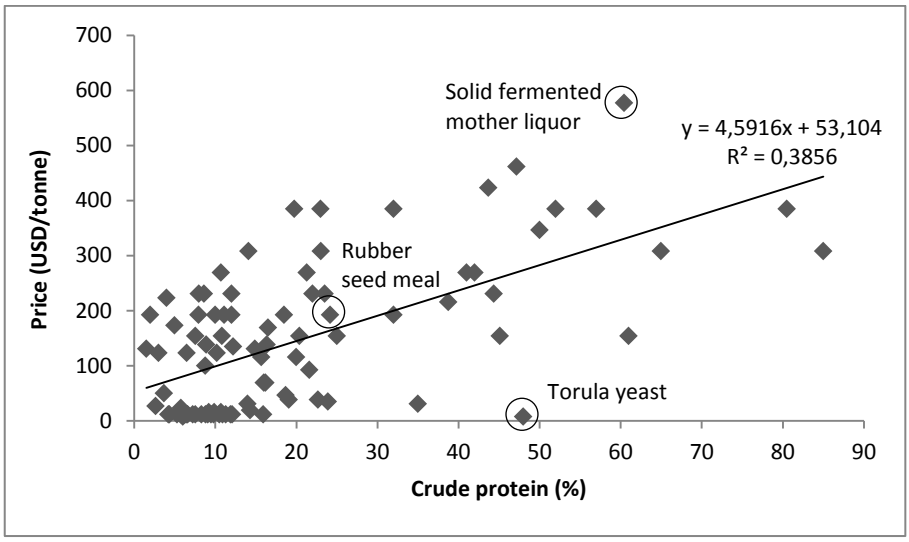

Figure 6.12 Protein content and price of animal feed protein sources in Indonesia[60]. Dataset for this graph is presented in Table B.12 in the Appendix.

Figure 6.12 shows the price of animal feed ingredients in Indonesia. It is poorly correlated to its protein content. The dataset (Table B.12 in the Appendix) lists 90 protein-containing ingredients out of 158 feed ingredients[60]. Demand and continuous availability of these ingredients are not indicated; combination of these factors may also determine the price.

For instance beer yeast has high protein content but the supply is not reliable, therefore lowering the price. Rubber seed meal (24% crude protein) is already listed in the dataset even though, to the best of the author's knowledge, there is currently no commercial rubber seed processing plant in Indonesia that can provide the meal in reliable amounts. Furthermore, the indicated dry weights for some materials seem to refer to dried materials, while the assigned prices are for undried materials. This further adds to the inaccuracy. Even though the data may not be accurate and gives poor correlation, it can be used to couple the increase in price to an increasing protein content, and is valuable for preliminary price estimation.

Using the estimated price from Figure 6.12, the annual profits from oil pressing followed by extraction of whole seed and dehulled seed press cakes were estimated at USD 4583 and USD 6755, respectively. The price for detoxified press cake was estimated at the price of rubber seed meal (USD 192/tonne), and the annual profit was estimated at USD 6828. A sensitivity analysis was performed on the processes and the results are presented in Figure 6.13a-6.13c.

For the Indonesian case, all processes are also highly sensitive to changes in seed price and oil price (Figure 6.13a-6.13c) just as the previously optimised general process. The process with pressed whole seed (Figure 6.13a) was estimated to give the lowest profit. Profit from detoxified press cake (Figure 6.13c) was estimated to be slightly higher than from protein concentrate from dehulled press cake (Figure 6.13b), however, the latter is less sensitive to product price. For all three processes, protein product price and labour cost have smaller influence compared to the optimised general case discussed in subchapter 6.3.4.

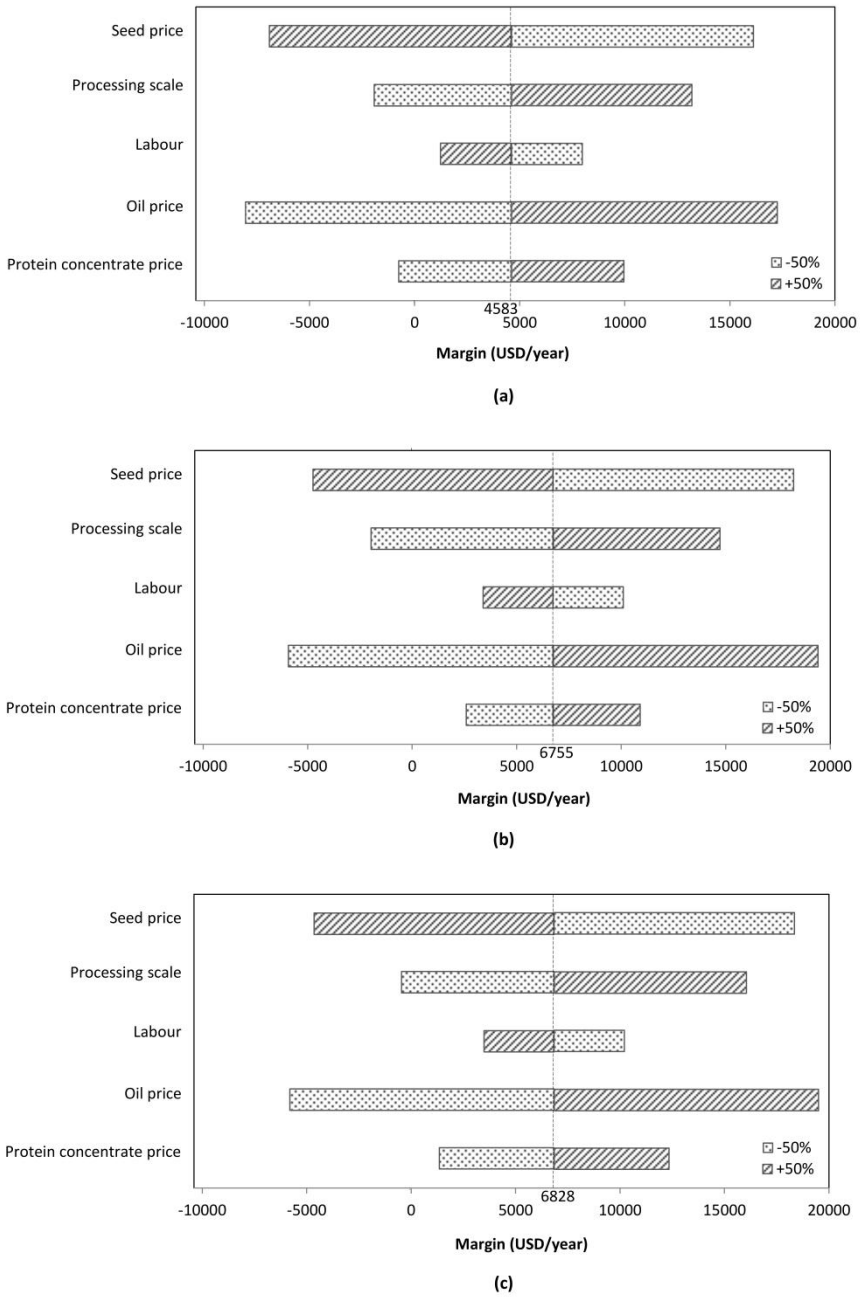

Figure 6.13 Sensitivity analysis for the optimised Indonesian case: (a) whole seed pressing + extraction, (b) dehulled seed pressing + extraction, and (c) dehulled seed pressing + detoxification.

Table 6.6 Potential applications of rubber seed proteins.

Fraction	Potential application	Progress	Bottlenecks	Potential benefit[a]
Native protein	Animal feed	Protein can be isolated from rubber seed kernel or press cake to get concentrate with 19-57% protein (21-63% crude protein), depending on the process. The residue after protein extraction also can be applied as low-protein feed for ruminants.	Rubber seed biorefinery is currently non-existent. Protein extraction is necessary to improve the economics of oil/biodiesel production.	People, planet
	Technical application	Rubber seed proteins are soluble in water at pH up and above 8.5. Emulsifying and adhesive properties have been observed qualitatively.	Technical properties, other than solubility, have not been quantitatively measured.	Profit
Peptides	Food/ pharmaceuticals	Enzymatic hydrolysis can be directed to yield short peptides that may have functional properties for food or even pharmaceutical applications.	The type of peptides and their properties have not been identified. Enzyme price may be restrictive.	Profit
Amino acid	Animal feed	Enzymatic hydrolysis can be directed to yield a mixture that is rich in essential amino acids (most of them are hydrophobic).	Lysine content is low. Enzyme price may be restrictive.	People, planet, profit
	Bulk chemicals	Rubber seed protein can be hydrolysed chemically, enzymatically, or combination of both to obtain mixture of amino acid.	Separation to single amino acids is still difficult.	Planet, profit

[a] Potential benefit is framed within the three pillars of sustainability: people (society), planet (ecology), and profit (economy).

6.4 Rubber seed protein applications

Figure 1.6 shows different routes to isolate proteinaceous fractions from agricultural residues. Table 6.6 shows the different routes applied to rubber seed proteins, based on the technological aspects discussed in chapter 3-5 and on the economical aspect discussed in this chapter.

In the setting of the Indonesian rural area, the use of native protein for animal feed is the most straightforward and economically feasible. It can provide animal feed, especially for small scale/household farmers. Using locally available agricultural residues also diverts the use of food harvest or imported feed ingredients. Central Kalimantan, the targeted area of this study, has 268,800 hectares of rubber plantations. If optimised seed collection can be applied, 32,200 tonnes protein is available annually. This can potentially provide 51% of the required protein source for animal feed in Central Kalimantan. This approach also potentially reduces the negative environmental impact from agriculture and transportation.

When hydrolysed, mixtures that are rich in essential amino acid also can be applied as supplement to increase essential amino acid content in compound feed. In this case, separation is not crucial. However, even though the proteins contain 34% essential amino acid, which can be increased up to 55% via hydrolysis, the lysine content is only 1.6-2.5%. As lysine is usually the limiting amino acid, mixtures with low lysine content have modest value as supplements.

Enzymatic hydrolysis also can be directed to yield short peptides. The use of protease in hydrolysis enables mild processing, thereby avoiding formation of unwanted compounds or even racemisation of amino acids, as well as increasing digestibility and reducing allergenic reactions. The peptides can be applied as nutritive supplement in food, drink, or clinical treatment. These applications have potentially the highest economic value with price in the order of USD 1-20/kg. However, more research is still required to reach this stage.

Next to enzymatic hydrolysis, chemical hydrolysis or a combination of both can be applied to obtain a mixture of amino acids. Amino acids can be used as feedstock for bulk chemicals production, however, a complete separation into single amino acids is necessary. The separation is complex, as hydrolysates contain multiple amino acids, and in a relatively dilute aqueous system. Ethanol precipitation can be applied as a pre-treatment to separate mixtures into groups of amino acid or a polishing step to increase purity. However, series of separation steps are needed to get pure compounds. If the amino acids can be separated in an energetically efficient way, the application for bulk chemicals

production can partially substitute the petrochemicals equivalents. The price for these applications is in the order of USD 500-900/tonne.

Other potential applications of rubber seed proteins in industrial setting are based on the technical properties, e.g. for adhesives or emulsifier. Identification of these technical properties is still required. Once identified, however, the technology to utilise these properties is already available. The price for these applications is also in the order of USD 500-900/tonne.

6.5 Perspective for protein-based biorefinery in Indonesia

Several biorefinery plants that include protein production are currently in (pilot) operation, for instance rapeseed biorefinery in Denmark and grass biorefinery in the Netherlands and Germany[61,62]. We can obtain valuable information from these examples, particularly on technical aspects. Application in different settings, however, sometimes needs not only some adjustments but even a different approach altogether.

Next to technological approach, there are broad socio-economic conditions that need to be taken into account in applying protein-based bioefinery in Indonesian setting. The following remarks are far from a complete list, but considered the most relevant:

1. Protein consumption in Indonesia ranges from 41 to 63 g/person/day. Animal proteins account for 16-40% (average 25%) of total protein in the human diet[63]

2. Agricultural sector is dominated by smallholder farmers[64]. Some of these farmers only own e.g. 1-2 hectares plantation or 1-2 cattle. Another existing business model is plasma-nucleus partnership, in which large companies provide smallholder farmers in surrounding areas with inputs e.g. capital, seeds, fertilizers, pesticides, and technical expertise, and in return the farmers produce the commodities according to the companies' standard. This business model is largely applied by e.g. oil palm[65].

3. Processing industries of agricultural products exist, mainly for food applications.

4. Due to the large area, disparities between regions can be striking in term of infrastructure and available skills and resources. Most industries are still located in Java Island as the centre of economic activities. Large plantations are located mostly in Sumatera and Kalimantan Islands.

The recommended protein intake for adults with 60 kg-body weight is 50 g/person/day[66]. Based on the first remark, one of the goals of protein-based biorefinery should be to increase protein consumption in regions where protein consumption is still low by providing alternative protein sources at affordable prices.

For areas where infrastructure is lacking, local processing and consumption can be preferable over transporting the biomass to central processing plants. The type of biomass, processing technology, and type of product should be adjusted to local conditions[67]. Utilisation of existing crops should be prioritised over introduction of new crops, as the former poses less risk and resistance from the farmers. The required capital investment may not be locally available and requires external investment. The required workers may also not be available due to (one of) these reasons: lack of skill, (perception of) low wage, seasonal employment, or cultural reasons e.g. the perception that farming is a low-status job for the poor. In societies where men traditionally provide for the family, some low-skilled jobs e.g. seeds collecting may provide opportunities for women employment as secondary provider. The downside is that this opportunity may also be exploited for children employment.

In some cases, partnership between industries and smallholder farmers may be beneficial. The farmers' constrains in processing their products are often the lack of technological skills and capital, and these can be bridged with small (plantation- or community-) scale pre-processing plants. Established companies can provide capital, aid technology transfer, and ensure market for the products. Furthermore, industry involvement enables more complex processing that yields products with higher values e.g. chemicals.

6.6 Conclusions and recommendations

The results of this study confirm that rubber seeds can be an alternative source of proteinaceous products within the framework of biorefinery. Utilisation of rubber seed proteins might increase the economics of rubber tree plantation. The most likely potential application for the farmers is using the rubber seed protein concentrate for animal feed. For industrial setting, the proteins can potentially be used for technical applications. Experiments were also performed on other materials e.g. wheat gluten and grass juice, suggesting some of the conclusions from this study might be extended to other agricultural residues with similar properties.

Two restrictions still apply when using rubber seed proteins for amino acid production: the price of enzymes and separation of amino acid from the mixture. The separation may be easier if hydrolysis selectivity can be improved, an area where enzyme plays an

important role. Hence even though the enzyme price is high, the whole process may still be beneficial if the separation can be done efficiently and the amino acids can be processed into final product(s) with sufficiently high price. The recommendations for future research are as follows:

1. Increasing the selectivity of the hydrolysis process to obtain hydrolysates with a significantly high fraction of a group of amino acids or even a specific amino acid. This might be achieved by careful selection of biomass and protease. Furthermore, selectivity can be increased by controlling the extent of hydrolysis.

2. Improving the process to separate amino acids from the hydrolysate, and from dilute aqueous mixture in general. The complex interactions between amino acids, as well as interactions between amino acids and other components that are possibly present in the mixture, have not been understood very well. Understanding these interactions may be one of the keys to design a better separation process.

3. Investigate intermediate process(es) that potentially integrate protein hydrolysis and amino acids separation. The use of non-aqueous solvent, enzyme combination, and (thermo-)chemical treatment can influence free amino acids liberation from protein. It can also convert the amino acids into intermediate product(s) with properties that may be beneficial for separation.

6.7 References

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Appendix A

Supplementary Information to Chapter 5

Table A.1 Amino acid concentration (µmol/g) in the starting mixtures of Protamylasse™, experiment at fixed starting material concentration and varying ethanol concentration.

Amino acid	Ethanol concentration (%w/w)										
	0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**95**
Asn	6.8	6.7	6.9	6.8	7.0	6.8	6.9	6.8	6.9	6.9	6.9
Gln	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ser	0.6	0.6	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.7
Thr	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Asp	3.5	3.4	3.5	3.5	3.6	3.5	3.5	3.5	3.5	3.5	3.5
Glu	1.6	1.5	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Arg	1.0	1.0	1.0	1.0	1.1	1.0	1.0	1.0	1.1	1.0	1.1
His	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Lys	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ile	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Leu	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Val	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Phe	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Trp	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Met	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Pro	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Ala	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Gly	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Cys	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	**18.5**	**18.3**	**18.7**	**18.6**	**19.0**	**18.5**	**18.7**	**18.5**	**18.8**	**18.7**	**18.9**

Table A.2 Amino acid concentration (µmol/g) in the supernatant of Protamylasse™, experiment at fixed starting material concentration and varying ethanol concentration.

Amino acid	Ethanol concentration (%w/w)										
	0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**95**
Asn	6.9	6.9	7.0	7.0	7.2	6.9	6.6	4.0	1.9	0.7	0.1
Gln	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ser	0.7	0.6	0.7	0.7	0.7	0.7	0.6	0.4	0.2	0.1	0.0
Thr	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.0
Asp	3.6	3.5	3.6	3.6	3.7	3.5	3.3	1.7	0.4	0.1	0.0
Glu	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.1	0.5	0.2	0.0
Arg	1.1	1.0	1.1	1.1	1.1	1.1	0.9	0.5	0.3	0.2	0.0
His	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0	0.0
Lys	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.2	0.1	0.0	0.0
Ile	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1
Leu	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1
Val	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.7	0.2
Phe	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.1
Trp	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tyr	0.5	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.2	0.0
Met	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
Pro	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.0
Ala	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.3	1.1	0.7	0.1
Gly	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0
Cys	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	**19.1**	**18.7**	**19.1**	**19.2**	**19.5**	**19.0**	**18.1**	**12.1**	**7.1**	**4.0**	**0.8**

Table A.3 Amino acid concentration (µmol/g) in the starting mixtures of RSP enzymatic hydrolysate, experiment at fixed starting material concentration and varying ethanol concentration.

Amino acid	Ethanol concentration (%w/w)										
	0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**95**
Asn	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Gln	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Ser	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Thr	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Asp	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glu	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Arg	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
His	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Lys	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Ile	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Leu	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2
Val	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1
Phe	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Trp	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Tyr	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Met	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pro	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
Ala	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Gly	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Cys	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Total	**20.6**	**20.5**	**20.5**	**20.6**	**20.5**	**20.5**	**20.6**	**20.5**	**20.6**	**20.5**	**20.5**

Table A.4 Amino acid concentration (µmol/g) in the supernatant of RSP enzymatic hydrolysate, experiment at fixed starting material concentration and varying ethanol concentration.

Amino acid	Ethanol concentration (%w/w)										
	0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**95**
Asn	0.8	0.8	0.9	0.8	0.8	0.8	0.8	0.8	0.6	0.3	0.2
Gln	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.0	0.9	0.7	0.5
Ser	0.8	0.8	0.8	0.7	0.8	0.7	0.7	0.7	0.6	0.4	0.2
Thr	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.4	0.5	0.4	0.3
Asp	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.3	0.1	0.0
Glu	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	0.4	0.2
Arg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
His	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.2
Lys	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.3	0.2	0.1
Ile	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.3	1.3	1.3	1.2
Leu	3.1	3.2	3.2	3.1	3.2	3.3	3.2	3.2	3.2	3.1	3.0
Val	4.0	4.1	4.1	3.9	4.0	4.0	3.9	3.8	3.7	3.6	3.4
Phe	1.3	1.4	1.4	1.3	1.4	1.4	1.4	1.4	1.4	1.3	1.2
Trp	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Tyr	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
Met	0.5	0.5	0.5	0.4	0.5	0.5	0.5	0.4	0.4	0.4	0.4
Pro	1.0	1.0	1.1	1.0	1.0	1.0	1.0	1.0	0.9	0.9	0.8
Ala	1.8	1.8	1.8	1.7	1.8	1.8	1.7	1.7	1.6	1.5	1.2
Gly	0.7	0.7	0.7	0.6	0.7	0.7	0.6	0.6	0.5	0.4	0.3
Cys	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
Total	**20.1**	**20.5**	**20.5**	**19.6**	**20.1**	**20.3**	**19.8**	**19.2**	**17.9**	**15.6**	**13.8**

Table A.5 Amino acid concentration (µmol/g) in the starting mixtures of Protamylasse™, experiment at varying starting material concentration and fixed (60% w/w) ethanol concentration.

Amino acid	Protamylasse™ concentration (%w/w)				
	5	**10**	**20**	**30**	**40**
Asparagine	7	14	27	41	54
Glutamine	0	0	0	0	0
Serine	1	1	3	4	5
Threonine	0	1	1	2	3
Aspartic acid	4	7	14	21	28
Glutamic acid	2	3	6	9	12
Arginine	1	2	4	6	8
Histidine	0	0	1	1	1
Lysine	0	1	2	3	4
Isoleucine	0	1	1	2	3
Leucine	0	0	1	1	2
Valine	1	2	3	5	7
Phenylalanine	0	1	1	2	2
Tryptophan	0	0	0	0	0
Tyrosine	0	1	1	2	2
Methionine	0	0	0	1	1
Proline	0	1	1	2	2
Alanine	2	3	6	9	12
Glycine	0	0	1	1	1
Cysteine	0	0	0	0	0
Polar uncharged	8	15	31	46	62
Negative	5	10	20	30	40
Positive	2	3	7	10	13
Hydrophobic	2	5	9	14	19
Special	2	3	7	10	13
Total	**19**	**37**	**73**	**110**	**147**

Table A.6 Amino acid concentration (µmol/g) in the supernatant of Protamylasse™, experiment at varying starting material concentration and fixed (60% w/w) ethanol concentration.

Amino acid	Protamylasse™ concentration (%w/w)				
	5	**10**	**20**	**30**	**40**
Asparagine	6.6	8.9	8.5	6.2	3.9
Glutamine	0.0	0.0	0.0	0.0	0.0
Serine	0.6	0.8	0.8	0.7	0.4
Threonine	0.2	0.4	0.5	0.5	0.4
Aspartic acid	3.3	4.0	2.8	1.4	0.7
Glutamic acid	1.6	2.1	2.0	1.3	0.7
Arginine	0.9	1.2	1.4	1.4	1.1
Histidine	0.1	0.1	0.1	0.1	0.1
Lysine	0.4	0.4	0.4	0.3	0.3
Isoleucine	0.3	0.6	1.3	1.8	2.3
Leucine	0.2	0.5	0.9	1.4	1.7
Valine	0.9	1.6	3.0	4.1	4.6
Phenylalanine	0.3	0.6	1.1	1.6	1.9
Tryptophan	0.0	0.0	0.0	0.1	0.1
Tyrosine	0.4	0.4	1.0	1.2	1.2
Methionine	0.1	0.3	0.5	0.6	0.7
Proline	0.3	0.6	1.1	1.3	1.3
Alanine	1.6	2.6	3.7	3.9	3.4
Glycine	0.1	0.2	0.2	0.1	0.1
Cysteine	0.0	0.0	0.1	0.1	0.1
Polar uncharged	7	10	10	7	5
Negative	5	6	5	3	1
Positive	1	2	2	2	1
Hydrophobic	3	5	9	12	14
Special	2	3	4	4	4
Total	**18**	**25**	**30**	**28**	**25**

Appendix B

Supplementary Information to Chapter 6

B.1 Material balance

Table B.1 Composition of streams involved in the process (Figure 6.1) for the capacity of 1 tonne (wet) seed per day, seeds are not dehulled, extraction at 25°C, centrifugation[a].

Mass stream	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Seed	834 (95)	-	834 (95)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Water	166	103	63	-	17	46	-	2634	890	-	503	394	387	7	1790	1773	17
Oil	-	-	-	203	-	-	-	-	-	-	-	-	-	-	-	-	-
Impurities	-	-	-	-	19	-	-	-	-	-	-	-	-	-	-	-	-
Press cake	-	-	-	-	-	612 (95)	-	-	-	-	-	-	-	-	-	-	-
NaOH	-	-	-	-	-	-	14	-	-	-	-	-	-	-	-	-	-
Extract	-	-	-	-	-	-	-	-	311 (76)	-	-	-	-	-	-	-	-
34% HCl	-	-	-	-	-	-	-	-	-	13	-	-	-	-	-	-	-
Supernatant	-	-	-	-	-	-	-	-	-	-	184 (27)	-	-	-	-	-	-
Protein concentrate	-	-	-	-	-	-	-	-	-	-	-	131 (49)	-	131 (49)	-	-	-
Residue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	316 (19)	-	316 (19)

[a] Numbers in kg. Numbers between brackets indicate crude protein in kg.

Table B.2 Composition of streams involved in the process (Figure 6.1) for the capacity of 1 tonne (wet) seed per day, seeds are not dehulled, extraction at 25°C, microfiltration[a].

Mass stream	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Seed	834 (95)	-	834 (95)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Water	166	103	63	-	17	46	-	2634	890	-	440	459	443	16	1790	1773	17
Oil	-	-	-	203	-	-	-	-	-	-	-	-	-	-	-	-	-
Impurities	-	-	-	-	19	-	-	-	-	-	-	-	-	-	-	-	-
Press cake	-	-	-	-	-	612 (95)	-	-	-	-	-	-	-	-	-	-	-
NaOH	-	-	-	-	-	-	14	-	-	-	-	-	-	-	-	-	-
Extract	-	-	-	-	-	-	-	-	311 (76)	-	-	-	-	-	-	-	-
34% HCl	-	-	-	-	-	-	-	-	-	13	-	-	-	-	-	-	-
Filtrate	-	-	-	-	-	-	-	-	-	-	9 (0)	-	-	-	-	-	-
Protein concentrate	-	-	-	-	-	-	-	-	-	-	-	306 (76)	-	306 (76)	-	-	-
Residue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	316 (19)	-	316 (19)

[a] Numbers in kg. Numbers between brackets indicate crude protein in kg.

Table B.3 Composition of streams involved in the process (Figure 6.2) for the capacity of 1 tonne (wet) seed per day, seeds are dehulled, extraction at 25°C, centrifugation[a].

Mass stream	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Seed	834 (95)	125 (14)	-	125 (14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Water	166	152	114	38	-	15	23	-	1300	439	-	249	195	191	3	883	875	8	14	14	-
Kernel	-	383 (71)	-	383 (71)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oil	-	-	-	-	203	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Impurities	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Press cake	-	-	-	-	-	-	302 (86)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NaOH	-	-	-	-	-	-	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-
Extract	-	-	-	-	-	-	-	-	-	153 (68)	-	-	-	-	-	-	-	-	-	-	-
34% HCl	-	-	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-	-	-	-
Supernatant	-	-	-	-	-	-	-	-	-	-	-	91 (24)	-	-	-	-	-	-	-	-	-
Protein concentrate	-	-	-	-	-	-	-	-	-	-	-	-	65 (45)	-	65 (45)	-	-	-	-	-	-
Residue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	156 (17)	-	156 (17)	-	-	-
Hull	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	326 (10)	-	326 (10)

[a] Numbers in kg. Numbers between brackets indicate crude protein in kg.

Table B.4 Composition of streams involved in the process (Figure 6.3) for the capacity of 1 tonne (wet) seed per day, seeds are dehulled, simultaneous extraction at 25°C, centrifugation[a].

Mass stream	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Seed	834 (95)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Water	166	150	-	1800	638	205	-	-	205	433	-	287	151	352	4	1312	1300	12	16	16	-
Kernel	-	450 (84)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NaOH	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soluble protein	-	-	-	-	92 (66)	-	-	-	24 (4)	92 (66)	-	-	50 (43)	-	75 (47)	-	-	-	-	-	-
Emulsion	-	-	-	-	137 (4)	137 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Solvent	-	-	-	-	-	-	888	888	-	-	-	-	-	-	-	-	-	-	-	-	-
Oil	-	-	-	-	-	-	-	113	-	-	-	-	-	-	-	-	-	-	-	-	-
34% HCl	-	-	-	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-	-	-	-
Supernatant	-	-	-	-	-	-	-	-	-	-	-	44 (23)	-	-	-	-	-	-	-	-	-
Residue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	232 (14)	-	232 (14)	-	-	-
Hull	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	384 (12)	-	384 (12)

[a] Numbers in kg. Numbers between brackets indicate crude protein in kg.

Table B.5 Material balances (on dry weight basis) for processing 1 tonne seed/day with

Treatment				Input[b]			
Oil separation	**Extraction T (°C)**	**Chemi-cals**	**Sepa-ration**	**Seed**	**Kernel**	**Hull**	**Chemi-cals**
Whole seed pressing	25	Na + Cl	C	834 (95)	0	0	19
	25	Ca + SO_4	C	834 (95)	0	0	19
	25	K + PO_4	C	834 (95)	0	0	24
	25	Na + Cl	F	834 (95)	0	0	19
	25	Ca + SO_4	F	834 (95)	0	0	19
	25	K + PO_4	F	834 (95)	0	0	24
	60	Na + Cl	C	834 (95)	0	0	21
	60	Ca + SO_4	C	834 (95)	0	0	22
	60	K + PO_4	C	834 (95)	0	0	26
	60	Na + Cl	F	834 (95)	0	0	21
	60	Ca + SO_4	F	834 (95)	0	0	22
	60	K + PO_4	F	834 (95)	0	0	26
Dehulled seed pressing	25	Na + Cl	C	125 (14)	383 (71)	326 (10)	9
	25	Ca + SO_4	C	125 (14)	383 (71)	326 (10)	9
	25	K + PO_4	C	125 (14)	383 (71)	326 (10)	12
	25	Na + Cl	F	125 (14)	383 (71)	326 (10)	9
	25	Ca + SO_4	F	125 (14)	383 (71)	326 (10)	9
	25	K + PO_4	F	125 (14)	383 (71)	326 (10)	12
	60	Na + Cl	C	125 (14)	383 (71)	326 (10)	10
	60	Ca + SO_4	C	125 (14)	383 (71)	326 (10)	11
	60	K + PO_4	C	125 (14)	383 (71)	326 (10)	13
	60	Na + Cl	F	125 (14)	383 (71)	326 (10)	10
	60	Ca + SO_4	F	125 (14)	383 (71)	326 (10)	11
	60	K + PO_4	F	125 (14)	383 (71)	326 (10)	13
Dehulled seed extraction	25	Na + Cl	C	0	450 (84)	384 (12)	13[c]
	25	Na + Cl	F	0	450 (84)	384 (12)	13[c]
	60	Na + Cl	C	0	450 (84)	384 (12)	14[c]
	60	Na + Cl	F	0	450 (84)	384 (12)	14[c]

[a] Numbers in kg. Numbers between brackets indicate crude protein in kg.

[b] Pre-drying and dehulling are regarded as pre-treatment. For whole seed and dehulled seed pressings, seed and kernel refer to input to the oil press. For dehulled seed extraction, kernel refers to input to extraction process. Hull refers to input to briquetting.

[c] Not including solvent

different processing options[a].

Output					
Oil	Oil impurities	Protein concentrate	Low protein feed	Supernatant/ filtrate	Briqu-ette
203	19	131 (49)	316 (19)	184 (27)	0
203	19	131 (49)	315 (19)	181 (27)	0
203	19	131 (49)	320 (19)	185 (27)	0
203	19	306 (76)	316 (19)	9 (0)	0
203	19	306 (76)	315 (19)	6 (0)	0
203	19	306 (76)	320 (19)	5 (0)	0
203	19	208 (62)	252 (19)	173 (14)	0
203	19	208 (62)	252 (19)	169 (14)	0
203	19	208 (62)	255 (19)	172 (14)	0
203	19	367 (76)	252 (19)	19 (0)	0
203	19	367 (76)	252 (19)	9 (0)	0
203	19	367 (76)	255 (19)	7 (0)	0
203	3	65 (45)	156 (17)	91 (24)	326 (10)
203	3	65 (45)	156 (17)	89 (24)	326 (10)
203	3	65 (45)	158 (17)	90 (24)	326 (10)
203	3	151 (68)	156 (17)	5 (0)	326 (10)
203	3	151 (68)	156 (17)	3 (0)	326 (10)
203	3	151 (68)	158 (17)	2 (0)	326 (10)
203	3	103 (56)	124 (17)	85 (13)	326 (10)
203	3	103 (56)	124 (17)	83 (13)	326 (10)
203	3	103 (56)	126 (17)	85 (13)	326 (10)
203	3	181 (68)	124 (17)	10 (0)	326 (10)
203	3	181 (68)	124 (17)	5 (0)	326 (10)
203	3	181 (68)	126 (17)	3 (0)	326 (10)
113	0	75 (47)	232 (14)	44 (23)	384 (12)
113	0	112 (70)	232 (14)	3 (0)	384 (12)
113	0	100 (47)	185 (14)	67 (23)	384 (12)
113	0	159 (69)	185 (14)	3 (0)	384 (12)

Na + Cl = NaOH (alkali source) + HCl (acid source)

Ca + SO_4 = Lime (alkali source) + H_2SO_4 (acid source)

K + PO_4 = KOH (alkali source) + H_3PO_4 (acid source)

C = Centrifugation

F = Microfiltration

B.2 Energy calculation

The main energy consumption in the process is from drying. To simplify the calculation, only thermal energy requirement was calculated. Electricity requirement was estimated at 2% of total thermal energy requirement.

Specific heat capacity of water = 0.0042 MJ/kg.K

Specific heat capacity of seed = 0.0017 MJ/kg.K

Heat of evaporation at 60°C = 2.36 MJ/kg-water

Table B.6 Thermal energy requirement for processing 1 tonne (wet) seed per day, seeds are not dehulled, extraction at 25°C, centrifugation.

Unit process	Mass going in and out (kg)				T (°C)		Thermal energy (MJ)
	Inlet		Outlet		Inlet	Outlet	
Pre-drying	Seed	1000	Dry seed	897	25	60	391
			Water (loss)	103			
Pressing	Dry seed	897	Oil	203	25	95	107
			Oil impurities	19			
			Press cake	658			
			Water (loss)	17			
Sub-total oil production							**497**
Mixing tank	Press cake (93% dw)	658	Slurry	3306	25	25	0
	NaOH	14					
	Water	2634					
Filter	Slurry	3306	Extract	1201	25	25	0
			Residue	2105			
Mixer-settler	Extract	1201	Wet concentrate	526	25	25	0
	34% HCl	10	Supernatant (27% dw)	686			
Drying-1	Wet concentrate	526	Protein concentrate (95% dw)	138	25	60	991
			Water (loss)	387			
Drying-2	Residue	2105	Low protein feed (95% dw)	332	25	60	4494
			Water (loss)	1773			
Sub-total protein production							**5485**
Total							**5982**

Table B.7 Energy requirements for processing 1 tonne seed per day with different

Treatment				Energy requirements			
				Oil production			
Oil separation	Extraction T (°C)	Chemicals	Separation	Pre-drying	Pressing	Oil extraction	Electricity
Whole seed pressing	25	Na + Cl	C	391	107	0	10
	25	Ca + SO_4	C	391	107	0	10
	25	K + PO_4	C	391	107	0	10
	25	Na + Cl	F	391	107	0	10
	25	Ca + SO_4	F	391	107	0	10
	25	K + PO_4	F	391	107	0	10
	60	Na + Cl	C	391	107	0	10
	60	Ca + SO_4	C	391	107	0	10
	60	K + PO_4	C	391	107	0	10
	60	Na + Cl	F	391	107	0	10
	60	Ca + SO_4	F	391	107	0	10
	60	K + PO_4	F	391	107	0	10
Dehulled seed pressing	25	Na + Cl	C	367	65	0	9
	25	Ca + SO_4	C	367	65	0	9
	25	K + PO_4	C	367	65	0	9
	25	Na + Cl	F	367	65	0	9
	25	Ca + SO_4	F	367	65	0	9
	25	K + PO_4	F	367	65	0	9
	60	Na + Cl	C	367	65	0	9
	60	Ca + SO_4	C	367	65	0	9
	60	K + PO_4	C	367	65	0	9
	60	Na + Cl	F	367	65	0	9
	60	Ca + SO_4	F	367	65	0	9
	60	K + PO_4	F	367	65	0	9
Dehulled seed extraction	25	Na + Cl	C	0	0	833	17
	25	Na + Cl	F	0	0	833	17
	60	Na + Cl	C	0	0	1187	24
	60	Na + Cl	F	0	0	1187	24

Na + Cl = NaOH (alkali source) + HCl (acid source)

Ca + SO_4 = Lime (alkali source) + H_2SO_4 (acid source)

K + PO_4 = KOH (alkali source) + H_3PO_4 (acid source)

processing options.

(MJ/tonne-raw material)				
Protein production				Total
Drying	Protein extraction	Briquetting	Electri-city	
5485	0	0	110	6102
5475	0	0	109	6091
5540	0	0	111	6158
5651	0	0	113	6272
5641	0	0	113	6261
5706	0	0	114	6328
5159	486	0	113	6265
5151	486	0	113	6257
5200	487	0	114	6308
4977	486	0	109	6080
4969	486	0	109	6072
5018	487	0	110	6123
2707	0	138	57	3342
2702	0	138	57	3337
2734	0	138	57	3370
2789	0	138	59	3426
2784	0	138	58	3421
2816	0	138	59	3454
2546	240	138	58	3422
2542	240	138	58	3418
2567	240	138	59	3444
2457	240	138	57	3331
2453	240	138	57	3327
2477	240	138	57	3352
4190	0	162	87	5289
4144	0	162	86	5241
3720	0	162	78	5170
3656	0	162	76	5106

C = Centrifugation

F = Microfiltration

B.3 Economic calculation

Table B.8 Estimated equipment cost for processing 1 tonne seed per day (Figure 6.1).

Equipment	Capacity	Estimated price[a] (USD)
Seed dryer	4 kg-H_2O/h	5 000
Oil press	120 kg/h	10 000
Stirring tank 1	3.5 m^3	6 000
Stirring tank 2	1.5 m^3	3 500
Filter press	450 kg-slurry/h	8 000
Centrifuge	140 kg/h	18 500
Product dryer	275 kg-H_2O/h	30 000
Total		81 000

[a] Estimated based on an online equipment cost estimator http://www.matche.com/equipcost/Default.html[1], except for oil press that was estimated based on Abdul *et al.*[2].

The following assumptions were applied for the calculation of processing cost:

- The plant processes 200 tonnes seed/year.
- Heat energy is 50% recycled.
- Coal is used as energy source, heating value 24 GJ/tonne, coal price USD 63/ton[3].
- Electricity price was estimated from Ulrich and Vesudevan[4].
- Price of protein-containing fractions was estimated based on crude protein content[5].
- Chemical price was taken from ICIS[6].
- Capital cost was calculated for annual 10% depreciation.
- Labour cost was calculated as 20% of the total processing cost in the process with whole seed press, 25°C, NaOH+HCl, centrifugation. The cost was normalised to the amount of raw material and similar value was used for all processes.
- Liquid fraction (filtrate/supernatant) is treated as wastewater at the cost of USD 1/tonne (estimated from Ulrich and Vesudevan[4]).
- Oil price was estimated at USD 786/tonne based on rapeseed oil price[7].

Table B.9 Processing costs and potential products from processing 200 tonnes seed per year (not dehulled, extraction at 25°C, centrifugation).

Processing cost					Product				
Item	**Unit**	**Quantity**	**Unit cost (USD)**	**Cost (USD)**	**Item**	**Unit**	**Quantity**	**Market price (USD/tonne)**	**Revenue (USD)**
Seed	tonne	200	198	39 666	Protein concentrate	tonne	28	434	12 019
Chemicals:					Low protein feed	tonne	66	149	9 916
• NaOH	tonne	3	400	1 159	Oil	tonne	41	786	31 833
• 34% HCl	tonne	3	90	233	Supernatant (27% dw)	tonne	138	-1	-138
Heat	GJ	598	3	1 795					
Electricity	MWh	7	100	665					
Capital cost	- (bulk)	1	8 100	8 100					
Labour	person-year	4	3 250	13 000					
Total cost				64 547	Total revenue				53 630
Margin									**-10 917**

Table B.10 Processing costs and potential products from protein extraction of 200 tonnes seed

Treatment				Processing cost (USD)					
Oil separation	**Extraction T (°C)**	**Chemicals**	**Separation**	**Raw material**	**Chemicals**	**Energy**	**Capital cost**	**Labour**	**Total**
Whole seed pressing	25	Na + Cl	C	39 666	1 392	2 459	8 100	13 000	64 618
	25	$Ca + SO_4$	C	39 666	270	2 455	8 100	13 000	63 492
	25	$K + PO_4$	C	39 666	4 312	2 482	8 100	13 000	67 560
	25	Na + Cl	F	39 666	1 392	2 528	6 750	13 000	63 336
	25	$Ca + SO_4$	F	39 666	270	2 524	6 750	13 000	62 210
	25	$K + PO_4$	F	39 666	4 312	2 550	6 750	13 000	66 279
	60	Na + Cl	C	39 666	1 508	2 525	8 100	13 000	64 800
	60	$Ca + SO_4$	C	39 666	312	2 522	8 100	13 000	63 600
	60	$K + PO_4$	C	39 666	4 521	2 542	8 100	13 000	67 830
	60	Na + Cl	F	39 666	1 508	2 450	6 750	13 000	63 375
	60	$Ca + SO_4$	F	39 666	312	2 447	6 750	13 000	62 175
	60	$K + PO_4$	F	39 666	4 521	2 468	6 750	13 000	66 405
Dehulled seed pressing	25	Na + Cl	C	39 666	687	1 347	7 700	13 000	61 650
	25	$Ca + SO_4$	C	39 666	133	1 345	7 700	13 000	61 095
	25	$K + PO_4$	C	39 666	2 128	1 358	7 700	13 000	63 103
	25	Na + Cl	F	39 666	687	1 381	6 500	13 000	60 484
	25	$Ca + SO_4$	F	39 666	133	1 379	6 500	13 000	59 928
	25	$K + PO_4$	F	39 666	2 128	1 392	6 500	13 000	61 937
	60	Na + Cl	C	39 666	744	1 379	7 700	13 000	61 740
	60	$Ca + SO_4$	C	39 666	154	1 378	7 700	13 000	61 148
	60	$K + PO_4$	C	39 666	2 231	1 388	7 700	13 000	63 236
	60	Na + Cl	F	39 666	744	1 343	6 500	13 000	60 503
	60	$Ca + SO_4$	F	39 666	154	1 341	6 500	13 000	59 911
	60	$K + PO_4$	F	39 666	2 231	1 351	6 500	13 000	61 999
Dehulled seed extraction	25	Na + Cl	C	39 666	9 543	2 132	9 450	13 000	73 041
	25	Na + Cl	F	39 666	9 543	2 113	8 250	13 000	71 822
	60	Na + Cl	C	39 666	9 622	2 084	9 450	13 000	73 073
	60	Na + Cl	F	39 666	9 622	2 058	8 250	13 000	71 847

Na + Cl = NaOH (alkali source) + HCl (acid source)
$Ca + SO_4$ = Lime (alkali source) + H_2SO_4 (acid source)
$K + PO_4$ = KOH (alkali source) + H_3PO_4 (acid source)

per year.

Revenue (USD)					
Protein concentrate	**Low protein feed**	**Oil**	**Briquette**	**Supernatant/ filtrate**	**Total**
12 019	9 916	31 833	0	-138	53 630
12 019	9 902	31 833	0	-137	53 616
12 019	9 993	31 833	0	-132	53 713
20 529	9 916	31 833	0	-90	62 189
20 529	9 902	31 833	0	-89	62 175
20 530	9 993	31 833	0	-84	62 273
15 900	8 646	31 833	0	-162	56 216
15 900	8 635	31 833	0	-161	56 207
15 900	8 704	31 833	0	-157	56 279
21 737	8 646	31 833	0	-145	62 071
21 737	8 635	31 833	0	-144	62 061
21 738	8 704	31 833	0	-140	62 134
9 777	6 373	31 833	2 871	-68	50 787
9 777	6 366	31 833	2 871	-68	50 780
9 777	6 411	31 833	2 871	-65	50 827
16 049	6 373	31 833	2 871	-44	57 081
16 049	6 366	31 833	2 871	-44	57 075
16 049	6 411	31 833	2 871	-42	57 123
12 654	5 746	31 833	2 871	-80	53 024
12 654	5 741	31 833	2 871	-79	53 020
12 654	5 775	31 833	2 871	-78	53 056
16 645	5 746	31 833	2 871	-72	57 023
16 645	5 741	31 833	2 871	-71	57 018
16 645	5 775	31 833	2 871	-69	57 055
10 363	7 339	17 685	3 377	-66	38 765
15 494	7 339	17 685	3 377	-61	43 896
10 875	6 408	17 685	3 377	-109	38 346
16 415	6 408	17 685	3 377	-100	43 885

C = Centrifugation

F = Microfiltration

B.4 Price Estimation

Table B.11 Oil content and price of plant oil feedstocks.

Plant oil feedstock	Oil content[a] (% dw)	Price (USD/tonne)	References
Cottonseed	18-25	195	8
Linseed	40-44	349	8
Jatropha seed	35-40	100	9
		190	10
Oil palm fresh fruit bunch	30-60	153	10
		56	11
Peanuts	45-55	480 – 1 339	7
		2 023	3
Rapeseed	38-46	412	7
Soybean	15-20	361 –428	7
		355	3
		169	10
Sunflower seed	25-35	447	7

[a] Atabani *et al.*[12]. Median values were used for Figure 6.7.

Table B.12 Feed ingredients prices in Indonesia[13,a].

Ingredient	Dry weight (%)	Crude protein (%)	Price (USD/kg)
Alfalfa leaves	91	20	115
Bamboo leaves	91	4	12
Banana frond	85	4	12
Banana leaves	95	6	12
Beer yeast	89	35	31
Beet molasse	90	7	123
Bermuda grass	91	12	12
Blood meal	91	81	385
Bone flour	97	12	192
Broken rice	90	9	138
Brown rice	90	8	231
Buffelgrass	89	11	12
Cane molasse	89	3	123
Cashew seed coat	97	12	231

Continued on next page

Table B.12 *Continued from previous page*

Ingredient	Dry weight (%)	Crude protein (%)	Price (USD/kg)
Cassava leaves	89	20	154
Cassava peel	92	6	12
Chicken manure (dried)	91	16	69
Chicken manure (fermented)	91	14	31
Coconut leaves	92	7	12
Coconut meal	91	19	192
Coconut residue	86	22	92
Concentrate for cattle (beef)	87	15	131
Concentrate for cattle (dairy)	88	16	115
Concentrate for chicken (broiler)	89	41	269
Concentrate for chicken (layer)	91	32	192
Corn bran (coarse)	94	11	154
Corn bran (fine)	97	8	154
Corn cob meal	91	4	223
Corn epiderm	87	8	192
Corn germ meal	99	20	385
Corn gluten feed	95	23	308
Corn husk	93	8	12
Corn stalk 34-56	91	11	15
Corn stalk 56-70	92	10	15
Corn stalk 99-112	91	9	15
Corn stover	94	3	27
Corn straw	86	7	12
Corn (whole, yellow)	89	9	231
Cottonseed meal	92	25	154
Elephant grass	90	9	12
Feather meal	93	85	308
Fermented mother liquor (liquid)	39	47	462
Fermented mother liquor (solid)	83	60	577
Fish (dried)	98	44	231
Fish flour (imported)	91	65	308
Fish flour (local)	91	50	346
Gaplek (dried cassava)	91	2	131
Gliricidia bud	90	23	38
Gliricidia flower	90	19	38
Grass (*Brachiaria decumbens*)	91	10	12
Grass (*Brachiaria mutica*)	89	11	12
Green bean	87	24	231
Groundnut hull	91	6	23

Continued on next page

Table B.12 *Continued from previous page*

Ingredient	Dry weight (%)	Crude protein (%)	Price (USD/kg)
Groundnut meal	92	42	269
Groundnut straw	91	11	12
Guinea grass	92	9	12
Imperata grass	92	7	12
Jaragua grass	92	6	12
Mealworm	92	24	35
Meat bone meal	90	52	385
Meat flour	91	57	385
Mung bean	89	21	269
Mung bean straw	91	16	12
Onggok (cassava residue)	90	4	50
Pangola grass	92	8	12
Pollard	91	17	169
Promix	80	23	385
Palm kernel meal	91	14	308
Rhodes grass	90	10	12
Rice bran A	91	12	135
Rice bran B	88	10	123
Rice bran C	89	9	100
Rice straw	90	6	8
Rubber seed meal	90	24	192
Rumen	93	16	138
Rumen (hydrolysed)	90	16	69
Sesame seed meal	90	45	154
Snail flour	91	61	154
Snap pea	88	22	231
Sorghum	90	10	192
Sorghum straw	90	5	12
Soy sauce residue (solid)	87	32	385
Soy sauce residue (liquid)	81	2	192
Soybean curd residue	90	19	46
Soybean groat	88	39	215
Soybean meal	86	44	423
Soybean seed coat	n.a.	11	192
Soybean straw	90	12	12
Sweet potato leaves	87	14	19
Torula yeast	90	48	8
Water spinach (dried)	89	5	173
Wheat	88	11	269

[a] USD 1 = IDR 13000.

B.5 Optimised Routes

Table B.13 Assumptions for the optimised cases for three type of process: (A) whole seed pressing + extraction, (B) dehulled seed pressing + extraction, and (C) dehulled seed pressing + detoxification.

Parameter	Unit	General case	Indonesian case
Rubber seed price	USD/tonne	145[a]	115[2]
Natural gas price	USD/GJ	7[b]	9[c]
Electricity cost	USD/MWh	146[d]	100[14]
Capital costs:			
- Process A	USD/year	5000	5000
- Process B		4250	4250
- Process C		3750	3750
Labour costs	USD/year	13000	6720[e]
Oil price	USD/tonne	656[f]	625[f]
Protein concentrate price:			
- A (25% protein)	USD/tonne	319	167
- B (45% protein)		505	261
Low protein feed price			
- A (6% protein)	USD/tonne	149	81
- B (11% protein)		194	104
Detoxified press cake price	USD/tonne	351	192[g]
Wood chip price (for fibreboard)	USD/dry-tonne	100[15]	70[16]
N-containing filtrate price	USD/tonne-N	635	301

[a] Median price for estimated Jatropha seed price.

[b] Price for Russian natural gas[3].

[c] Price for Indonesian natural gas[3].

[d] Estimated from Ulrich and Vesudevan[4] for electricity from natural gas.

[e] Labour cost was estimated based on Central Kalimantan minimum regional wage[17]: USD 140 per month/worker and 4 workers employed.

[f] Palm oil price[7,16].

[g] From Table B.12[13].

Table B.14 Processing costs and potential products from processing 200 tonnes seed per (B) dehulled seed pressing + extraction, and (C) dehulled seed pressing + detoxification.

Case	Process[a]	Processing cost (USD)					
		Raw material	Chemi-cals	Energy[b]	Capital cost	Labour	Total
General	A	29 000	270	1 821	5 000	13 000	49 091
	B	29 000	133	544	4 250	13 000	46 927
	C	29 000	0	276	3 750	13 000	46 026
Indonesia	A	23 000	270	1 743	5 000	6 720	36 733
	B	23 000	133	373	4 250	6 720	34 476
	C	23 000	0	189	3 750	6 720	33 659

[a] Extraction is performed at 25°C, separation using microfiltration. Sun or air drying is applied to protein concentrate, detoxified press cake, and low protein feed until 30% moisture content.
[b] Natural gas was used as the energy source for process with pressed whole seed and rubber seed hull was used as the heat source for process with pressed dehulled seed.
[c] Protein concentrate for process with protein extraction and detoxified press cake for process with detoxification.
[d] The remaining hull that is not used for burning is sold as wood chip for fibre board production.

year, optimised cases for three type of process: (A) whole seed pressing + extraction, **dehulled seed pressing + extraction, and (C) dehulled seed pressing + detoxification.**

Revenue (USD)						Margin (USD)
Protein product[c]	Low protein feed	Oil	Fibre-board[d]	Filtrate	Total	
20 529	9 902	26 568	0	-89	56 910	7 819
16 049	6 366	26 568	6035	-44	55 017	8 090
20 095	0	26 568	5971	174	52 808	6 782
10 737	5 356	25 313	0	-89	41 316	4 583
8 301	3 394	25 313	4224	-44	41 231	6 755
10 994	0	25 313	4180	82	40 487	6 828

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Summary

Biorefinery is the sustainable processing of biomass into a spectrum of biobased products and bioenergy. With a biorefinery approach, the existing use of biomass for food, feed, traditional biofuels, and biomaterials is expanded to biobased chemicals, advanced biomaterials, and transportation biofuels. Proteins are available as a fraction in food and non-food biomass sources. The latter includes agricultural residues e.g. biofuels production residues, leaves, grass, stover, microalgae, and animal slaughter waste. Protein-based biorefinery using agricultural residues can increase protein availability from non-food sources for multiple applications. The objective of this research was to design a process for the recovery of proteinaceous fractions from rubber tree. The aimed applications were expected to be suitable for local use, particularly in Indonesia, being one of the world's largest rubber producers.

Rubber trees are mainly grown for their latex that can be processed into various rubber products in well-established industrial processes. Utilisation of protein fraction from rubber tree has not yet received much attention. The objective of chapter 2 was to identify the availability, possible applications, and economic potential of proteins that are present in different parts of the rubber tree. Streams with promising protein content were identified based on field visits, interviews, and literature. Utilisation of protein in the latex is not economically feasible at this moment, but may be feasible when specific use of the latex protein(s) with high value can be identified. Next to latex, the seeds and leaves have promising protein contents. It was estimated that annually, 21-144 kg-protein/ha can be obtained from seeds and 380 kg-protein/ha can be obtained from leaves. Commercial processes to obtain proteins from these parts are not yet available and processes to isolate proteins from the seeds and the leaves are therefore proposed. In the subsequent chapters, rubber seeds were selected as the model biomass since the protein extraction can be incorporated within a biorefinery concept that produces biodiesel as its main product.

Rubber seed kernel contains 48-50% oil and 17% protein (nitrogen-to-protein conversion factor = 5.7). The objective of chapter 3 was to obtain high amounts of protein from rubber seed kernel, without major losses in oil recovery. Prior to protein extraction, the oil can be separated either by pressing yielding press cakes (20-23% protein) as residue or by solvent extraction yielding meals (24-29% protein) as residue. Influences of process variations in pre-treatment, oil separation method, and protein extraction temperature on protein recovery were investigated. Using alkaline extraction, up to 80% protein from the total original amount of protein in the kernel could be recovered in the extract, comparable to protein recoveries from other oilseeds and oilseed cakes. Seed type and pre-treatment had the most influence on protein recovery. Due to the high moisture content in the kernel, pre-drying is a necessary step before oil separation. Increasing the pre-drying temperature from 60°C to 105°C tends to decrease protein recovery from press

cakes and meals. This decrease in protein recovery may be attributed to protein denaturation at high temperature, resulting in protein coagulation and a decrease in solubility. Solubility decrease may also have caused the low protein recovery from meals, due to the use of high temperature and solvent during oil recovery. Increasing the protein extraction temperature from 25°C to 60°C slightly increased protein recovery from the press cake. Oil and protein can also be extracted simultaneously during alkaline extraction of the full-fat kernel, albeit at lower oil recovery compared to solvent extraction and even compared to pressing. Protein recovery from combined extraction, on the other hand, was not hindered by oil presence and comparable to protein recovery from the press cakes and higher than protein recovery from the meal. This can be an alternative for processes aiming only for protein in the kernel.

Following protein extraction, the extracted proteins were recovered via isoelectric precipitation, resulting in rubber seed protein concentrate that can be used as such or can be processed further. Enzymatic protein hydrolysis is a method that can be used to obtain amino acids from protein-rich materials. Amino acids can be applied in food and feed, or used in production of nitrogen-containing chemicals. For these applications, not only degree of hydrolysis, but also hydrolysis selectivity is important. Selectivity can be achieved by selection of starting material, selection of hydrolysis conditions, and separation of the amino acids in the hydrolysate. In chapter 4, hydrolysis selectivity towards hydrophobic amino acids was investigated using different protease mixtures. Hydrophobic selectivity was defined as the amount (on molar-base) of free hydrophobic amino acids: phenylalanine, leucine, isoleucine, tyrosine, tryptophan, valine, methionine, and proline, relative to the total free amino acids. Experiments with rubber seed protein concentrate (48% protein) were performed using different combinations of proteases: Alcalase 2.4L FG, Validase FP Concentrate, Validase FP Concentrate + Peptidase R, and Pronase + Peptidase R. After 24 h hydrolysis of rubber seed protein, 52-53% degree of hydrolysis and 32-35% protein recovery as free amino acids were observed. Only the experiment with Alcalase yielded lower values. The highest hydrophobic amino acid selectivity was obtained after hydrolysis with Pronase + Peptidase R. Selectivity increased from 0.35 mol-hydrophobic/mol-total amino acids in the starting material to 0.6 mol/mol in the hydrolysate after 24 h. Hydrolysis in 10-50% ethanol was also performed to establish ethanol influence on hydrophobic selectivity. No difference in hydrophobic selectivity was observed, however, a different hydrolysate profile was achieved that may be interesting to investigate further.

The result of protein hydrolysis is a mixture containing multiple amino acids. A separation process is required to obtain pure amino acids, e.g. for bulk chemicals production. The objective of chapter 5 was to develop an energy-efficient method for amino acids separation from aqueous systems containing a mixture of amino acids. Ethanol was used

as an anti-solvent for selective precipitation of amino acids. In a water-ethanol system, some amino acids had lower solubility in mixtures than as a single component, thereby facilitating precipitation. Ethanol (90% or higher) was able to selectively increase the hydrophobic amino acid fraction in rubber seed protein hydrolysate from 59% (mol/mol) in the starting material to 76% in the supernatant. Leucine and valine contributed most to this increase. The results show that ethanol precipitation can be applied as a pre-treatment to separate mixture into groups of amino acid or as a polishing step to increase purity.

The results of this study confirm that rubber seed can be an alternative source of proteinaceous products within the framework of biorefinery. In chapter 6, the techno-economic feasibility of rubber seed processing and the applications of protein fractions were discussed. It shows that processing the seeds only for the oil is not economically feasible and additional revenue from the protein fraction is needed to improve the economics of oil production. The most likely potential application of the protein fraction for the farmers is using the rubber seed protein concentrate for animal feed. In an industrial setting, the proteins can potentially be used for technical applications. Experiments were also performed on other materials e.g. wheat gluten and grass juice, suggesting some of the conclusions from this study might be extended to other agricultural residues with similar properties.

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When I started my PhD, I also happened to start reading Douglas Adams' The Hitchhiker's Guide to the Galaxy. The most important lesson I learned from the book was printed on the cover: Don't Panic. It is an important advice when the Earth is about to be demolished and it is also an important advice for a PhD student. My journey was not panic-free, nor was it as adventurous as Arthur Dent's, but it was an exciting journey nonetheless. And for the people who made the journey possible, I want to express my gratitude.

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About the Author

List of Publications

Peer reviewed

Widyarani; Ratnaningsih, E.; Sanders, J. P. M.; Bruins, M. E. Biorefinery methods for separation of protein and oil fractions from rubber seed kernel. *Ind. Crops Prod.* **2014**, *62*, 323–332.

Widyarani; Sari, Y. W.; Ratnaningsih, E.; Sanders, J. P. M.; Bruins, M. E. Production of hydrophobic amino acids from biobased resources: wheat gluten and rubber seed proteins. Accepted for publication.

Widyarani; Coulen, S.; Sanders, J. P. M.; Bruins, M. E. Valorisation of proteins from rubber tree. Submitted.

Widyarani; Bowden, N. A.; Kolfschoten, R. C.; Sanders, J. P. M.; Bruins, M. E. Precipitation of amino acids from agro-industrial residues. Submitted.

Oral/poster presentations

Widyarani; Sanders, J. P. M.; Bruins, M. E. Isolation and valorization of peptides and amino acids from the rubber, oil palm and Jatropha tree. Agriculture beyond Food PhD Conference; Wageningen, the Netherlands, **2011**.

Widyarani; Ratnaningsih, E.; Sanders, J. P. M.; Bruins, M. E. Biorefinery methods for separation of protein and oil fractions from rubber seed kernel. 9th International Conference on Renewable Resources & Biorefineries; Antwerp, Belgium, **2013**.

Widyarani; Coulen, S.; Ratnaningsih, E.; Sanders, J. P. M.; Bruins, M. E. Valorisation of rubber tree proteins. 7th Open Science Meeting; Makassar, Indonesia, **2014**.

Overview of Completed Training Activities

Discipline-specific courses and meetings

- Thermodynamics for the Process Technology Course, OSPT, Delft, 2010.
- International Conference on Jatropha Curcas, University of Groningen, Groningen, 2010.
- Biorefinery products: Renewable resources for the bulk chemical industry, WUR/ORC, Wageningen, 2011
- Agriculture beyond Food PhD Conference, NWO-WOTRO, Wageningen, 2011.
- Chemical Product Centric Sustainable Process Design Course, OSPT, Enschede, 2011.
- Sustainability Analysis in Food Production Course, VLAG, Wageningen, 2011.
- Advanced Course on Downstream Processing, BSDL, Delft, 2011.
- 9th International Conference on Renewable Resources & Biorefineries, Ghent University, Antwerp, 2013.
- 7th Open Science Meeting, KNAW, Makassar, 2014.

General courses

- Advanced Statistics, WUR/MAT, 2010.
- PhD Week, WUR/VLAG, 2011.
- Competence Assessment, WUR/WGS, 2011.
- Mobilising your scientific network, WUR/WGS, 2011.
- Techniques for Writing and Presenting Scientific Papers, WUR/WGS, 2012.
- C2R- from Concept to Reality: Technology Introduction in Rural Areas, University of Groningen, 2014.

Optional activities

- Writing of research proposal, WUR/VLAG, 2010-2011.
- Theme Meeting (discussion group BCH), WUR/BCH, 2010-2013.
- AbF-Breakthrough in Biofuels Project meeting, 2010-2014.
- Agriculture beyond Food Mid-term Workshop, NWO-WOTRO, 2012.
- PhD excursion to Brazil, WUR/BCH, 2013.

Curriculum Vitae

Widyarani was born on 21 March 1979 in Sumedang, West Java, Indonesia. She studied Environmental Engineering in Institut Teknologi Bandung and received her bachelor degree in 2003. After trying several jobs including building manager assistant, trainer for water sanitation personnels, and television writer, in 2005 she decided to join Indonesian Institute of Sciences (LIPI) as a research staff at Research Centre for Chemistry. Her research was mainly on wastewater treatment. In 2007, she started her Master study in Wageningen University with a scholarship from Nuffic. She graduated in 2009 with a final thesis on anaerobic digestion and a minor thesis on microalgae photobioreactor. Starting 2010, she conducted her PhD research within Biobased Chemistry and Technology Group (formerly Valorization of Plant Production Chains), Wageningen University, and the results of the research are presented in this thesis. She currently returns as a research staff at LIPI and she will do research on products recovery from waste streams. She can be reached at widyarani@lipi.go.id.

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Sankey diagrams (Figure 1.3–1.5) were created using SankeyMATIC (www.sankeymatic.com).
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