Biorefinery of leafy biomass using green tea residue as a model material

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CHAPTER 1: INTRODUCTION

"TWICE THE FOOD PRODUCTION AT HALF THE ECOLOGICAL FOOTPRINT" BY 2050

1.1 "Twice the food production at half the ecological footprint" by 2050

Food and environment play very important roles in human survival and development. With the rapidly growing world population and improving living standards, food demand is increasing daily at a high rate. Today, for a better environment, human activities must have less impact on the earth's ecosystems. "Twice the food production at half the ecological footprint" is the goal for 2050 to feed the world (Nellemann et al., February 2009); however, in fact, not all countries need a food production boost.

The two major factors that influence food demand are population and economy, suggesting that the increase in food demand is mainly from developing countries. African countries are expected to have a population boost, followed by countries in Asia and America. Nigeria and Kenya, for example, are expected to have a population increase of 176% and 138%, respectively by 2050 (2013). India, Mexico, and Brazil are expected to have relatively low increases in population growth rate, ranging from 17% to 34% (2013). However, owing to their large populations, an increase of 3 billion people is still expected from these three countries by 2050. Other than an expected population boost, African, Asian, and Latin American countries also have rapid economic growth rates (Table 1.1) that are above the world average, which further suggests an increased food demand in these countries in the future. With the implementation of the "one-child

Table 1.1 Variation of gross domestic product in selected regions (% change compared to the previous year)

Regions	2011	2012	2013	2014	2015
World	4.2	3.4	3.4	3.4	3.5
Emerging and developing Asia	7.7	6.8	7.0	6.8	6.6
European Union	1.8	-0.4	0.1	1.4	1.8
Latin America and the Caribbean	4.9	3.1	2.9	1.3	0.9
Emerging and developing Europe	5.4	1.3	2.9	2.8	2.9
Middle East and North Africa	4.5	4.9	2.3	2.4	2.7
Sub-Saharan Africa	5.0	4.2	5.2	5.0	4.5
Australia	2.7	3.6	2.1	2.7	2.8
USA	1.6	2.3	2.2	2.4	3.1
UK	1.6	0.7	1.7	2.6	2.7
China	9.3	7.8	7.8	7.4	6.8
India	6.6	5.1	6.9	7.2	7.5

Source: International Monetary Fund. Download URL: http://knoema.com/IMFWEO2015Apr/imf-world-economic-outlook-weo-april-2015.

policy", China is expected to have only a 2% increase in population by 2050 (2013). Neverthelss, the driving force of food demand is the improving living standards of the Chinese population, because of an increased average economic rate of 7.8% over the past five years (OECD and FAO Secretariats). Developed countries, except the USA, are expected to experience a decline in population with steady economic increase, indicating a small contribution to the increasing food demand in the future.

Attitudes of governments to the "food crisis" debate depend not only on the increasing food demands, but also on food productivity. The agriculture gross production values of selected regions and countries are presented in Table 1.2. As shown, developed countries in Europe, Oceania, and North America produce more food than they demand. To balance food prices in worldwide trade, some agricultural products are used to produce fuels, thereby generating a debate on food and biofuel (Pimentel et al., 2008). Agriculture gross product values of China and Brazil are quite similar. However, owing to the potential for increase in food production, Brazil (which has a higher potential) tends to develop biofuel technologies, whereas China, which has limited farmlands, is still looking for additional food sources to feed the next generation (OECD and FAO Secretariats, 2013). Most countries in Asia and Africa have low agricultural production, which is only half of the world average. Owing to the limited number and poor quality of farmlands, these countries suffer from a food shortage. Increased food production is therefore a priority of these countries.

Table 1.2 Agricultural gross production value USD/person

	2011	2012	2013
World	306	307	312
Oceania	846	898	878
Europe	526	499	521
Asia	266	272	273
Americas	435	435	453
Africa	171	175	180
USA	605	585	605
Brazil	432	422	453
China	432	446	451
India	152	153	157

Source: FAO.

Nevertheless, although only some counties require a boost in food production by 2050, "twice the food production at half the ecological footprint" is the goal for all countries worldwide. Looking back on the history of agriculture, a boost in agricultural production resulted from an increase in either productivity or farm lands, and it also came with its share of environmental and health threats. The three primary factors that led to recent

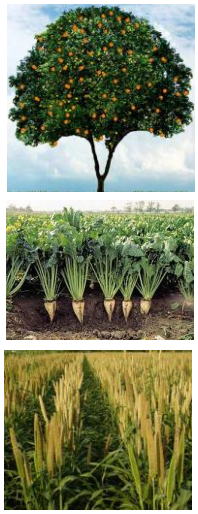
increases in worldwide crop production are increased cropland and rangeland area (15%), increased yield (78%), and greater cropping intensity (7%) (Alexandratos and Bruinsma, 2012; Diouf, 2002). Over-farming and overuse of fertilizer have already resulted in a 5% degradation of arable land (Nellemann et al., February 2009). Overuse of pesticide not only affects biodiversity, but also poses a threat to human health (Nellemann et al., February 2009). The effects of some agricultural practices on the environment in developing countries can spread to all other countries in terms of climate change, air/ocean pollution, etc.

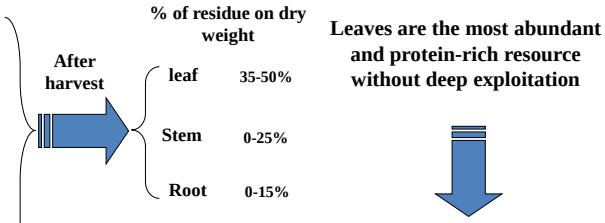
In fact, many developed countries are assuming the responsibility of looking for new food sources and technologies that can increase food production with less environmental impact. The concept of a "Bio-economy" was therefore proposed to fulfil the target of "twice the food production at half the ecological footprint". Using biorefinery technologies on unused biomass to produce more food or animal feed could be a solution to compensate for the inadequacy of the food supply.

1.2 To fulfil the demand – Biorefinery of leafy biomass

Residual biomass, including leaf, stem, and/or root, are often abandoned after agricultural products are harvested. Leaves, which may account for 35–50% dry weight of the whole plant, are the most valuable biomass that can be obtained from all types of plants (Fig. 1.1).

The composition of leaves differ as a result of adaption to environmental conditions, including climate and available light, and other factors such as grazing animals, available nutrients, and ecological competition from other plants (James and Bell, 2000). Through the process of photosynthesis, leaves become rich in protein, minerals, and sugars, whereas through the structure and transportation of nutrients, leaves also require large amounts of lignocellulose for biofuel production. To adapt to various environmental conditions, structural and functional components of leafy biomass are needed, such as pigments, lipid, polyphenol, and pectin (Dashek and Harrison, 2006a; Haslam and Cai, 1994).





Plant Species	Dry matter yield Mg ha^{-1} $year^{-1}$	Protein% of dry matter
Rapeseed (Telek, 1983)	28	25.4
butterfly pea (Telek, 1983)	28	24
Goa bean (Telek, 1983)	20	22.2
Mung bean (Telek, 1983)	16	22.9
Cassava leaf (Ravindran, 1993)	4.64	20.5
Sugarcane leaf (Chandel et al., 2012; Devi et al., 1965)	6-8	10
Olive leaf (Martín García et al., 2003)	-	7

Fig. 1.1 Residual biomass after harvesting and protein production yield of different leaf species.

Leafy biomass is considered a good food source due to its production yield and high protein content. Dry matter yield of bean leaves range from 10–30 ton ha^{-1} $year^{-1}$, with protein content between 18% and 30% (Telek, 1983) (Fig. 1.1). In comparison, soybean production is 3 ton ha^{-1} $year^{-1}$ (Cordonnier, November 1, 2013) with protein content of 30–40% (Wolf et al., 1982), which is only about a quarter of the production of their leaves. In addition to soybean, protein production yields of leaves from other commercial agricultural plants, such as cassava (Ravindran, 1993), sugarcane (Chandel et al., 2012; Devi et al., 1965), and olive (Martín García et al., 2003), are also considerable (Fig. 1.1). The leaves of many species can be found in developing countries (Telek, 1983), in which developments of leaf biorefinery technologies can directly contribute to the food supply, where a food production boost is required to feed an increasing population.

1.3 Products from leafy biomass

1.3.1 Upgrading the value of leaf components

Current and potential applications of leaf components are illustrated in Fig. 1.2. More than 90% of leafy biomass, such as fallen leaves from trees, is used in burning or compositing, which are among the lowest categories of biomass applications. Although

leafy biomass can be applied to the production of second-generation ethanol (Taherzadeh and Karimi, 2008), few efforts have been successful, which is because of the difficulties encountered in the hydrolysis of lignocellulose (Taherzadeh and Karimi, 2008). Leaf biomass that is used for animal feed or human food is often used entirely for its digestible protein and carbohydrates, which account for only 30% of the total biomass. The functional components of herbs, which likely represent less than 10% on a dry weight basis, are used in medicine and functional beverage. The residues obtained after extraction of functional components are then abandoned or used for their energy through burning. Development of leaf biorefinery technologies by which leafy components are solubilized and fractionated, and used for their highest value can augment the supply of food, animal feed, and chemicals and fuels.

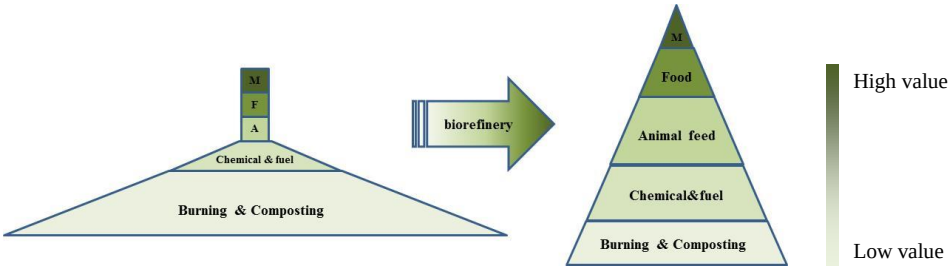


Fig. 1.2 Current (Left) and potential (Right) applications for leaf components.

M: medicine; F: food; A: animal feed.

Although the compositions of leafy biomass are very diverse depending on the plant species and growing period, there are four major components in all species: protein, pectin, lignin, and (hemi-) cellulose (Table 1.3). These four major components account for more than 70% of total dried biomass and can be considered as target products of all leafy biomass. Among these four components, protein and pectin can be used in food and animal feed, which are the key components to supplementing food production. Some leaf biomass, such as tea and olive leaves contain high amount of polyphenols, which can be used in food additives or in medicine as anti-oxidants (Altiok et al., 2008; Figueirinha et al., 2008; Harold N, 1992). Some leaves contain lipid (Bals et al., 2007a; Latif and Müller, 2015), which can be used in food or biodiesel. In addition, minerals in leafy biomass should be considered for recycling as fertilizer.

Table 1.3 Composition of leafy biomasses of several selected plant species (% in dry weight)

Plant species	Protein	Pectin	(Hemi-) cellulose	lignin	Other components
Premna microphylla turcz (Chen et al., 2014)	13	30	34	17	
Peat moss *(Ballance et al., 2012)*	20*	20	31	16.5	
Green tea (Harold N, 1992)	15	5	20	6.5	Polyphenol 36
Switchgrass (Bals et al., 2007a; DeMartini et al., 2013)	7	10*	43	11	Lipid 7
Cassava (very young) (Latif and Müller, 2015)	38	14*	18	1	Lipid 4
Cassava (young) (Latif and Müller, 2015)	29	12*	28	4	Lipid 6
Cassava (mature) (Latif and Müller, 2015)	18	10*	38	8	Lipid 7
Olive (Martín García et al., 2003)	7	7	25	19	Polyphenol 25

* Estimation based on the content of other components.

1.3.2 Protein and pectin

Protein can be used in food (Ghaly and Alkoaik, 2010), animal feed (Kondo et al., 2004), or, when hydrolysed to amino acids, for other applications such as bulk chemicals (Sanders et al., 2007). Proteins are large biological molecules or macromolecules, consisting of one or more long chains of amino acid residues. In food applications, the functionalities of leaf protein, including its foaming, water absorbing, emulsifying, and gelling properties, determine the protein value. In animal feed applications, the digestibility and amino acid composition of leaf protein are very important. Owing to the absence of key enzymes that synthesize certain amino acids, most animals (including humans) must obtain some amino acids from their diet, which can be synthesized by plants (Donald Voet and Voet, 2011). These amino acids, which cannot be synthesized by animals, are referred to as essential amino acids, and they also determine the value of leaf protein. The non-essential amino acids can be separated from animal feed to produce other useful chemicals, such as nitriles for polyamides like Stanyl® (Lammens et al., 2011).

Other than protein, pectin is another major component in leaves that can be used in food applications. Pectin is a structural heteropolysaccharide in the primary cell walls of terrestrial plants. It can be roughly divided into three types: homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII) (Ridley et al., 2001). Owing to its gelling properties, pectin is mainly used as a food additive. Current commercial pectin is mainly obtained from apple pomace and citrus peel (Wang et al.,

2014; Willats et al., 2006). Although leaf pectin constitutes approximately 15% of dried leafy biomass, it is rarely used because of its poor gelling properties and high production costs compared to commercial pectin. Therefore, to improve the market value of leaf pectins, either their structure or molecules should be modified to improve their functionalities, or alternative applications should be found, based on their constituent chemicals, such as galacturonic acid (Lavilla et al., 2012; Lavilla et al., 2011; Muñoz-Guerra, 2012) .

1.3.3 Polyphenol and lignocellulose

The White–Bate–Smith–Swain–Haslam definition (Haslam and Cai, 1994) describes the polyphenol class as: moderately water-soluble compounds with more than 12 phenolic hydroxyl groups and molecular weight of 500–4000 Da, with 5–7 aromatic rings per 1000 Da. Polyphenols have high values in practical uses. Herbal polyphenols are used in food additives or in medicine. Other polyphenols, such as tannins, were used traditionally for tanning leather and today they are used as precursors in green chemistry (Hillis and Urbach, 1959; Pizzi et al., 1994; Polshettiwar and Varma Rajender, 2008).

Lignocellulose, which consists of lignin and (hemi-) cellulose, may account for 30–50% of leafy biomass. Currently, applications of lignocellulose are limited; board, paper, or combustible energy are its major applications (Anwar et al., 2014; Pothiraj et al., 2006). It has huge potential for production of biofuel and bulk chemicals (Anwar et al., 2014; Pothiraj et al., 2006), but has not been very successful owing to the complexity of the structure of lignin and the difficulties encountered in its hydrolysis (Boerjan et al., 2003; Grabber, 2005).

1.4 Challenges of leaf biorefinery

The concept of leaf biorefinery to supplement the food supply had been proposed 70 years ago, but few commercial cases have been successful. The main challenge of leaf biorefinery is its low cost-effectiveness. Developments of leaf biorefinery are not only limited scientifically from unstable raw materials, complex components, and rigid plant cell walls, but also influenced by social factors such as underdeveloped leaf economics.

1.4.1 Variation of leaf composition

Compositions of leafy biomass differ not only by species, but also by growing period (Nagy et al., 1978). Generally, young shoots, as compared to old leaves, have higher concentrations of protein and soluble sugars, whereas old leaves contain higher concentrations of lignocellulose (Latif and Müller, 2015; Waring et al., 1985). Leaves of deciduous plants, for example, change colour from green to yellow, bright-orange, or red, as various accessory pigments (carotenoids and xanthophylls) become evident when the plant responds to cold and reduced sunlight by curtailing chlorophyll production (Feild et al., 2001). The variation in leaf composition of various plant species leads to inconsistencies in leaf biorefinery research and leaf product quality through biorefinery.

1.4.2 Rigidity of plant tissues

Leaves have three major tissue systems: epidermal, mesophilic, and vascular. Vascular tissues are located in mesophyll tissues, covered by epidermal tissue. Those tissues are adhered by the lamella layer, and they contain a large quantity of pectin. The epidermis is a tabular and layered sheet of cells on the surface of the leaf, covered by a waxy cuticle that functions as mechanical protection for mesophyll tissue (Dashek and Harrison, 2006b; Mauseth, 2009). Furthermore, organelles in mesophyll tissues are well protected by the cell walls, which each consist of middle lamella, primary wall, and secondary wall. Besides protein, carbohydrate (including pectin, hemi-cellulose, and cellulose) and lignin are two major components of the cell wall (Dashek and Harrison, 2006b). Pectin is a family of complex polysaccharides located in the primary plant cell wall and middle lamella (Dashek and Harrison, 2006b; Somerville et al., 2004). Hemi-cellulose and cellulose are mainly found in both primary and secondary plant cell walls, and they both have simpler compositions than pectin. In comparison, lignin could be the most complicated component located in the secondary plant cell wall (Dashek and Harrison, 2006b). It is a complex phenolic polymer that drives out water and strengthens the cell wall. The rigidity of the epidermis and the two-layer cell wall increase the difficulty of leaf biorefinery.

1.4.3 Complexity of components

Although the diverse components indicate the potential of leaves for high value applications in food or medicine, they also result in relatively high costs of production (extraction). Unlike commercial agricultural products, such as potato, which may comprise only one or two major components, the amounts of the four major components, protein, pectin, polyphenol, and lignocellulose, are quite similar in leaves (see Table 1.3). The similarity of these four components result in relatively low amounts compared to that of the same components in commercial agricultural products. Furthermore, these four components are entwined in leaf tissues resulting in difficulties in separation. The production cost of obtaining one component from leaves would be 2–3 times higher than that from commercial crops. In addition, during processing, these components may be hydrolysed (Sari et al., 2015a) or may react mutually (Osawa and Walsh, 1993; Whitmore, 1978; Zahedifar et al., 2002) to form new unexpected products.

1.4.4 Undeveloped leaf economics

For commercial crops, the whole chain from plantation to the final product in market is mature. The cost of logistics and the impact on the environment (waste management) are low, whereas the market demands are high. Development of these commercial crops in all fields from the plantation steps to the final products can be fully supported by current mature economics. By contrast, for most species, leaf economics, from harvesting to sales, need to be established.

The main challenge in the undeveloped leaf economics is the logistics system. Logistics is the process of planning, implementing, and controlling the effective and efficient flow of goods and services from the point of origin to the point of consumption (Vitasek, 2013). Considering potato as an example, information and machines for its growth and harvesting can be obtained from related bureaus or companies, and related factories have been built at a reasonable distance to reduce the cost of transportation. Information on potato related products is clear enough for customers, that no extra effect is required to convince them to purchase. Generating products from leaves however, does not establish the production chain only, but the logistics chain as well, encompassing harvest

machinery, transportation, and design to sales. Currently, the poor logistics of leaf biorefinery limit its development and distract investors from relevant research.

1.5 Extraction technologies for leaf components

To overcome the challenges of leaf biorefinery, extraction technologies for specific components and/or specific species of leafy biomass have been investigated (Badar and Kulkarni, 2011; Nie and Xie, 2011; Willats et al., 2006), but only few cases have been transferred successfully to practical production. These successful cases are mainly in the extraction of functional components, such as tea polyphenol or taxol, which can be used as functional food or medicine (Day and Frisvold, 1993; Khan and Mukhtar, 2007). Production of other components, including leaf protein, which may account for 25% of total leafy biomass in dry weight and can be used in food or animal feed, are not economically feasible yet.

1.5.1 Protein extraction

For protein extraction, many technologies are tested, including mechanical press, alkaline extraction, enzymatic extraction, and steam explosion. According to the conditions of processing, pH, temperature, and pressure, these techniques are divided into mild, medium, and severe. Protein products obtained by these conditions can be accordingly applied in food, animal feed, and amino acid related products. Protein yield obtained by these technologies can be higher than 80% from seed biomass (Sari et al., 2015a), but is at present not cost-efficient for leaf protein extraction, which severely impedes the applications of leaf protein. The efficiencies of current protein extraction technologies for the leaves of several species are listed in Table 1.4.

Protein extraction yield is different depending on the species and the practical conditions (Chiesa and Gnansounou, 2011; Dale et al., 2009b; Kammes et al., 2011; Sari et al., 2015b; Telek, 1983). Extraction yield by mechanical pressing is relatively low and varies from 15 to 45% of total protein (Fasakin, 1999; Fasuyi, 2005; Kammes et al., 2011). However, as they are treated by mild conditions, these proteins are regarded as food grade. Alkaline extraction has a better extraction yield that varies from 30 to 55%, which could

be further improved by enzyme assistance or the combination of mechanical disruption (Huang et al., 1971). Proteins can be denatured and hydrolysed in alkaline conditions; therefore, proteins extracted under these conditions are of lower quality and are difficult to be recovered (Bals and Dale, 2011; Chiesa and Gnansounou, 2011). These proteins are suitable to be used as animal feed, or for its amino acids through hydrolysis. Ammonia fibre explosion (AFEX) was suggested to use as a pre-treatment for protein extraction, which may increase protein to higher than 60% (Bals et al., 2007a). Nevertheless, its capital costs are very high due to the requested high pressure and ammonia recycle system. In addition, other techniques have been also tested for protein extraction, such as ultra-sonic and pulsed electric field. All these methods of protein extraction are energy intensive with a low cost-efficiency.

Table 1.4 Protein extraction yield obtained by various technologies on different leafy biomass

Plant species	Method	Protein yield (%, Wprotein)
Orchardgrass	Screw press	15~25 (Kammes et al., 2011)
Water fern	Press and heat	11 (Fasakin, 1999)
Duck weed	Press and heat	10 (Fasakin, 1999)
Alfalfa leaves	Press and heat	38 (Edwards et al., 1975)
Cassava leaf	Screw-press	50 (Fasuyi, 2005)
Switchgrass	Screw press	15~25 (Kammes et al., 2011)
Tea residue	Alkaline extraction enzymatic	58 (Shen et al., 2008) 48 (Shen et al., 2008)
Comptonia peregrina	Alkaline extraction	27.2 (Jones et al., 1989)
Dwarf Elephant Grass	Ammonia	35 (Davison et al., 2005)
Tobacco	Phosphate buffer	18 (Fu et al., 2010)
Orthosiphon aristatus	Tris–sucrose buffer	50 (Koay and Gam, 2011)
Alfalfa leaves	Milling in buffer, acid coagulate	61 (Huang et al., 1971)
Monochoria hastata Solms	Homogenized and filtered	60 (Pandey and Srivastava, 1991)
Switchgrass	Ammonia fiber explosion	68 (Bals et al., 2007a)

1.5.2 Extraction of components other than protein

Pectin extraction with the use of acid, alkaline, buffer solutions, ionic solutions, enzymes, and subcritical water has been studied (Lim et al., 2012; Methacanon et al., 2014; Seixas et al., 2014; Sengkhamparn et al., 2010; Wang et al., 2014; Westereng et al., 2008; Zykwinska et al., 2006). Generally, more than 90% pectin can be extracted by acid extraction, which can be recovered by ethanol precipitation. Pectin can be extracted in its

native form by acid extraction, which means its gelling property can be preserved. Alkali has also been applied in pectin extraction, by which more than 80% of pectin can be extracted (Renard et al., 1990; Zykwinska et al., 2006). Although alkaline extracted pectin retains its structure and the length of its molecular chains, the loss of its degree of esterification reduces the gelling property, and therefore reduces its value in food applications. In addition, enzymatic methods may have the potential for obtaining high yield chemicals derived from pectin, such as galacturonic acid, rhamnose, galactose, and arabinose (Garna et al., 2006; Ridley et al., 2001).

Research on the extraction of lignocellulose is limited. Peer research is more focusing on hydrolysis of lignocellulose using concentrated acid, H_2O_2, or enzymes (Doner and Hicks, 1997; Pothiraj et al., 2006; Taherzadeh and Karimi, 2008). After hydrolysis, lignocellulose is often used as a substrate in a fermentation system to produce biodiesel, bioethanol, or other bulk chemicals (Pothiraj et al., 2006; Taherzadeh and Karimi, 2008).

1.5.3 Integrated biorefinery for multiple products

Integrated processes were considered the best option for leaf biorefinery (Badar and Kulkarni, 2011). The extra costs generated from multiple processes could be covered by higher product quality and relatively low production costs. By obtaining multiple products from various processes, it is possible to reach the highest value of all components in leafy biomass. For example, producing animal feed protein along with fuels and chemicals in a biorefinery context, increased total economic value with relatively lower production costs (Dale et al., 2009b).

The difficulties of integrated biorefinery include the incompatibilities of processes. Extractions of different components from leafy biomass are often studied separately; therefore, their compatibility in an integrated process is rarely tested. Pigments and polyphenol have been extracted by organic solvents (Arvayo-Enriquez et al., 2013; Sybesma et al., 1984; Turkmen et al., 2006); pectin is usually extracted by acid (Lim et al., 2012); and leaf protein extraction by alkali has been suggested (Shen et al., 2008; Zhang et al., 2014). Combining above processes to obtain highest yields of protein, pectin, and polyphenols is technically possible, but it will not be economically feasible.

This is because of the cost of organic solvents, the influence of the solvent in pectin/protein extraction, and the generation of salts from the neutralization reaction. The compatibilities of processes should be investigated and adapted in the research of leaf biorefinery.

1.6 General description of this thesis

1.6.1 Using green tea residue as a starting material

In this study, green tea residue (GTR) was used as a starting material. This decision was made mainly for four reasons: it is produced in developing countries with relatively high yields, its components have high potential economic value, it can be easily recycled from tea factories where it is already gathered and pre-processed for tea production, and it can be supported by local economics.

Table 1.5 Tea production in different countries (k ton dry leaves)

Country	2011	2012	Country	2011	2012
China	1623	1700 F	**Malawi**	52 F	54 F
India	967	1000 F	**Uganda**	35	51
Kenya	378	369	**Tanzania**	32	33
Sri Lanka	328	330	**Myanmar**	31*	32 F
Turkey	222	225	**Mozambique**	27	22
Vietnam	207	217	**Rwanda**	24	23
Indonesia	150	150	**Zimbabwe**	18 Im	19 F
Iran	104	158 F	**Nepal**	17	19
Argentina	97	100 F	**Taiwan**	17	15
Japan	82	86	**Malaysia**	17	17
Thailand	73	75 F	**Azerbaijan**	11	12
Bangladesh	61	62 F			

Source: http://faostat.fao.org/. *: Unofficial figure; F: FAO estimate; Im: FAO data based on imputation methodology.

Tea production is relatively high and it is produced mainly in developing countries (Table 1.5), in which food shortage remains a challenge. In 2011, world tea production reached over 4.7 million tons after having increased by 5% between 2010 and 2011. Production rose by 4% between 2011 and 2012. The greatest amounts of tea were produced in developing countries such as China, India, Kenya, Sri Lanka, and Turkey. Traditionally, tea has been consumed individually, and the residue was considered unrecyclable. However, thanks to the appearance of colossal beverage companies, such

as Coca Cola and Lipton, tea residues are now produced centrally. It is estimated that 350k tons of dry tea residues are generated and can be collected from instant tea factories in China alone .

Tea has been made for hundreds of years, and therefore the tea leaf economic chains have been already formed. Although these chains focus only on the beverages that mainly contains tea polyphenols, it offers a relatively stable raw biomass and convenient logistics if other tea products can be produced. Green tea residue (GTR), used as a model material for this study, contains more than 25% protein represent a waste stream from tea factories, which amounted to 50,000 ton $year^{-1}$ from Damin Company, Fujian Province, China, in 2010. Currently, GTR is only used for energy generation through burning. The aforementioned company is looking for methods to increase the value of GTR that scaling-up production of GTR products that found in this study can be fully supported.

GTR is also an excellent model of a starting material that can be used as an example for the leaves of other species. It has a relatively high protein content (Jayasuriya et al., 1978; Shen et al., 2008) in which at least seven different types of proteins have been discovered, including RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) and glycoproteins (Lin et al., 2009; Shen et al., 2008; YuXiang; and Liu ShenKui, 2005). The superior quality of tea leaf protein in terms of amino acid profile, as compared to soy bean meal, has been documented (Shen et al., 2008). Tea residue also contains relatively high amounts of valuable components other than protein, such as pectin, phenolic compounds (polyphenol and lignin) and (hemi-) cellulose. The value of these compounds makes an integrated biorefinery attractive from both economic and sustainability perspectives.

1.6.2 Objective and research questions

The aim of this study is to develop new processes and applications to optimally utilize all components, particularly protein, of leafy biomass in the feed and/or food industry using green tea residues as a starting material.

The research questions are:

1. Can we use alkaline protein extraction cost-effectively for GTR?

2. How does alkali aid protein extraction?

3. Can protein products be used for animal feed?

4. Are there any pre-treatments that can be used to further improve the cost-efficiency of alkaline protein extraction with the yield of other GTR components?

5. Are these processes technically and economically feasible for sustainable production?

6. Is it possible to upgrade the applications of protein and pectin for human food?

7. Do pre-treatments and alkaline extraction remove or degrade plant toxicants in protein products for animal feed?

8. Can the knowledge gained from work on GTR be applied to the leaves of other species?

1.6.3 Thesis layout

Chapter 1 is the introduction of this thesis.

Chapter 2 describes the critical parameters in alkaline protein extraction that are related to protein extraction yield or production cost from GTR. Nutritional value based on amino acid profile of alkaline protein extracts is investigated. The chapter also shows the applicability of alkaline extraction on the leaves of various species. This chapter will answer the questions 1, 3, and 7.

Chapter 3 shows the variations of GTR in terms of leaf tissues, protein properties, and extraction of non-protein components during alkaline protein extraction. Limiting factors for protein extraction and the possible components that can be extracted prior to protein are discussed. This chapter will answer question 2.

Chapter 4 presents the efficiency of pre-treatments that target selected components according to Chapter 3 and their influence on the subsequent alkaline protein extraction, including the production(yield, purity, and alkali consumption) and protein quality (colour, digestibility, composition, and amino acid profile). This chapter answers

questions 3 and 4.

Chapter 5 introduces the production of leaf pectin and its integration with alkaline protein extraction. Influence of processing techniques on pectin structure and properties are discussed. This chapter supplement the answer to question 4.

Chapter 6 discusses improvements in alkaline extraction by using potassium and/or calcium alkali instead of sodium alkali. Cost-efficiency and sustainability of the alkaline extraction scheme are analysed. This Chapter answers question 5.

Chapter 7 is a general discussion of all our unpublished data. It refers to our understanding of the biorefinery concept, the alkaline extraction mechanism, possible improvements for extraction techniques, and product quality. In addition, the prospects of leaf biorefinery are presented based on our findings and state-of-the-art techniques. This chapter answers questions 1-8.

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CHAPTER 2: CRITICAL PARAMETERS IN COST- EFFECTIVE ALKALINE EXTRACTION FOR HIGH PROTEIN YIELD FROM LEAVES

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Abstract:

Leaves are potential resources for feed or food, but their applications are limited due to a high proportion of insoluble protein and inefficient processing. To overcome these problems, parameters of alkaline extraction were evaluated using green tea residue (GTR). Protein extraction could be maximized to 95% of total protein, and, after precipitation by pH adjustment to 3.5, 85% of extracted protein was recovered with a purity of 52%. Temperature, NaOH amount, and extraction time are the protein yield determining parameters, while pH and volume of extraction liquid are critical parameters for production cost. The cost of energy and chemicals for producing 1 ton GTR proteins is minimized to 102 €, and its nutritional value is comparable to soybean protein. Furthermore, this technology was successfully applied to other sources of biomass and has potential to be used as a part of an integrated biorefinery process.

2.1 Introduction

Leaf protein has been regarded as an additional protein source since 1960s (Akeson and Stahmann, 1965; Badar and Kulkarni, 2011; Gerloff et al., 1965). These proteins can be used in food (Badar and Kulkarni, 2011; Ghaly and Alkoaik, 2010), animal feed (Kammes et al., 2011; Kondo et al., 2004), or when hydrolyzed to amino acids for other applications, such as bulk chemicals (Sanders et al., 2007). Tea leaf residue is one example of a potential new protein source. As a major agro product in China, 1.6 Million tons (dry weight) of tea leaf products were produced in 2011 (FAOSTAT, 2012). Tea producers estimate that around one fifth (dry weight) of the tea residues are produced centrally and can be collected from instant tea factories .Tea residues, which are the waste of tea leaves after hot water extraction, contain 20-30% protein (Jayasuriya et al., 1978; Shen et al., 2008). There are at least seven different types of protein, including Rubisco and glycoproteins (Lin et al., 2009; Shen et al., 2008; YuXiang; and Liu ShenKui, 2005). The superior quality of tea leaf protein in terms of amino acid profile compared to soy bean meal has been documented (Shen et al., 2008).

However, although huge economic potential lies in leaf protein, its applications are severely impeded by low cost-efficient production. Protein extraction yield is relatively low that varies from 15% to 60% of total protein, depending on species and processing methods (Chiesa and Gnansounou, 2011; Dale et al., 2009b; Kammes et al., 2011; Telek, 1983). Furthermore, protein production yields are reduced during recovery, particularly for those processes, such as alkaline extraction, that generate protein hydrolysates (Bals and Dale, 2011; Chiesa and Gnansounou, 2011). Conventional alkaline extraction has already been studied decades ago, but no significant improvement was made in leaf protein extraction. Despite the lower cost, alkaline extraction has the lowest profit among all extraction techniques for leaves primarily due to low protein yield (Bals and Dale, 2011). If protein yield can be increased without increasing cost for extraction, the economic value of leaf protein can be exploited.

The low productivity of alkaline extraction may result from overlooking two points. Firstly, applying high temperature in alkaline extraction has shown to increase protein

yield in some cases (Choi and Markakis, 1981), which is conflicting with the general knowledge that heating results in protein precipitation. Secondly, the influence of solution to raw material ratio (v/w) and alkaline concentration (pH) on protein yield were always studied independently (Harnedy and FitzGerald, 2013; Lestari et al., 2010; Shen et al., 2008), but the influence of alkali amount, which is determined by both v/w and alkaline concentration, was never considered.

In this study, the possibility to increase protein yield at elevated temperature was investigated, followed by an evaluation of the influence of v/w and alkali amount on protein extraction yield. The parameters that involves in alkaline extraction were grouped to protein yield related and cost related, and its economic value was estimated. In addition, the general applicability of new parameter setup was tested by using other materials, like oolong tea residue, grass, and barley straw.

2.2 Materials &Methods

2.2.1 Materials

Green tea residue (GTR) is our main material, which is a gift from Damin Company, Fujian Province, China. This residue from tea lemonade production was collected from *camellia sinensis* trees in Zhejiang province, and it was sun-dried after soaking green tea leaves in water at 85 °C for 45 min. The dried residue was then ground into powder. Its protein content is 26.5%, which was determined by the method of Kjeldahl (Kjeldahl, 1883).

Oolong tea residue (leaves collected from the *Camellia sinensis* trees in Fujian province, processed by Damin company, Fujian, China), and Barley straw (*Hordeum vulgare* L., from Cargill B.V., the Netherlands) were sundried, collected, and stored at room temperature for further use. Grass (*Poa pratensis*, from Wageningen, The Netherlands) was freshly harvested and used immediately.

NaOH, HCl, and other chemicals for analysis were of analytical grade, purchased from Sigma, the USA.

2.2.2 Protein extraction

Protein extraction was performed by soaking 0.5 g GTR in alkaline solution. NaOH concentration (0-0.1 M), temperature (25-95 °C), extraction time (5 min - 24 h), and v/w (8-60 mL g^{-1}), were varied. After subsequent centrifugation, which was always performed at 15000 g for 10 min (Sorvall centrifuge, Thermo Fisher Scientific, the USA), supernatants were collected and stored at -20 °C until further analysis.

In two-step protein extractions, 0.5 g GTR was first extracted with 0.1 M NaOH at v/w of 40 mL g^{-1} and 40 °C for 4 h. After centrifugation, the supernatant was obtained and stored at -20 °C until further analysis while the precipitate was then soaked in 0.1 M NaOH at v/w of 40 mL g^{-1} and 95 °C for 2 h. The supernatant from the second extraction and final cake were separated by centrifugation and stored at -20 °C until further analysis.

2.2.3 Protein precipitation

Protein supernatants obtained from two experiments: 1) 0.1 M NaOH at v/w of 40 mL g^{-1} and 40 °C for 4 h, and 2) 0.1M NaOH at v/w of 40 mL g^{-1} and 95 °C for 4 h, were concentrated to 5 g protein L^{-1} by a rotary evaporator (IKA, Labortechnick, Germany). The pH of each sample (10 mL) was adjusted to pH 3-5, by the addition of 1 M HCl, and left still at 4 °C for 24 h. Protein precipitates were collected by centrifugation and stored at -20 °C until further analysis.

All the experiments were performed in triplicate, and the errors were calculated using standard deviation.

2.2.4 Sample analysis

2.2.4.1 Protein content:

Protein content (g L^{-1}) was determined by Kjeldahl method (Kjeldahl, 1883), using Kjeldahl equipment from Gerhardt, which consist of digestion unit (Gerhardt Kjeldahlterm) and rapid distillation unit (Gerhardt Vapodest). Kjeldahl measures all nitrogen, including non-protein N-containing components, such as caffeine, chlorophyll, and theobromine (Harbowy; and Balentine, 1997). However, we assumed that it is all

protein and used a conversion factor of 6.25 to calculate protein concentrations.

Protein extraction yield was calculated as ***extracted protein / total protein (TP) × 100%***.

Protein recovery was calculated as ***precipitated protein / extracted protein × 100 %***.

2.2.4.2 Weight of extracted GTR

Weight of extracted GTR was determined by analytical balance after oven drying at 60 °C for 24 h in glass containers, corrected for the amount of NaOH added, and subtracted by the amount of OH^- that was consumed to H_2O due to pH decrease. *GTR extraction yield* was calculated as ***extracted GTR / total GTR × 100%***.

2.2.4.3 Amino acid composition

Amino acid compositions were determined via UPLC (Meussen et al., 2013). Samples were obtained from extraction with 0.1 M NaOH (40 v/w, for 4 h) at 40 °C and 95 °C. They were first hydrolyzed in 6 N HCl containing 1% (w/v) phenol at 110 °C for 24 h and the amino acids were separated by using an Acquity UPLC BEH C18 reserved phase column. Detection was performed at the wavelength of 338 nm and 263 nm using Dionex RSLC (Dionex Corporation, Sunnyvale, CA, USA).

2.2.5 Protein extraction process for cost calculation

GTR (10 g) was soaked in 80 mL 0.4M NaOH with thorough stirring at 95 °C for 4 h, and the solid-liquid mixture was separated by centrifugation. The pellet was washed with 40 mL water twice with sequential centrifugations, and the supernatants were then mixed. The pH was adjusted to 3.5 using 1 M HCl to precipitate the protein. The results were used to calculate the cost of producing 1 ton leaf protein product.

2.3 Results and discussion

2.3.1 Alkaline protein extraction in elevated temperature

2.3.1.1 Protein yield

To test the influence of temperature on protein extraction yields from GTR, 0.1 M NaOH was tested at v/w of 40 mL g^{-1} and different temperatures. As shown in Fig. 2.1a,

approximately 20% of protein can be easily extracted in 15 min and the increase in protein yield is temperature dependent. At 95°C, about 95% protein can be extracted in 4 h, which is almost twice the amount using peer technology in green tea leaves or other leaf species (Badar and Kulkarni, 2011; Bals et al., 2007a; Chen et al., 2012; Dale et al., 2009b; Fernández et al., 1999; Fu et al., 2010; Schwenzfeier et al., 2011; Shen et al., 2008).

In protein extraction, high temperature is not favorable, for it may not only reduce protein yield because of protein coagulation (Tangka, 2003) and protein hydrolysis (in recovery step) (Bals and Dale, 2011), but may also reduce protein quality as a result of denaturation, hydrolysis, or amino acid racemization (Bals and Dale, 2011; Tangka, 2003; Zhu et al., 2010). However, at low temperature (25 °C), only 35% of protein was extracted even when extraction was extended to 3 days (data not shown). This barrier in alkaline protein extraction for leafy biomass can only be overcome by applying high temperature.

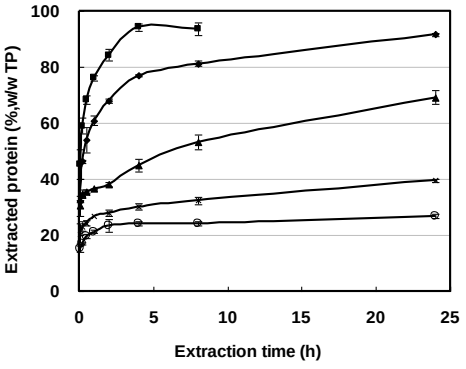

Fig. 2.1a Protein extraction yield (%, w/w Total Protein) with 0.1 M NaOH and v/w of 40 at different temperatures: 95 °C; 80 °C; 60 °C; 40 °C; 25 °C.

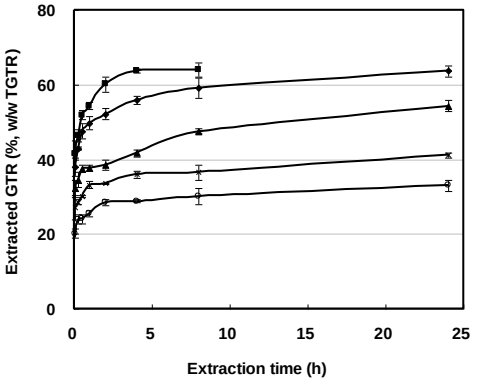

Fig. 2.1b Total mass extracted from GTR (%, w/w Total GTR) with 0.1M NaOH and v/w of 40 at different temperatures: 95 °C; 80 °C; 60 °C; 40 °C; 25 °C.

For better understanding of the extraction process and to enable calculation of protein purity, extracted dry matter from GTR was also measured. Similar to protein extraction, yield of extracted GTR increased over time and its increase was also temperature dependent (see Fig. 2.1b). Approximately 25% of GTR was dissolved within 15 min, and roughly 63% of GTR was extracted in 4h at 95 °C.

2.3.1.2 Amino acid composition

Since Kjeldahl determines total N content, real protein content is less than the calculated value (Kjeldahl, 1883). For comparisons, total amino acid composition was determined via UPLC. In the extract obtained at 40 °C, total amino acid amount is only 50% of Kjeldahl protein, while 88% of N-containing compound consists of amino acid in the 95 °C sample. The proportion of real protein in the 95 °C sample (without purification) is even higher than the purified protein product obtained by peer studies (Shen et al., 2008) (See Table 2.1). It is suggested that non-protein N-containing compounds, such as chlorophyll and caffeine, are easier to extract, leading to a low nutrition value of the GTR protein product extracted at mild conditions (Lin et al., 2003).

Table 2.1. Amino acid composition of 100 g protein extracts (%)

Amino acid	40 °C GTR extract	95 °C GTR extract	GTR extract [a]	Soy bean meal [b]
His	1.3 ± 0.1	1.9± 0.1	1.96	2.7
Arg	2.0 ± 0.1	3.4± 0.2	4.83	7.5
Thr	2.6 ± 0.1	3.1± 0.2	3.83	4.0
Val	2.9 ± 0.2	5.5± 0.2	4.63	4.8
Met	0.8 ± 0.1	1.9± 0.1	1.12	1.4
Try*	-	-	3.05	1.4
Ile	3.2 ± 0.2	6.3± 0.3	3.98	4.6
Phe	2.1 ± 0.2	4.4± 0.2	4.46	5.1
Leu	4.2 ± 0.3	9.0± 0.3	7.78	7.7
Lys	1.8 ± 0.1	2.8± 0.2	5.62	6.3
Asp+Asn	5.8 ± 0.5	11.6± 0.7	8.01	11.6
Glu+Gln	7.7 ± 0.4	12.4± 0.5	10.12	18.3
Ser	3.0 ± 0.1	4.3± 0.1	3.94	5.1
Gly	3.9 ± 0.2	5.9± 0.2	4.59	4.3
Tyr	2.3 ± 0.2	4.3± 0.1	-	3.5
Ala	3.4 ± 0.2	6.0± 0.1	4.90	4.4
Pro	3.1 ± 0.2	4.8± 0.2	3.62	5.2
Cys*	-	-	0.80	1.5
Total	50.3 ± 2.3	87.7± 2.1	77.2	99.3

a, Amino acid composition of soy bean meal based on (Shen et al., 2008)
b, Amino acid composition of soy bean meal based on (Frikha et al., 2012)

The nutritional value of proteins for monogastric animals and humans is often limited to the amount of essential amino acids, being arginine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Dale et al., 2009a). Demonstrated in Table 2.1, the extract obtained at 40 °C contained 21% of essential amino acids in N-containing compounds. In the 95 °C sample, the number was 38%,

suggesting a higher nutrition value. Compared to the amino acid composition of soybean protein (Frikha et al., 2012), GTR extracts from the 95 °C process has higher leucine, valine, isoleucine, methionine content, similar histidine, threonine, phenylalanine content, and less arginine and lysine content.

2.3.1.3 Protein purity

To quantify protein purity, the yield of extracted GTR (W_{GTR}, %) was plotted against the corresponding extracted protein (W_{GTR}, %) in Fig. 2.2, based on the data obtained from 3.1. Here, two constant slopes can be seen. When GTR extraction yield is below 33%, the slope is 0.25, which is less than half of the slope (0.57) above 33%. This was caused by a relative increase of extracted protein compared to extracted GTR, which indicates two regimes for extraction with protein purities of 25% and 57%. The first part may only contain easy solubilizable protein, while the second part also contains protein that is harder to solubilize. The lower slope corresponds to the experiments performed at 25 °C, 40 °C, and some at 60 °C, while the steeper slope covers the measurements of 60 °C and higher temperatures. It indicates that temperatures of 60 °C and higher may cause this change in the extraction mechanism, leading to higher yields.

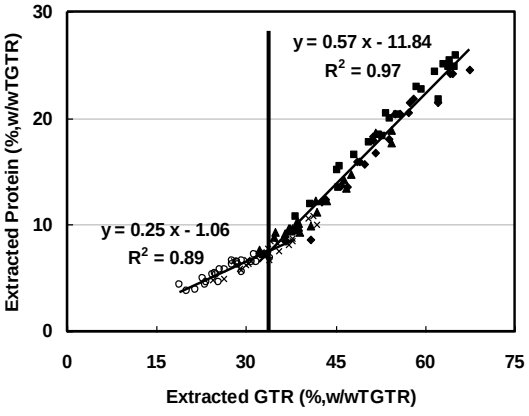

Fig. 2.2 The variation of protein purity (%, w extracted protein / w extracted GTR) after extraction: ○: 25°C; ×: 40°C; ▲: 60°C; ◆: 80°C; ■: 95°C. Lines are fitted to 25, 40 and 60°C (left part), and to 60, 80 and 95°C (right part).

A two-step extraction could separate the easy solubilizable protein from harder to solubilize ones. An advantage from using two steps is the reduction the amount of non-protein components in the second fraction, since they are already extracted in the first step. Based on the above assumption, a two-step extraction was performed, yielding two separate protein fractions. About 34% GTR was extracted in the first fraction with a protein purity of 23%, while 31% of GTR was extracted in the second fraction with a protein purity of 52%. The absolute amount of extracted protein in the second fraction

was over twice as that in the first one showing that temperatures above 60 °C are needed for high protein yield.

2.3.1.4 Protein recovery

As protein may be hydrolyzed in alkaline solution at high temperature, leading to a low recovery of subsequent acid precipitation (Pedroche et al., 2004), the efficiency of acid precipitation was tested by lowering pH of GTR extracts to pH-range of 3 to 5. As shown, protein recovery of the extracts obtained at 40 °C was generally lower than those obtained at 95 °C (Fig. 2.3). The optimal pH for recovering protein from the 95 °C sample is 3.5, at which approximately 85% protein was precipitated. Whereas, the optimal pH of the 40 °C sample was 3 or lower, yielding a protein recovery of 75%. Protein content of the 95 °C precipitates obtained by pH 3.5 was 52%，which is almost twice the highest protein content of the precipitates from 40 °C. This phenomenon may be caused by the presence of non-protein N-containing compounds, such as caffeine and chlorophyll (Lin et al., 2003), which are easier to extract and more difficult to precipitate. Combined with the extraction, approximately 80% of protein can be recovered. This number is higher than those from other studies (Badar and Kulkarni, 2011; Bals et al., 2007a; Chen et al., 2012; Fernández et al., 1999; Fu et al., 2010; Schwenzfeier et al., 2011; Shen et al., 2008).

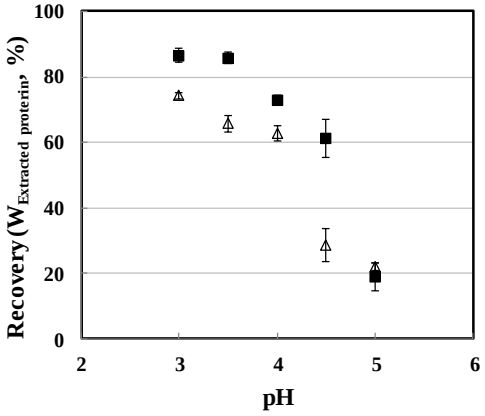

Fig. 2.3 Protein recovery (%, w/w Initial Protein) at different pH: ■ 95 °C sample; Δ 40 °C sample.

2.3.2 Influence of v/w, [alkali], and alkali amount on protein yield

In alkaline extraction, the ratio of the volume of solvent and the weight of material (v/w), as well as alkaline concentration, is regarded as a key parameter for protein yield (Harnedy and FitzGerald, 2013; Lestari et al., 2010; Shen et al., 2008). To test its influence on protein yield, varied v/w was tested using 0.1 M NaOH, at 95 °C for 2 h.

Fig. 2.4 illustrates that protein extraction yield and v/w are positively related until extraction reached 82% at around v/w of 32 mL g^{-1}. When varied alkaline concentrations were tested, it showed a similar pattern (data not shown). However, this influence cannot simply be attributed to a change in volume (or alkaline concentration), for the total NaOH amount also varied proportionally. Therefore, protein extraction was also tested by varying the volume with a fixed total amount of NaOH (4 mmol g^{-1} GTR) at 95 °C for 2 h. This means that an indirect parameter like alkaline concentration decreases with an increase in volume and a constant NaOH amount. As can be seen in Fig. 2.4, almost constant protein yields are obtained at varying combinations. This demonstrates that the absolute amount of applied alkali is a critical parameter for protein extraction yield, which can be indirectly influenced by both alkaline concentration (pH) and v/w. Additionally, it sums up with that approximately 3.2 mmol NaOH is needed to process 1 g GTR to obtain high protein yield.

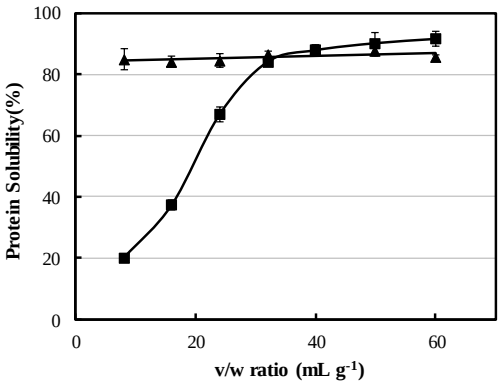

Fig.2.4 Influence of v/w at 95 °C for 2 h on protein yield ($W_{Protein}$,%). ■ by using 0.1 M NaOH; ▲ by using a fix amount of 4mmol NaOH to 1 g GTR

2.3.3 Grouping parameters to protein yield and production cost

Based on the above analysis, five parameters are involved in alkaline extraction, in which temperature, amount of alkali, and extraction time can be attributed to protein yield determining parameters, while v/w and alkali concentration can be considered as factors that affect production cost.

2.3.3.1 Protein yield related parameters

To profile the combined influence of NaOH amount, temperature, and extraction time on protein yield, these parameters were analyzed in pairs. Influence of extraction time on protein yield is only within the first 2 hours (Fig. 2.5a, Fig. 2.5b). NaOH amount and

temperature both influenced protein extraction yield in a positive way, consolidating each other. To reach high protein yield (>80%), 4 mmol NaOH was needed at 60 °C to process 1 g GTR (Fig. 2.5a); where at 80 °C the minimal NaOH amount can be 3 mmol (Fig. 2.5b). Their combined influence is also demonstrated in Fig. 2.5c.

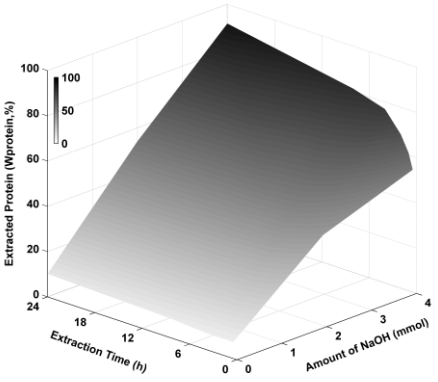

Fig. 2.5a Influence of added amount of NaOH (mmol) and extraction time (h) on extracted protein (Wprotein, %) by using v/w of 40 mL g^{-1}at 80°C.

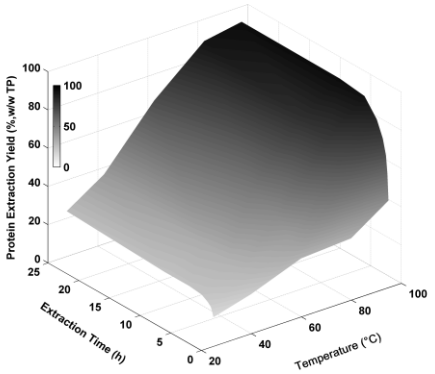

Fig. 2.5b Influence of temperature (°C) and extraction time (h) on extracted protein (Wprotein, %) by using 0.1 M NaOH and v/w of 40 mL g^{-1}.

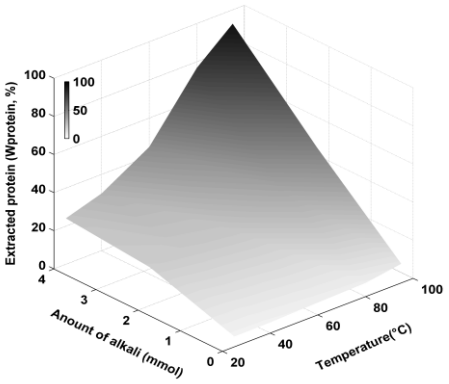

Fig. 2.5c Influence of temperature (°C) and added amount of NaOH (mmol) on extracted protein (Wprotein, %) by extracting 1 g of GTR with v/w of 40 mL g^{-1} for 4 h.

Choosing appropriate parameters and their scopes is critical for optimization. Unfortunately, pH, v/w, temperature, and extraction time are almost always selected as parameters (Bals et al., 2007a; Harnedy and FitzGerald, 2013; Shen et al., 2008), and the scope of temperature is normally lower than 60 °C. Low protein yield was therefore always demonstrated in alkaline protein extraction from leaves, even though an experimental designed was carried out (Shen et al., 2008). By using temperature, alkali amount, and extraction time as parameters with using temperature scope higher than 60 °C, a better optimization can be obtained.

2.3.3.2 Production cost related parameters

Although v/w and alkali concentration are proved less important in the protein yield, a decent combination of v/w and alkali concentration is critical to production cost. Protein concentration in the extracts is inversely proportional to v/w. At v/w of 8 mL g^{-1}, in which 0.5 M NaOH was applied, protein concentration can be up to 28 mL g^{-1}. As lower v/w and high protein concentration means less cost on heating, transmission, and protein recovery steps, the cost-efficiency of NaOH extraction is hereby improved.

The chemical cost and energy cost were calculated for the optimized process (Bals and Dale, 2011). The total cost of chemicals and energy for producing 1 ton protein product is only 148 € ton^{-1} with 2.5 ton GTR as starting material (Table 2.2). Furthermore, cost of heating can be decreased by applying cheaper energy sources or incorporating other processes. For instance, making full use of the heating energy from tea production (GTR is made by using water at 85 °C for 45 min) and the energy derived from dissolving NaOH, the heating cost can be reduced to less than 24 € ton^{-1} protein product, resulting in a final cost of only 102 € ton^{-1} protein product.

Table 2.2. Cost of energy and chemical for obtaining one ton protein product

	NaOH	HCl	Heating [a]	**Total**
Cost (€)	76	2	70 (24)	**148 (102)**

a, 70€ is the cost of using electric energy, while 24 € is the estimation of using other cheap energy sources.

Others also optimized alkaline extractions, leading to different optimized conditions (Bals and Dale, 2011; Chiesa and Gnansounou, 2011; Shen et al., 2008). However, using the above mentioned calculation method, our optimized alkaline extraction has the lowest chemicals and energy cost with highest protein yield among peer studies (Bals and Dale, 2011; Chiesa and Gnansounou, 2011; Shen et al., 2008). Also the capital cost of alkaline extraction is low compared to other techniques (Bals and Dale, 2011). The total cost (including logistics) of producing 1t protein product from GTR may even be less than one third of the selling price of soy bean meal (around 360 € t^{-1} in 2012 (WorldBank, March 2014)), showing a competitiveness to protein products from other sources.

2.3.4 Application

To test the general applicability of using alkaline extraction at elevated temperature, other sorts of biomass were tested as substrates. Application of 0.1 M NaOH at v/w of 40 mL $g^{-1}$and 95 °C for 4h extracted 92%, 95%, and 95% of total protein from oolong tea residue, grass, and barley straw respectively. The corresponding alkali consumption was 3.3, 2.0, and 0.8 mmol g^{-1} biomass, which can be further decreased after optimization. These results show that alkaline protein extraction has a wide applicability in various materials. It can be not only applied on material from same species of residues with different treatments (oolong tea residue), but also on fresh leaf species (grass), and even on the stem of plants (barley straw).

The positive results on our current high protein yield stimulate an interest in the underlying mechanism. Determining the influence of alkaline extraction on leaf tissues, cell wall and cell components may clarify how alkali aid protein extraction for leafy biomass. This knowledge can then be used to further develop extraction techniques.

Next to protein, other valuable components, such as polyphenols (Li et al., 2005), pectin (Ele-Ekouna et al., 2010), or pigments (Vencl et al., 2009) can be obtained by an integrated processing based on the core process of alkaline extraction. The alkaline pre-treated residue, which contains more than 70% cellulose, has for instance been proven to be good substrate for producing second generation ethanol with promising cost-efficiency (Knill and Kennedy, 2003).

2.4 Conclusion

In this study, we proved that high protein yield can be obtained by alkaline extraction from leaves in an economic way. In the extraction, temperature, alkaline amount, and extraction time were crucial for high extraction yields, while pH and v/w could be varied to limit production cost. Protein product, which is comparable to soy bean meal in protein content and nutritional value, can be then obtained at low cost. This technology can be universally applied to other leaf species, such as grass, which brings promising prospects for leaf protein products.

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CHAPTER 3: HOW DOES ALKALI AID PROTEIN EXTRACTION IN GREEN TEA LEAF RESIDUE: A BASIS FOR INTEGRATED BIOREFINERY OF LEAVES

Published as: Zhang, C., Sanders, J.P.M., Xiao, T.T., Bruins, M.E., 2015. How does alkali aid protein extraction in green tea leaf residue: A basis for integrated biorefinery of leaves. PLoS One 10, 1-14.

Abstract:

Leaf protein can be obtained cost-efficiently by alkaline extraction, but overuse of chemicals and low quality of (denatured) protein limits its application. The research objective was to investigate how alkali aids protein extraction of green tea leaf residue, and use these results for further improvements in alkaline protein biorefinery. Protein extraction yield was studied for correlation to morphology of leaf tissue structure, protein solubility and hydrolysis degree, and yields of non-protein components obtained at various conditions. Alkaline protein extraction was not facilitated by increased solubility or hydrolysis of protein, but positively correlated to leaf tissue disruption. HG pectin, RGII pectin, and organic acids were extracted before protein extraction, which was followed by the extraction of cellulose and hemi-cellulose. RGI pectin and lignin were both linear to protein yield. The yields of these two components were 80% and 25% respectively when 95% protein was extracted, which indicated that RGI pectin is more likely to be the key limitation to leaf protein extraction. An integrated biorefinery was designed based on these results.

3.1 Introduction

Leaf protein has been considered as an additional protein source since 1960s (Akeson and Stahmann, 1965; Gerloff et al., 1965). These proteins can be used in food (Ghaly and Alkoaik, 2010), animal feed (Kondo et al., 2004), or when hydrolysed to amino acids for N-chemicals bulk chemicals (Sanders et al., 2007). However, applications of these proteins are limited by its low cost-efficient production (Bals and Dale, 2011), particularly in extraction processes. This limitation was preliminarily solved by using alkaline conditions at higher than 60 ℃ (Zhang et al., 2014). Drawbacks of this technique are overuse of chemicals and low quality of (denatured) protein. To overcome these drawbacks and design an integrated process for protein and other products, the basis of alkaline protein extraction in leaf should be better understood.

Alkali might aid leaf protein extraction as a result of leaf tissue disruption. Leaf has three major tissue systems: epidermis, mesophyll, and vascular. Vascular tissues are located in mesophyll tissues covered by epidermis tissue. Those tissues are adhered by lamella layer embracing a large quantity of pectin. Epidermis is a tabular and layered sheet of cells on surface of leaf covered by a waxy cuticle functioning as mechanical protection of mesophyll tissue (Dashek and Harrison, 2006b; Mauseth, 2009). As most leaf proteins, including lectins, enzymes (Rubisco), storage proteins, cell wall proteins and some toxins (Feller et al., 2008) are located in mesophyll tissues (Dashek and Harrison, b), disruption of epidermis and lamella might aid protein release from mesophyll tissues.

Alkali might also aid leaf protein extraction by increasing protein solubility or / and hydrolysis degree. Most leaf proteins are considered insoluble (Badar and Kulkarni, 2011; Chiesa and Gnansounou, 2011; Lamsal et al., 2007). These protein are hydrophobic in neutral solution, because they are bound to other compounds, such as polyphenol (Brovko and Zagranichnaya, 1998) or polysaccharides (membrane protein) (Henriques and Park, 1976). Solubility of these proteins can be increased with the increase of pH by adding alkali. At high temperature, alkali can even hydrolyse protein into small peptides (Jamdar et al., 2010; Yalçın and Çelik, 2007), which reduces protein molecular size, and therefore increases protein solubility and accelerates protein

diffusion.

Finally, alkali might aid in disruption of cell wall and thus leading to high protein yield. Leaf proteins within cells are well protected by the cell walls, which consist of middle lamella, primary wall, and secondary wall (Dashek and Harrison, 2006b). In cell wall, carbohydrates (including pectin, hemi-cellulose, and cellulose) and lignin are two major components other than protein (Dashek and Harrison, 2006b). Pectin is a family of complex polysaccharides located in primary plant cell wall and middle lamella (Dashek and Harrison, 2006b; Somerville et al., 2004). It can be roughly divided into three types: homogalacturonan (HG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII) (Ridley et al., 2001). Hemi-cellulose and cellulose are mainly found in both primary and secondary plant cell wall, and they have a simpler composition than pectin. In comparison, lignin could be the most complicated component located in secondary plant cell wall (Dashek and Harrison, 2006b). It is a complex phenolic polymer that drives out water and strengthens the cell wall (Dashek and Harrison, 2006b). Solubilisation of carbohydrates and lignin performs differently under different alkaline conditions (Carvalheiro et al., 2008; Knill and Kennedy, 2003; Renard et al., 1990; Xiao et al., 2001). Correlating yields of these components with protein yield may profile how alkali disrupts cell wall, and offer a basis for integrated leaf biorefinery.

Green tea residue (GTR) is used as a model material for research on leaf biorefinery. It was demonstrated earlier that the concept of protein extraction using GTR as a model material can also be applied in Oolong tea leaf residue, Jatropha leaf, barley straw (Zhang et al., 2014), and even on algae. GTR is the waste of tea leaves after hot water extraction, containing high value components, such as polyphenols (10-15%), proteins (20-30%), and carbohydrates (30-40%) (Jayasuriya et al., 1978; Shen et al., 2008). It is now only used for energy generation through burning. An integrated biorefinery targeting on high value components of GTR will increase its value.

In this study, GTR was again used as a model material, and protein extracts were obtained at various alkaline extraction conditions based on our previous work (Zhang et al., 2014). Alkaline treated leaf tissues were analysed by microscope; solubility and

hydrolysis degree of extracted protein and the composition of extracted carbohydrates was determined; extracted amounts of lignin were estimated. These results were all correlated to protein yield to analyse how alkali aids protein extraction in GTR. Based on these results, potential of integrated biorefinery of protein and other components for leaves was discussed.

3.2 Methods and materials

3.2.1 Materials

Green tea residue (GTR) is our main material, which is a gift from Damin Company, Fujian Province, China. This residue from tea lemonade production was collected from *C. sinensis* trees in Zhejiang province, and it was sun-dried after soaking green tea leaves in water at 85 °C for 45 min. Chemicals used for analysis were purchased from Sigma (USA, analytic grade) if not stated otherwise.

3.2.2 Preparation of alkaline extracts

Protein extraction was performed by soaking 0.5 g GTR in 20 mL 0.1 M NaOH at 25 °C, 60 °C, and 95 °C for 2h. After subsequent centrifugation, which was always performed at 15000 g for 10 min (Sorvall centrifuge, Thermo Fisher Scientific, the USA), the supernatants were then stored at -20 °C for further analysis. The corresponding solid residues were collected and washed with water for 3 times, and then they were immersed in water and analysed by microscopy immediately.

For analysing the correlations of carbohydrates or lignin with protein, samples were made by soaking 0.5 g GTR in 20 mL 0.1 M NaOH at 25 °C, 60 °C, and 95 °C over time (5 min-24 h). Samples were freeze-dried and stored at room temperature till further use.

3.2.3 Visualization of leaf tissues

Solid samples obtained after alkaline treatment for 2 h, as well as a control of untreated material and a control treated by 0.05M NaOH, were examined under the microscope (SMZ-U, Nikon, Japan). Pictures were taken with a BCE-C050 Camara (Mightex, US)

that was fitted on to the microscope.

3.2.4 Determination of protein properties

3.2.4.1 Protein content of extracts

Protein content (g L^{-1}) was determined by Kjeldahl method (Kjeldahl, 1883), using Kjeldahl equipment from Gerhardt, which consist of digestion unit (Gerhardt Kjeldahlterm) and rapid distillation unit (Gerhardt Vapodest). Kjeldahl measures all nitrogen, including non-protein N-containing components, such as caffeine, chlorophyll, and theobromine (Harbowy; and Balentine, 1997). However, we assumed that it is all protein and used a conversion factor of 6.25 to calculate protein concentrations. Protein extraction yield was calculated as ***extracted protein / total protein * 100%.***

3.2.4.2 Protein hydrolysis degree

Protein hydrolysis degree is defined as the percentage of cleaved peptides bonds. As the amount of fully hydrolysed protein from GTR is the same for each sample, here we use the difference of -NH_2 residue before and after hydrolysis to discuss the hydrolysis degree. The content of -NH_2 residue was determined by a modified o-phthaldialdehyde (OPA) method (Nielsen et al., 2001). 1.5 mL OPA reagent (OPA 0.88 g L^{-1}, dithiothreitol 0.88 g L^{-1}, SDS 1 g L^{-1}, and $Na_2B_4O_7 \cdot 10H_2O$ 38.1 g L^{-1}) was mixed with 200 µl serine (0.1 g L^{-1}), sample, or water (as a blank). After exactly 2 min, absorbance of the mixture was determined spectrophotometrically at 340nm. Concentration of -NH_2 residue was calculated using serine as a reference.

3.2.4.3 Protein solubility

Alkaline extracts were diluted to a protein concentration of 10g L^{-1}. Protein solutions pHs were adjusted to 2-11 using 0.5 M HCl, after which the solution was kept stirring at room temperature for 1 h. Samples were subsequently centrifuged at 15000 g for 10 min at room temperature and the supernatant was collected. The amount of soluble protein in the filtrate was determined by Lowry method (Sigma, Lowry total protein determination kit) using bovine serum albumin as standard. Solubility is presented as a percentage of total protein in weight.

3.2.5 Water and ash content

Samples were pre-weight and dried for 24 hours at 60 °C to evaporate all water. After cooling down in a desiccator, the residual weights of samples were measured. Then, samples were transferred to a 550 °C furnace for 16h to burn off all the organic matter. After cooling in a desiccator, the crucibles were weighed. Water content and ash content were calculated as weight percentage of the starting material.

3.2.6 Polyphenol content

Content of tea polyphenols in tea extracts was determined spectrophotometrically with scaling down reagent usage (Li et al., 2005; Turkmen et al., 2006). Polyphenols content was calculated assuming a concentration of polyphenols of 3.914g L^{-1}leads to an adsorption of 1 at 540 nm after reaction.

3.2.7 Galacturonic acid determination

Galacturonic acid content was determined as anhydro-uronic acid by an automated m-hydroxydiphenyl assay with an auto-analyzer (Skalar Analytical BV, Breda, The Netherlands) (Van Dongen et al., 2011). Galacturonic acid (Fluka AG, Buchs, Switzerland) was used as a reference in a concentration range from 12.5 to 200 mg L^{-1}.

3.2.8 Neutral sugar composition

Freeze dried alkaline extracts were pre-hydrolysed with 72% (w/w) sulphuric acid at 39 °C for 1 h, followed by hydrolysis in 1 M sulphuric acid at 100 °C for 3 h. Monosaccharides were analysed using GC according to Englyst's method (Englyst and Cummings, 1984). Inositol was used as internal standard. Response factor was determined using a standard sugar solution of L-(+)-rhamnose , L-(+)-arabinose, D-(+)-xylose, D-(+)-mannose, D-(+)-galactose (97%), D-(+)-glucose (99,5%) with concentrations of 1 g L^{-1}.

To quantify carbohydrates, indirect assays are often used. Samples are hydrolysed to mono-sugars, and these sugars are quantified by HPLC or GC (Englyst and Cummings, 1984).HG pectin consists only of galacturonic acid of which some of the carboxyl groups are methyl esterified (Ridley et al., 2001; Willats et al., 2006).HG pectin can be

analysed by galacturonic acid content. RGI consists of a backbone of repeating disaccharide of galacturonic acid and rhamnose. A variety of different glycan chains (principally arabinan and galactan) are attached to the rhamnose residues. As RGI pectin is predominated by side chains (Ridley et al., 2001; Willats et al., 2006) mainly constituted from arabinose and galactose, it can be analysed by the contents of these two mono-sugars. In comparison, RGII has a backbone of HG with complex side chains attached to the galacturonic acid (Ridley et al., 2001; Willats et al., 2006). Therefore RGII analysis needs information of all sugars contents. Hemi-cellulose's backbone comprises of xylose and glucose, while cellulose is a linear chain of glucose (Carvalheiro et al., 2008; Knill and Kennedy, 2003).

All the experiments were performed in duplicate, and all results were plotted in the figures.

3.3 Results

3.3.1 Influence of alkali on leaf tissues

As mentioned, leaf consists of three major tissues systems adhered by middle lamella. To study the disruption of leaf tissues under alkaline conditions, alkali treated GTRs were analysed microscopically. Although GTR is the leftover of green tea leaves treated by hot water, the structure of its tissue is still intact. Shown in Fig. 3.1a, the epidermal layer of untreated GTR was normally attached to the mesophyll tissue and the lamella was not solubilized, shown as a low transparency of leaf tissue and visible fragments suspended in the solution. After treatment with 0.1M NaOH, leaf tissues were transparent and solutions became clear (Fig. 3.1c, Fig. 3.1d, and Fig. 3.1e). At 25 °C, the epidermal layer was still attached to the mesophyll tissue (Fig. 3.1c), but it started to peel off when higher temperature was applied (Fig. 3.1d, Fig. 3.1e). As protein yield increased with the increase of temperature (23% obtained by 25 °C, 38% obtained by 60 °C, and 84% obtained by 95 °C), these figures indicate a correlation of leaf tissue disruption with protein yield under alkaline conditions.

When alkaline concentration was limited to 0.05M, GTR tissues (Fig. 3.1b) are similar

as the untreated one (Fig. 3.1a). The protein yield obtained by using 0.05M NaOH at 95 °C was about 40% (Zhang et al., 2014), which was similar to the yield obtained by 0.1M NaOH at 60 °C (38%). However, the transparency of the tissue treated with 0.05M NaOH was lower than that treated by 0.1M NaOH. This indicates that alkali was used to solubilize substances between cells, while high temperature was related to protein located inside cells or membrane, which is commonly identified as insoluble (Henriques and Park, 1976). To further understand how does alkali aids in protein extraction, influence of alkali on protein hydrolysis and solubility was tested, and yields of non-protein components were subsequently analysed and correlated to protein yields.

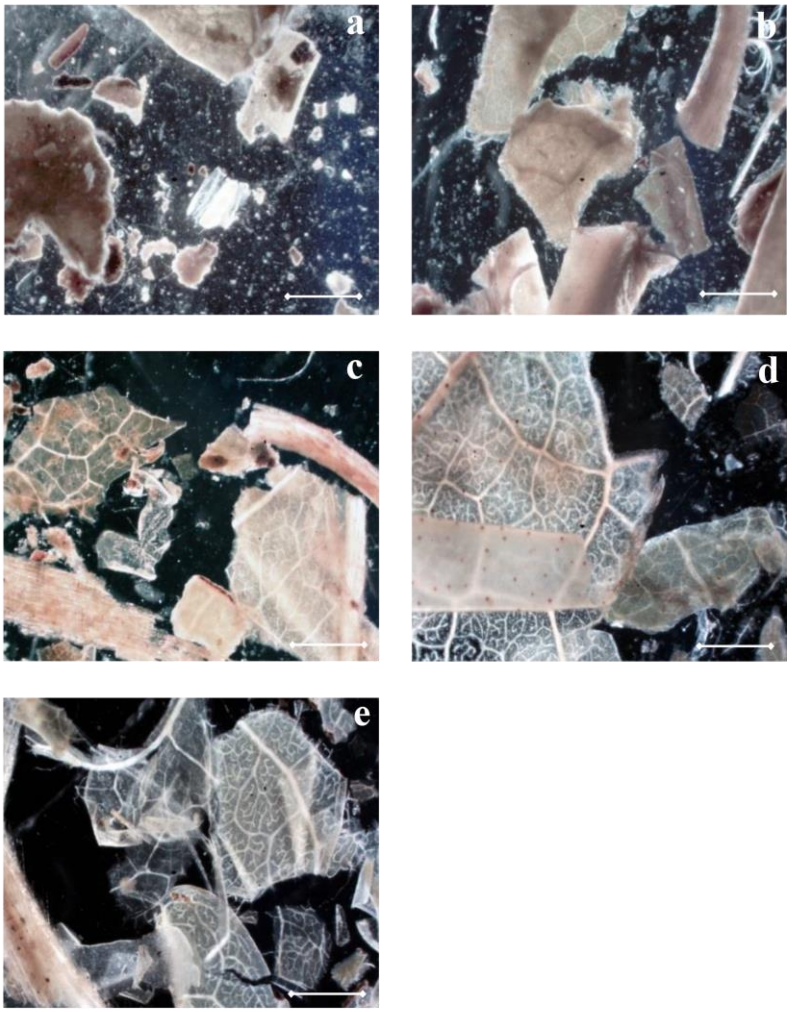

Fig. 3.1 Morphology of GTR tissues after a 2h treatment with 40 v/w solution (scale bar: 100μm). a, GTR treated with water at 25°C; b, GTR treated with 0.05M NaOH at 95°C; c, GTR treated with 0.1M NaOH at 25°C; d, GTR treated with 0.1M NaOH at 60°C; e, GTR treated with 0.1M NaOH at 95°C.

3.3.2 Composition of GTR

To calculate yields of all components in alkaline condition, composition of GTR was analysed. Carbohydrate and protein (or more accurate: all N-containing components) are the two major components, accounting for 31% and 27% of GTR. Other components are

polyphenol (8%), water (7%), and ash (6%). The residual undetermined part majorly consists of lipid (wax, organic acids) and lignin (Harold N, 1992). As lipid and lignin will not be extracted by the pre-treatment with hot water that was done in the factory, the ratio of lipid to lignin (2:6.5) presented in green tea leaf (Harold N, 1992) is estimated to remain constant in GTR, leading to 5% and 17% of dry GTR respectively. Carbohydrates were quantified by summing all mono-sugars. Glucose and galacturonic acid are the major sugar components constituting 13.4% and 7.6% of GTR, followed by galactose, arabinose, and xylose with percentages of 3.4%, 2.8%, and 2.1% of GTR respectively. Other less abundant sugars are mannose with 1.1%, rhamnose with 0.8%, and fucose with 0.4%.

3.3.3 Influence of alkali on protein hydrolysis and solubility

Generally, 1g native protein already contains 0.342-0.457 mmol $-NH_2$ groups, when protein is fully hydrolysed, $-NH_2$ content will increase to 8.6 mmol depending on amino acid composition (Nielsen et al., 2001; Zhang et al., 2014). To compare hydrolysis degree, protein extracts obtained using 0.1M NaOH with v/w of 40 for 2h at 25 °C, 60 °C, and 95 °C were tested by OPA method. The protein yields were 23% at 25 °C, 38% at 60 °C, and 84% at 95 °C. In these samples, $-NH_2$ content in 1g protein was 0.43 ± 0.03 mmol, 0.54 ± 0.07 mmol, 0.19 ± 0.02 mmol respectively. Those numbers are close to the value of native protein, suggesting that only very limited hydrolysis occurred. When 95 °C was applied, the $-NH_2$ content was even reduced. This may result from reactions of $-NH_2$ with other components, such as polyphenol (Ozdal et al., 2013). Protein hydrolysis degree did not correlate with protein yield.

To determine the correlation of protein solubility with protein yield, protein solubility was measured as a function of pH. Samples from three temperatures, as mentioned above, were used and the result is depicted in Fig. 3.2. At pH higher than 6, protein solubility of all samples remained at the maximal, added amount of 10g L^{-1}. This is over twice the amount of extracts from other research (Chen et al., 2012; Shen et al., 2008). Based on these results, we conclude that protein solubility was not a limitation to protein yield. Below pH 6, solubility of all three protein extracts decreased. The 95 °C sample

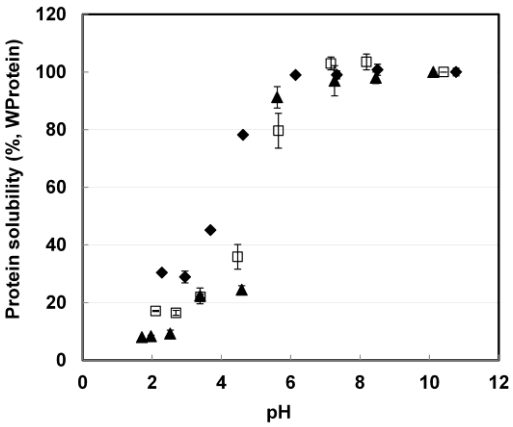

Fig.3.2 Solubility of protein extract as a function of pH obtained from experiments using 0.1 M NaOH with v/w of 40 for 2 h at different temperatures. ◆: 25 °C;□: 60 °C; ▲:95 °C.

had its lowest protein solubility at pH<4.5. In other cases solubility of hydrolysed protein increased between pH 2-6 when more severe hydrolysis had happened (Jamdar et al., 2010; Yalçın and Çelik, 2007). This again indicates that protein was not severely hydrolyzed after alkaline treatment at 95 °C. Alkaline protein extraction is therefore not facilitated by hydrolysis or increased solubility of protein.

3.3.4 Correlation of carbohydrates yields with protein yields

3.3.4.1 Pectin

As mentioned in introduction, galacturonic acid, rhamnose, galactose, and arabinose can be used to analyse pectin yields and types. Yields of galacturonic acid, rhamnose, galactose, and arabinose in protein extracts were plotted against protein yields in Fig. 3.3.

At mild conditions (all 25°C samples and samples of 60 °C treated less than 4h), yields of galacturonic acid were positively correlated with protein yield. Galacturonic acid yield was highest at 95% when around 40% of protein was extracted (Fig 3.3a), which occurred at 60 °C, after 2-4 hours of extraction. When higher temperature was applied (95 °C), galacturonic acid yield was less. This lower yield might result from the β-elimination of galacturonic acid under harsh alkaline conditions (Renard et al., 1996). As HG pectin consists of only galacturonic acids, HG pectin was suggested to be completely extracted at relatively mild conditions. Therefore, HG pectin is not the limitation for obtaining high protein yield.

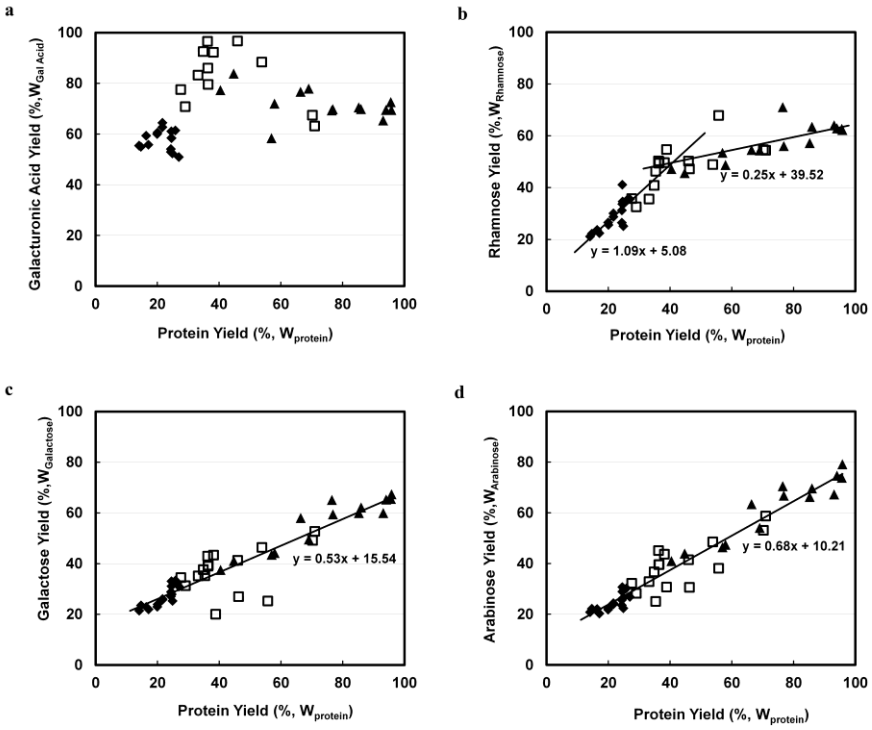

Fig.3.3 Weight based correlation of extracted pectin related sugars with extracted protein (%,$W_{Protein}$) by using 0.1M NaOH with 40 v/w at ◆: 25°C; □: 60°C ; ▲ 95°C. a, Galacturonic acid (%,$W_{Galacturonic\ Acid}$); b, Rhamnose (%,$W_{Rhamnose}$); c, Galactose (%,$W_{Galactose}$); d, Arabinose (%,$W_{Arabinose}$).

Rhamnose yields were also positively correlated to protein yields, but a bend was observed in the curve in Fig. 3.3b and two different lines and slopes were obtained. The coefficient of rhamnose yield to protein yield was 1.09 when less than 40% of protein was extracted, and their coefficient was 0.25 when more than 40% protein was extracted. The two coefficients indicate that rhamnose may originate from two pectin sources, RGI and RGII, and one of them might be already completely extracted at mild conditions.

Yields of galactose and arabinose were linear to protein yields throughout the entire time and temperature range (Fig. 3.3c & Fig. 3.3d). When 95% protein was extracted, the yields of galactose and arabinose were about 70% and 80% respectively. The coefficient of galactose yield to protein yield was 0.53, while the coefficient of arabinose yield to protein yield was 0.68. As galactose and arabinose majorly originate from RGI pectin (Ridley et al., 2001; Willats et al., 2006), these results indicate a linearity of RGI pectin yield to protein yield, and RGI may therefore be the limitation to protein extraction.

Combined with the result of the correlation on rhamnose yield to protein yield, RGII pectin was probably completely extracted at mild conditions. This conclusion could be confirmed by another study, in which RGII of green tea leaf was shown to be more soluble than RGI in water (Ele-Ekouna et al., 2010).

3.3.5 Hemi-cellulose& cellulose

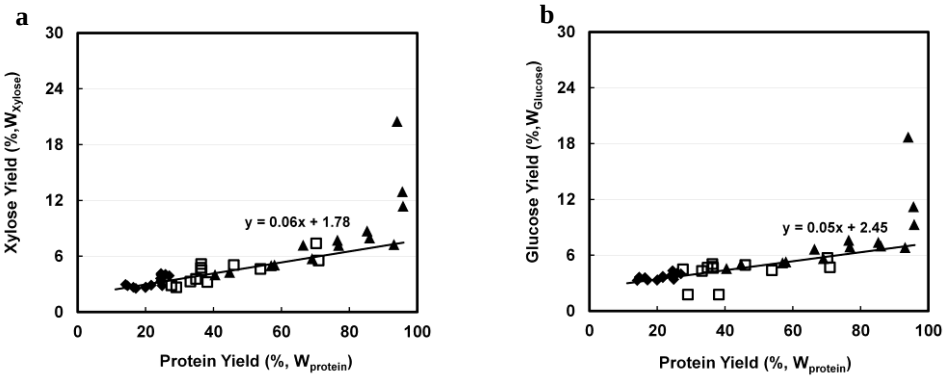

Fig.3.4 Correlation of extracted cellulose and hemi-cellulose related sugars with extracted protein (%,$W_{Protein}$) by using 0.1M NaOH with 40 v/w at ◆: 25°C; □: 60°C ; ▲ 95°C. a, Xylose (%,W_{Xylose}); b, Glucose (%,$W_{Glucose}$).

To analyse the correlation of the solubilised hemi-cellulose and cellulose with protein extraction, yields of xylose and glucose were measured and plotted against protein yields. Generally, yields of xylose and glucose are less than 8% (Fig. 3.4a, Fig. 3.4b) with a highest yield of around 20% only at the harshest condition applied, when all protein had already been extracted. The yield coefficients of these two sugars to protein are therefore relatively low, 0.06 for xylose and 0.05 for glucose. Considering some xylose and glucose may be emanated from side chains of RGI and RGII pectin, the correlation slopes of these two sugars with protein are close to zero. Therefore, high protein yield is probably not due to disruption of hemi-cellulose or cellulose.

3.3.6 Correlation of non-determined component yields with protein yields

To investigate the influence of lignin on protein extraction, yields of non-determined components (likely to be majorly lignin and lipid) were plotted against yields of protein (Fig. 3.5). Generally, yields of non-determined components were positively correlated to protein yields, but a bend was observed in the curve of Fig. 3.5 and two correlation slopes were calculated. When protein yield was less than 40%, the yield coefficient of

non-determined components to protein was 1.28, which was almost 3 times higher as when protein yield was above 40%. The two coefficients might indicate that the yields of two major non-determined components, lipid and lignin, have different correlation with yields of protein, and one of them was completely extracted at mild condition. As lipid can be released with mild alkaline while lignin was insoluble in the same condition (Carvalheiro et al., 2008; Parajó et al., 1996), lipid possibly contributed to the higher slope while lignin contributed to the lower (0.44). The extractability of lignin showed a maximum of only 25% of total lignin at 95% protein extraction. This means that it is either not necessary to completely dissolve lignin to facilitate protein extraction, or that another component is limiting protein extraction.

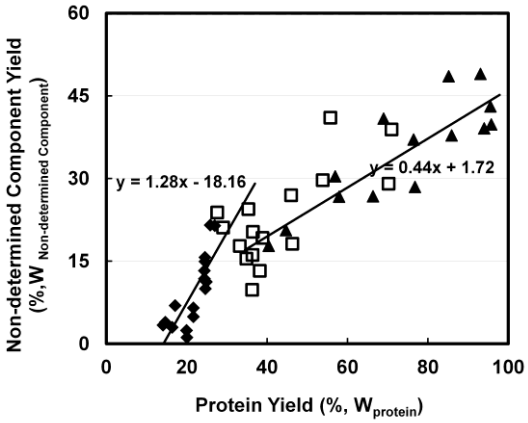

Fig.3.5 Correlation of extract undetermined components ($\%,W_{Non\text{-}determined\ Components}$) with extracted protein ($\%,W_{Protein}$) by using 0.1 M NaOH with 40 v/w at ◆: 25 °C; □: 60 °C ; ▲ 95 °C.

3.4 Discussion

3.4.1 How does alkali aid protein extraction in leaves

Although the role of lignin in protein extraction is still unclear, the correlation of RGI extraction yield with protein extraction yield is clearly shown. In leaf cell wall, RGI is rooted in a putative structure functioning as a scaffold in which galactan and arabinan occur as side chains, forming a kind of molecular brush (Vincken, 2003). It is often correlated with stages of cell development (Ridley et al., 2001) and can covalently-link to xylogulcan (hemi-cellulose) (Thompson and Fry, 2000; Zaidel and Meyer, 2012), phenolic acids (lignin) (Saulnier and Thibault, 1999; Zaidel and Meyer, 2012) and cellulose (Zykwinska et al., 2005). The mechanism of how RGI links to other components is still controversial, but how alkaline solutions can extract RGI has been

reported. It has been stated that extraction of pectin with side chains of arabinan and galactan is more sensitive to temperature than alkaline concentration (Zykwinska et al., 2006). Previous conditions for extracting 80% of arabinose and galactose (Zykwinska et al., 2006) were similar to our protein extraction condition for 95% protein (0.1M NaOH at 95 °C) again suggesting RGI pectin is the key to obtaining high protein yields.

3.4.2 Integrated biorefinery concept for leaves

An optimized alkaline protein extraction process that is based on our previous work (Zhang et al., 2014)has the lowest chemicals and energy cost with highest protein yield among peer studies (Bals and Dale, 2011; Chiesa and Gnansounou, 2011; Shen et al., 2008). To further reduce the use of chemicals and improve quality of protein, an integrated biorefinery concept is recommendable.

Phenolic components and organic acids in leaves can be removed by solvent extraction (Durling et al., 2007; Turkmen et al., 2006) in the first step. For example, using ethanol pre-treatment at ambient temperature can extract all caffeine and soluble polyphenols from GTR (data not shown). In the extracts, a yield of 10% N-containing components was detected (Zhang et al., 2014). These N however was not derived from protein, but from caffeine and chlorophyll (data not shown). This step does not only yield product, but the removal of phenolic compounds also prevents their reaction with protein under alkaline conditions, and may thereby also improve the digestibility of the final protein product (de Toledo et al., 2013).

Using relatively mild alkaline conditions (20 °C<T<60 °C), leaf components, such as HG pectin, RG II pectin, protein and lipids (or organic acids), can be obtained. When all HG pectin (galacturonic acid) was extracted, approximately 32% of N-containing components were also extracted (Fig. 3.3a). Assuming that from this 32%, 10% are non-protein N-containing components extracted by solvent extraction, in this step approximately 22% of protein (25% of total real protein) will be obtained. To reduce alkali consumption, relatively low pH (9-11) is recommended; for excess alkali will only be consumed by the buffering components, such as polyphenol or pectin. The products obtained in this second step can be applied in food industry as functional ingredients, as

they are obtained at relatively mild conditions.

When more severe conditions (95 °C, pH 13) are applied, the network of RGI pectin and lignin that makes up the cell wall starts to collapse, and membrane protein and proteins from inside the cells begin to liberate. When all protein is extracted approximately 80% of galactose and arabinose (Fig. 3.3), 8% xylose and glucose (Fig. 3.4), and 42% non-determined components (Fig. 3.5) can be extracted. After solvent extraction and subsequent mild alkaline extraction, 68% N-contain components (75% of total real protein), 60% RGI pectin (galactose and arabinose), 5% (hemi-) cellulose, and 25% lignin are obtained. Protein purity will be approximately 55%. Due to this high concentration, these protein products have a high value for application in animal feed.

The remainder, which majorly contains lignocellulose can be hydrolysed at extreme conditions with temperatures above 100 °C and alkali concentrations above 0.5M to release mono-sugars (Testova et al.; Xiao et al., 2001). Alternatively, the lignocellulose can be used as a feedstock for producing bio-ethanol, for which alkaline pre-treatment was proved to increase the conversion rate (Maas et al., 2008).

Based on the results presented here, and on previous results from literature that aimed for extracting pectin, lignin, hemi-cellulose, and cellulose (Testova et al.; Xiao et al., 2001; Zykwinska et al., 2006), an integrated leaf biorefinery scheme, which may consist of four steps, can be designed. This is illustrated in Fig. 3.6 with our results on green tea residues.

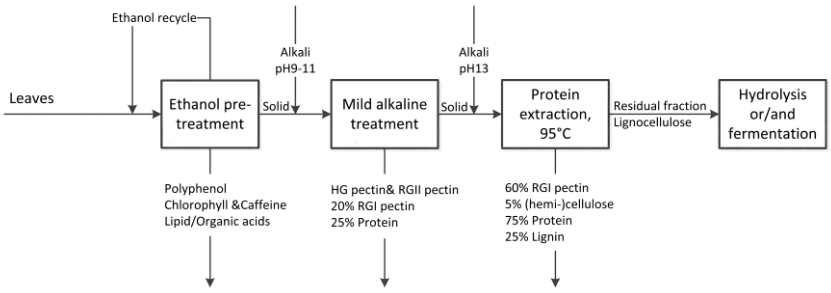

Fig. 3.6 Integrated leaf biorefinery concept. Numbers are estimated based on results with green tea residue.

3.4.3 Outlook

Next to the presented chemical approach to leaf biorefinery, new approaches can be developed. Using enzymes to specifically break down RGI pectin or mechanical disruption of the epidermal layer in plant cell tissue may improve protein yields in mild conditions such that native protein with higher quantity and quality may be obtained.

3.5 Conclusion

The use of the integrated biorefinery concept under alkaline conditions will add revenue because of the increased value of final products and reduction of production cost. Polyphenol, lipid, pectin, protein and lignocellulose can be obtained separately with higher purity and quality that improves their commercial value as final products or mediates further conversion. The integrated process can reduce alkali consumption for protein extraction, as buffering components such as polyphenol and pectin will be extracted priory. Alkaline treatment can be universally applied to other leaf species (Zhang et al., 2014), such as grass, which brings promising prospects for leaf biorefinery.

3.6 Reference

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CHAPTER 4: EFFECT OF PRE-TREATMENT ON THE EFFICIENCY OF ALKALINE PROTEIN EXTRACTION FROM GREEN TEA RESIDUE AND ON THE FEED QUALITY OF ITS PROTEIN PRODUCT

Submitted as: Zhang, C., van Krimpen, M.M., Sanders, J.P.M., Bruins, M.E., 2016. Improving yield and composition of protein concentrates from green tea residue in an agri-food supply chain: effect of pre-treatment.

Abstract:

Leaf protein for animal feed can be obtained cost-efficiently by alkaline extraction. However, alkali usage is high and the resulting low protein quality limits its application. The research objective was to investigate if pre-treatment with ethanol, Viscozyme® L, and/or H_2O_2 can improve the efficiency of alkaline extraction and at the same time improve the nutritious value of its protein products using green tea residue as a model material. The extraction yield of chlorophyll, carotenoids, polyphenols, carbohydrates, as well as the protein content in the extract, were measured to analyse the extraction efficiencies of these pre-treatments. After that, alkali consumption, protein yield, protein purity, color formation, dry matter digestibility, sample composition, and amino acid composition were measured as efficiency indicators of these pre-treatments on alkaline protein extraction and protein quality. Ethanol pre-treatment removed polyphenols and/or pigments depending on its concentration, but only the removal of polyphenols had a positive effect on protein yield, protein purity, alkali consumption, and reduction of colored components in the subsequent alkaline protein extraction. Viscozyme® L pre-treatment removed carbohydrates, including pectin and cellulose, which also had a positive effect on subsequent alkaline extraction except for the reduction of colored components. H_2O_2 didn't release lignin, and had a negative effect on the alkaline protein extraction. As alkaline protein extracts were fully digestible, the utility of protein in animal feed was 2 times improved. A combined pre-treatment of Viscozyme® L with 50% ethanol could further reduce alkali consumption and color formation by over 50%, and improve protein purity and animal feed nutritive value with less essential amino acid degradation. The suggested pre-treatment and subsequent alkaline protein extraction can be applied for other leafy biomass, which significantly increases protein sources for animal feed.

4.1 Introduction

Leaves have been fed to animals for hundreds of years. Protein is a major component of leaf, however, due to the low protein digestibility and the presence of anti-nutritional components in whole leaf (Hanczakowski and Skraba, 1989; Jayasuriya et al., 1978; Ravindran, 1993; Szymczyk et al., 1995), the use of leaf sources in diets of monogastrics is limited. To increase protein digestibility and to extend the application of leaf proteins, extraction techniques, such as mechanical pressing, alkaline extraction, and ammonia fiber explosion were studied (Bals and Dale, 2011; Chiesa and Gnansounou, 2011; Sari et al., 2015a). The nutritional quality of these protein extracts could be further improved by removing anti-nutritional components that are still present after extraction. The bottleneck of these techniques are their low cost-efficiencies (Bals and Dale, 2011). Although the cost-efficiency of the alkaline extraction technique was recently improved, the use of chemicals, the generation of large amounts of salts, and the low protein quality in terms of taste, digestibility and nutritional value, brings down its applicability (Zhang et al., 2014).

During extraction, alkali is first consumed by buffering components, such as polyphenol, pectin, and lignin, where after it is used in protein extraction(Zhang et al., 2014; Zhang et al., 2015). The requested alkali amount for obtaining high yield may be reduced when these buffering components are removed prior to protein extraction. Leaf protein quality decrease in alkaline extracts may due to the reaction of pigments, polyphenols, lignin, or carbohydrates with protein, which generates bitterness and decreases protein digestibility (Felicetti and Schrader, 2009) (de Toledo et al., 2013) (Rubanza et al., 2005; van Soest and Mason, 1991). These reactions can be detected by the degree of browning, as all the reaction products can result in brown color of protein products under alkaline conditions. Chlorophyll and carotenoids are the main pigments in leaves, which can turn brown at high temperature (Felicetti and Schrader, 2009). Soluble polyphenols and lignin form highly conjugated brown compounds when reacting with protein (Ozdal et al., 2013). Carbohydrates, containing reducing sugars can react with amino acids generating brown color, which is well-known as the Maillard reaction (van Soest and Mason, 1991). These

reactions can be accelerated when high temperature or pH are applied (Carvalheiro et al., 2008). Removal or prevention of coloring components can improve the protein quality.

Many chemicals and/or enzymes may be used during pre-treatments to remove pigments, polyphenols, carbohydrates, and lignins. Solvents (ethanol, acetone, hexane) are used for isolating pigments and polyphenols (Turkmen et al., 2006). Cell wall degrading enzymes, such as Viscozyme® L, pectinase, and Celluclast® are used to enhance protein extraction by degrading cell wall polysaccharides, such as pectin, and (hemi-) cellulose (Bals et al., 2007b; Jodayree et al., 2012; Rosset et al., 2014). H_2O_2, which breaks down lignin, is also used to increase the dry matter digestibility of biomass used as animal feed (Chaudhry, 1998). As all these pre-treatments can be carried out under mild conditions ($T<60$ °C), the integrity of other side products such as pigments, polyphenols, and carbohydrates are preserved.

Pre-treatments using ethanol, Viscozyme® L, and/or H_2O_2 may fit in an integrated bio-refinery concept for leafy material (Zhang et al., 2015), but three main questions remain to be answered. First, how efficient are these pre-treatments for isolating pigment, polyphenol, carbohydrate, and lignin from biomass? Second, how do these pre-treatments influence the efficiency of a subsequent alkaline protein extraction in terms of alkali consumption, protein yield, and protein purity? Finally, how do these pre-treatments influence the color, nutrient digestibility and nutritive value of the extracted proteins? To answer these questions, we tested ethanol, Viscozyme® L, and/or H_2O_2 as pre-treatments using green tea residue (GTR), a potential source for animal feed (Jayasuriya et al., 1978; Wu et al., 2014), thereby aiming to make leaf proteins available for monogastrics. The yield of chlorophyll, carotenoid, polyphenol, carbohydrate, as well as the protein content, were measured to analyse the extraction efficiencies of these pre-treatments. Alkali consumption, protein yield, protein purity, color formation, and dry matter digestibility were measured as efficiency indicators of these pre-treatments on alkaline protein extraction and protein quality.

4.2 Materials & Methods

4.2.1 Materials

Green tea residues, which are the waste of tea leaves after hot water extraction, contain 8% polyphenols, 25% proteins, and 31% carbohydrates. Currently, these tea residues are only used in a low value application, namely for energy generation through burning.

Viscozyme® L (Multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) was purchased from Sigma, USA. If not stated otherwise, other chemicals used for analysis were purchased from Sigma (USA) and of analytic grade.

4.2.2 Pre-treatments & protein extraction

4.2.2.1 Ethanol pre-treatment

To optimise ethanol extraction efficiency, GTR (200 mg) was first soaked in 4mL 0-100% v/v ethanol, and incubated in a Thermomixer (VWR International B.V., USA) at 60 °C and 1000 rpm for 2 h. In subsequent experiments, GTR (200 mg) was soaked in 4mL 50% ethanol or absolute ethanol in 10 mL tubes, and then they were incubated in a Thermomixer (60 °C, 1000 rpm) for 1, 5, 15, 30, 60, or 90 min. After centrifugation (7,000 g, 20 °C, 10 min), liquid extracts were collected and stored at 4 °C until further analysis.

4.2.2.2 Viscozyme® L pre-treatment

GTR (200 mg) was suspended in 4 mL 0.02 M sodium acetate – acetic acid buffer at pH 4.5, and then mixed with Viscozyme® L of 0, 12, 30, 60, or 120 UFBG g^{-1} GTR activity, and supplemented to 4.2 mL with demi-water. After incubation in Thermomixer (30 °C, 1000 rpm) for 20 h, 4 mL demi-water or ethanol was added to the mixtures, and then centrifuged (7,000 g, 20 °C, and 10 min). The liquid extracts were collected and stored at 4 °C until further analysis.

4.2.2.3 H_2O_2 pre-treatment

GTR (200mg) was suspended in 4 mL H_2O_2 solution with concentration of 0, 0.5, 1, or

2% v/v. After incubation in Thermomixer (60 °C, 1000 rpm) for 2 h, 4 mL demi-water or ethanol was added to the mixtures, and then centrifuged (7,000 g, 20 °C, and 10 min). The liquid extracts were collected and freeze-dried for the removal of excess H_2O_2. Dried samples were store at room temperature until further analysis.

4.2.2.4 Alkaline protein extraction

The residual pellets obtained after pre-treatment were washed with 4 mL solution that corresponded to the extraction liquid, and used for a subsequent alkaline protein extraction. The alkaline protein extraction protocol was based on our previous research (Zhang et al., 2014). Pipetting 7 mL 0.1 M NaOH to the pre-treated GTR, the mixture was homogenised and incubated in a thermo mixer (95 °C, 1000 rpm) for 2h. After centrifugation (7,000 g, 20 °C, 10 min), protein extracts were collected and stored at 4 °C until further analysis.

4.2.2.5 Samples for analysis of the nutritional value

Several samples were selected for analysing the influence of pre-treatments on the nutritional value of protein samples. For this, larger amounts of protein product had to be generated: 150 g GTR was used in pre-treatments with 1.5 L liquid, in which 50% ethanol, 10 mL Viscozyme® L with water, or 2% H_2O_2 with water were used. The extractions were carried out in 2 L Scott bottles, and incubating was done in a water bath with a shaking speed of 170 rpm min^{-1}. Temperature, extraction time, and combinations for two-step pre-treatments were the same as in previous experiments. Alkaline protein extraction was performed on 100 g pre-treated GTR samples that were mixed with 1.5 L 0.2 M NaOH in 2 L Scott bottles. After incubating in a water bath with a shaking speed of 170 rpm min^{-1} for 2.5 h, supernatants were collected by centrifugation. To obtain the final product, protein extracts were purified by acid precipitation at pH 4 at 4 °C overnight. After centrifugation, and neutralisation samples were freeze-dried and stored at room temperature until further analysis.

4.2.3 Determinations

4.2.3.1 Polyphenol

Content of tea polyphenols in GTR extracts was determined by ferrous tartrate method (Turkmen et al., 2006). Polyphenols content was calculated assuming a concentration of polyphenols of 3.914 g L^{-1} leads to an adsorption of 1 at 540 nm after reaction (Turkmen et al., 2006).

4.2.3.2 Total sugar

Total sugar content was determined by DuBois method at 492 nm (DuBois et al., 1956; Taylor, 1995). Glucose was used as a reference in a concentration range from 0 to 50 mg L^{-1}.

4.2.3.3 Galacturonic acid

Content of galacturonic acid was determined spectrophotometrically with scaling down reagent usage (Taylor, 1993). Galacturonic acid (Fluka AG, Buchs, Switzerland) was used as a reference in a concentration range from 0 to 50 mg L^{-1}.

4.2.3.4 Chlorophyll & Carotenoids

Chlorophyll that was extracted by ethanol was measured using a spectrophotometer (Ritchie, 2006). Data of absorbance at wavelengths of 470 nm, 649 nm and 664 nm were collected. Chlorophyll content (mg/L) was calculated by using $22.24 \cdot A_{649} + 5.24 \cdot A_{664}$, and carotenoid content was calculated as $4.78 \cdot A_{470} - 12.76 \cdot A_{649} + 3.66 \cdot A_{664}$ (Sumanta; et al., 2014).

4.2.3.5 Protein yield and protein purity

Protein content (g L^{-1}) was determined by Dumas analysis using a Nitrogen analyzer, FlashEA 1112 series from Thermo Scientific (Interscience, Breda, The Netherlands). Although Dumas measures all nitrogen, we assumed that it is all protein and used a conversion factor of 6.25 to calculate protein concentrations. Protein extraction yield was calculated as $W_{\textbf{Extracted protein}} / W_{\textbf{Total protein}} \cdot \mathbf{100\%}$.

Protein purity was calculated as $W_{\textbf{Extracted protein}} / W_{\textbf{Èxtracted GTR}} \cdot \mathbf{100\%}$. Here, weight of extracted GTR was determined by analytical balance after oven drying at 80 °C for 24 h

in glass containers, corrected for the amount of NaOH added, and subtracted by the amount of OH^- that was consumed to H_2O due to pH decrease.

4.2.3.6 Alkali consumption

Alkali consumption was calculated from the pH decrease after protein extraction, originally starting from pH 13 (0.1 M NaOH).

4.2.3.7 Color of protein extracts

Protein extracts were diluted 20 times, and the absorbance of diluted samples was scanning spectrophotometrically (DU®700, Beckman, USA) from 200 to 850 nm. The absorbance at 500 nm was finally used to determine the extent of browning in protein extracts.

4.2.3.8 Analysis of nutritional value

Sample composition: All samples were analyzed in duplicate. For determination of the DM content, feed was freeze-dried according to ISO 6496 (1998). Following freeze-drying, feed was ground to pass a 1 mm screen and kept for analysis. Air-dry feed was dried in a forced air oven at 103 °C to a constant weight according to ISO 6496 (1998). Kjeldahl nitrogen content was measured according to ISO 5983 (1997) in fresh feed. Crude protein content was calculated as nitrogen times 6.25. Crude fat content was determined after acid hydrolysis according to ISO 6492 (1999). For determining the crude ash content, samples were incinerated at 550 °C in a muffle furnace according to ISO 5984 (2002). The starch content was analyzed enzymatically as described by Brunt (1993). Reducing sugars were extracted from the feed samples, using 40% ethanol, and determined as described by Suárez et al. (2006).

Amino acid composition: To quantify the nutritional value, amino acid compositions of pre-treated GTR samples and their protein extracts were determined via UPLC (Meussen et al., 2014). Samples were first hydrolysed in 6N HCl containing 1% (g L^{-1}) phenol at 110 °C for 24 h and the amino acids were separated using an Acquity UPLC BEH C18 reversed phase column. The analysis was performed at the wavelength of 338 nm and 263 nm using Dionex RSLC (Dionex Corporation, Sunnyvale, CA, USA).

4.2.3.9 In vitro digestibility

In vitro incubations were performed according to a modified Boisen two-step method (Boisen and Fernandez, 1997), which simulates the digestive process in the stomach and small intestine of a pig and estimates the ileal nutrient digestibility. Substrates (1 g) were incubated in beakers with a phosphate buffer solution (75 mL, 0.1 M, pH 6.0) and a HCl solution (30 mL, 0.2 M). The pH was adjusted to 2.0 with 1 M HCl or 10 M NaOH. Fresh pepsin solution (1 mL, 25 g L^{-1}, porcine pepsin 2000 FIP U g^{-1}, Sigma P7000) was added and each beaker was covered with a glaze and placed in a heating chamber at 39 °C for 2 h under constant magnetic stirring. Then, 30 mL phosphate buffer (0.2 M, pH 6.8) and 12 mL of a 0.6 M NaOH solution were added. The pH was adjusted to 6.8 with 1 M HCl or 10 M NaOH. Fresh pancreatin solution (1 mL, 100 g L^{-1} pancreatin, Porcine pancreas grade VI, SigmaP-1750) was added and hydrolysis was continued for 4 h under the same conditions. Then 30 mL of a 0.2 M EDTA solution was added and the pH adjusted to 4.8 with 30% acetic acid. After hydrolysis, the residues were collected by filtration of the slurries on a nylon gauze (37 μm) folded in a Büchner porcelain funnel. The sample was washed twice by acetone (99.5 %) followed by ethanol (96 %). Then the cloth with the residue was temporarily placed on a clean paper to evaporate the remaining ethanol/acetone overnight. The residue was scraped off the nylon cloth and collected in a pre-weighed jar and dry matter digestibility was calculated.

4.3 Results & discussion

4.3.1 Extraction efficiencies of pre-treatments

4.3.1.1 Single pre-treatments

Ethanol, Viscozyme® L, and H_2O_2 were used aiming for removal of polyphenol, carbohydrates, and lignin, respectively. Polyphenols and carbohydrates were extracted efficiently in their respective pre-treatments, but lignin was not extracted by H_2O_2. To further clarify what can be extracted with the various pre-treatments, dry matter, N-containing components, galacturonic acid, polyphenol, and total sugar content of the extracts were determined and plotted in Fig. 4.1a, 4.1b, and 4.1c.

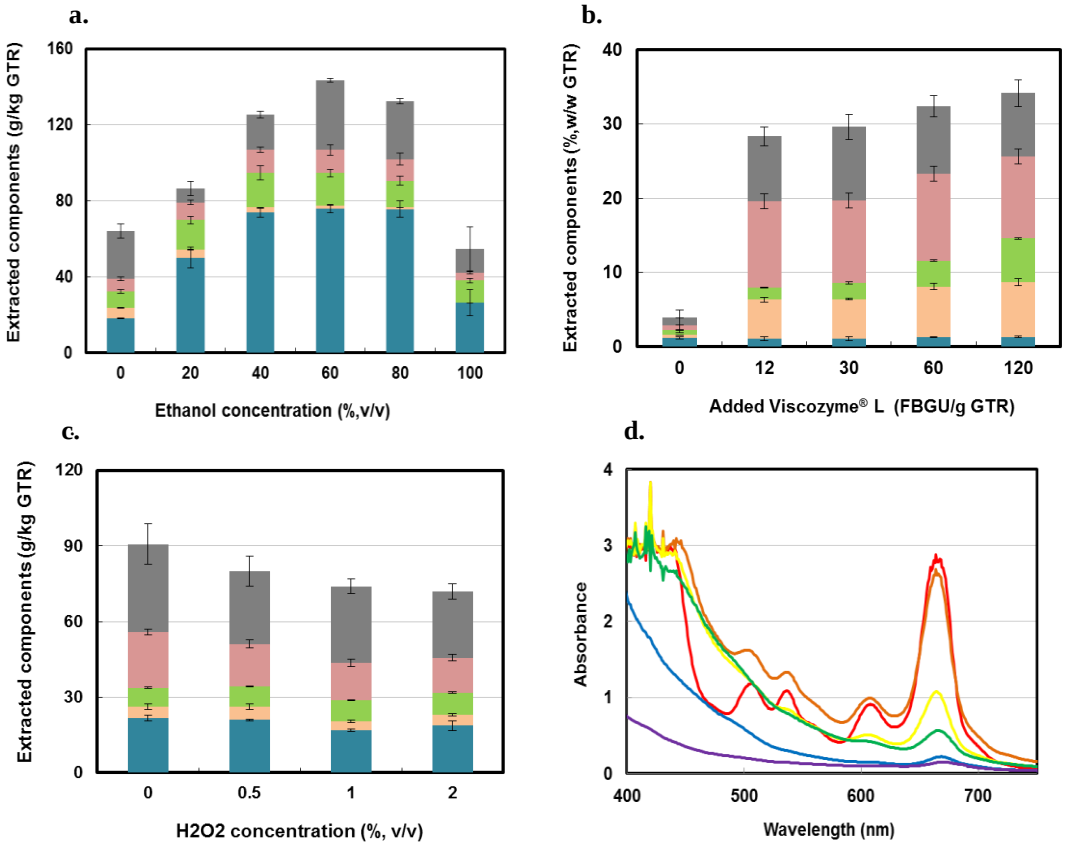

Fig. 4.1 Components extracted (g kg^{-1} GTR) by different methods. ■: Polyphenol; ■: Galacturonic acid; ■: N-containing components; ■: Sugars; ■: Other components. a, Ethanol with different concentration (%, v/v) at 60 °C for 1h; b, Viscozyme® L with different activity (UFBG g^{-1} GTR) treatment at 30 °C for 18h with water extraction; c, H_2O_2 at various concentrations (%, v/v) at 60 °C for 2h; d, Absorbance of extracts obtained by using various ethanol concentrations at 60 °C for 2h. Ethanol concentration: ——: 100%; ——: 80%; ——: 60%; ——: 40%; ——: 20%; ——: 0%.

In ethanol extraction, the amount of extractable dry matter showed a bell-shaped curve against the applied concentration of ethanol (Fig. 4.1a). The highest amount of dry matter (140 g kg^{-1} GTR) was extracted when 60% ethanol was used as solvent, which is higher than in the H_2O_2 pre-treated samples, but much lower than the Viscozyme® L treated samples. When 40-80% ethanol was applied, all polyphenols (78 g kg^{-1} GTR) were extracted (Fig. 4.1a). Under these conditions, very low amounts (<2% GTR) of galacturonic acid and glucose, and a fixed amount of protein (2-3% GTR), were extracted. The amount of non-determined components was high when 60% ethanol was

used, suggesting an extraction of complex compounds with both polar and non-polar groups (Harold N, 1992; Turkmen et al., 2006). Most pigments can only be extracted in high ethanol concentration. Looking at the wavelength scan of the ethanol extracted samples (Fig. 4.1d), absorption peaks in the 80% ethanol and pure ethanol extracts can be seen at 510nm, 540nm, 630nm, and 670nm, while these peaks are not present or smaller at lower ethanol concentrations. These absorption peaks were contributions from chlorophyll a, chlorophyll b, and carotenoid (Harbowy; and Balentine, 1997; Mantoura and Llewellyn, 1983; Ritchie, 2006).

Viscozyme® L had the highest extraction efficiency. Over 30% of GTR was extracted (Fig. 4.1b) at 30 °C, and its total extraction yield was positively dosage dependent. Upon addition of 12 FBGU of Viscozyme® L in 1g GTR, the extraction yield of galacturonic acids, sugars, and other components was at 50, 110, and 90 g kg^{-1} GTR correspondingly, which is 10 times higher than without adding enzyme. Addition of more enzyme had no influence on extraction yields of sugar and polyphenol, but led to higher yields of galacturonic acid and N-containing components. As polyphenols are more soluble in 50% ethanol (Brennan, 2005; Turkmen et al., 2006), while protein is not, a combined extraction with ethanol aided extraction after Viscozyme® L pre-treatment may extract non-protein components with low protein yield.

H_2O_2 had the lowest extraction efficiency with only 80 g kg^{-1} GTR extracted (Fig. 4.1c). Its extraction yield was not influenced by the H_2O_2 concentration and even lower than the blanc. Residual GTR was bleached (data not shown), indicating reactions of H_2O_2 with color related compounds. As lignin has a very low solubility in water, an ethanol extraction after H_2O_2 pre-treatment may aid extraction of lignin.

4.3.1.2 Viscozyme® L or H_2O_2 combined with 50% ethanol

To test the potential of combined Viscozyme® L or H_2O_2 treatment with ethanol extraction, the same volume of ethanol was added to the samples after Viscozyme® L or H_2O_2 extraction. Based on the previous results and other research, final ethanol concentration was 50%, by which most polyphenol can be extracted with least ethanol addition (Turkmen et al., 2006). The composition of Viscozyme® L and H_2O_2 extracts

combined with ethanol extraction were plotted in Fig. 4.2a and Fig. 4.2b.

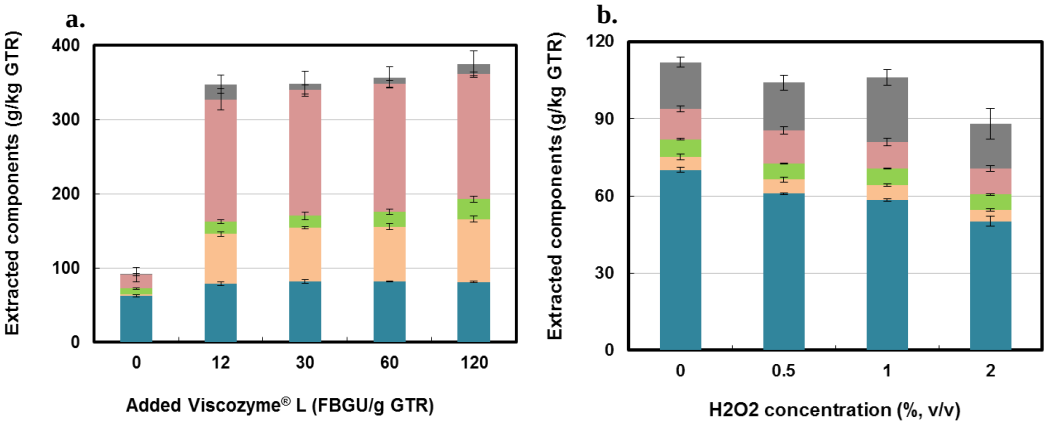

Fig. 4.2 Components (g kg^{-1} GTR) extracted by two combined methods. ■: Polyphenol; ■: Galacturonic acid; ■: N-containing components; ■: Sugars; ■: Other components. a, Viscozyme® L treatment at 30°C for 18h with 50% ethanol extraction; b, H_2O_2 at 60°C for 2h in 50% ethanol extraction.

The total amount that was extracted from GTR was increased due to additional polyphenol solubilisation compared to water extraction. As protein has a low solubility in 50% ethanol, less protein was extracted leading to a higher remaining protein content in the residue. This 340 g kg^{-1} protein content in residue will probably also lead to higher protein content in subsequent alkaline protein extracts. The amount of extractable polyphenol and non-determined components was less when higher H_2O_2 concentrations were applied under 50% ethanol conditions. This suggests that the bleaching effect of H_2O_2 on GTR that was observed did not lead to a solubilisation of lignin.

4.3.2 Influence of pre-treatments on efficiency of alkaline extraction

The influence of individual pre-treatments on a subsequent alkaline extraction was quantified by measuring alkali consumption, protein extraction yield, and protein purity. As shown in Table 4.1a, using ethanol or Viscozyme® L as pre-treatments could improve the efficiency of alkaline extraction, while using H_2O_2 as a pre-treatment had a negative effect.

Table 4.1a. Influence of three individual pre-treatments on subsequent alkaline protein extraction

		Alkali consumption (mmol g^{-1} GTR)	Protein yield (g kg^{-1} leaf protein)	Protein purity (g kg^{-1} dry matter)
Ethanol concentration (L/L)	0	2.9±0.05	685±18	349±20
	0.2	2.7±0.05	735±38	365±11
	0.4	2.4±0.1	764±12	396±10
	0.6	2.2±0.05	770±34	388±22
	0.8	2.1±0.1	781±41	411±10
	10	2.7±0.1	804±36	367±11
Viscozyme® L activity (FBGU/g GTR)	0	3.0±0.2	615±14	325±10
	12	2.6±0.1	742±34	416±51
	30	2.5±0.1	770±16	464±13
	60	2.6±0.1	799±40	451±42
	120	2.4±0.3	753±7	457±49
H_2O_2 concentration (mL/L)	0	3.0±0.2	722±60	351±3
	5	3.1±0.1	716±12	357±6
	10	3.1±0.2	667±3	338±2
	20	3.3±0.2	696±8	335±4

NaOH consumption could be reduced by 0.8 mmol g^{-1} GTR when ethanol was used as a pre-treatment and by 0.6 mmol g^{-1} GTR using Viscozyme® L as pre-treatment, while it slightly increased when H_2O_2 was used. The reductions were due to the removal of polyphenol and carbohydrates that were specifically targeted in the ethanol and Viscozyme® L pre-treatment. The increased alkali consumption with H_2O_2 pre-treatment may result from the reaction of residual H_2O_2 with alkali. Protein yields and purity in alkaline extracts were both influenced by the extraction of non-protein components and by protein loss in pre-treatments. Although Viscozyme® L can release protein from plant seeds due to the degradation of plant cell wall (Guan and Yao, 2008; Rosset et al., 2014), Viscozyme® L has only a limited effect on leaf protein extraction due to the low efficiency on hydrolysing rhamnogalacturonan I pectin and lignin, which probably hampered protein extraction (Zhang et al., 2015). A relatively low protein yield (75%) was obtained when as much as 120 FBGU Viscozyme® L was used, because 23% of the protein components were lost in the pre-treatment step. Improvements can probably be made combining pre-treatments. Using ethanol as solvent for Viscozyme® L or H_2O_2 pre-treatment to change the distribution of protein fractions may improve protein yield and purity in the alkaline extracts.

The influences of combined pre-treatment are shown in Table 4.1b. The maximum protein yield and purity were 89% and 53% respectively in protein extracts, obtained

after using Viscozyme® L of 120 FBGU g^{-1} GTR in 50% ethanol, which was higher than those obtained without addition of ethanol. The amount of alkali that was consumed by polyphenol was calculated as 0.7 mmol NaOH g^{-1} GTR and by carbohydrates was 0.6 NaOH g^{-1} GTR and in alkaline protein extraction without pre-treatment. H_2O_2 pre-treatment was not improved by 50% ethanol extraction. The bleaching reactions between H_2O_2 and color related components did not result in their solubilisation. Excess H_2O_2, which was not totally separated from solid GTR, even increased alkali consumption in the subsequent protein extraction step.

Table 4.1b. Influence of Viscozyme® L and H_2O_2 pre-treatments using 50% ethanol as solvent on subsequent alkaline protein extraction

		Alkali consumption (mmol g^{-1} GTR)	Protein yield (g kg^{-1} leaf protein)	Protein purity (g kg^{-1} dry matter)
Viscozyme® L activity (FBGU/g GTR)	0	2.3±0.1	706±9	373±12
	12	1.6±0.1	725±45	442±67
	30	1.6±0.05	766±43	454±46
	60	1.6±0.05	823±38	506±36
	120	1.5±0.1	890±15	531±20
H_2O_2 Concentration (mL/L)	0	2.5±0.1	692±12	367±7
	0.5	2.5±0.1	683±17	359±9
	1	2.5±0.1	662±2	373±2
	2	2.6±0.1	671±7	381±4

4.3.3 Influence of pre-treatments on protein color formation

To analyse the influence of pre-treatments on protein color formation, protein extracts obtained after various pre-treatments were scanned spectrophotometrically at wavelengths ranging from 400 to 750 nm (Fig. 4.3a, Fig. 4.3b, and Fig. 4.3c). Protein color derived from pigments, polyphenol, carbohydrates, and lignin were identified by associating amounts of these components removed in the pre-treatments and the change of color absorbance in alkaline protein extracts.

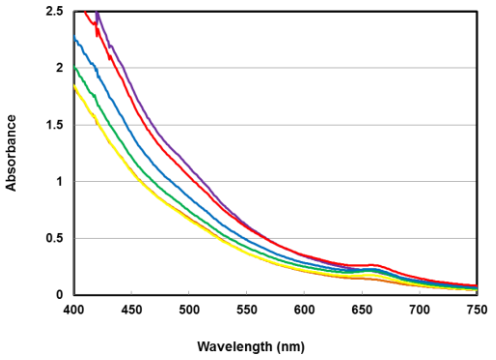

Fig. 4.3a Influence of ethanol pre-treatment on the color of protein extracts obtained by 0.1 M NaOH with 40 v/w and 95 °C for 2 h (10 times diluted). Ethanol concentration: ——: 100%; ——: 80%; ——: 60%; ——: 40%; ——: 20%; ——: 0%.

The lowest absorbance was found for protein extracts pre-treated by 60% ethanol and 80% ethanol (Fig. 4.3a), while the absorbance of protein extracts with pre-treatment of 40% ethanol was slightly higher. The absorbance of protein extracts with pure ethanol pre-treatment, which removed pigments, was similar as that with water pre-treatments, suggesting pigments had a minor influence on color formation. To quantify the influence of pigment and polyphenols on color formation, the amount of polyphenols and pigments (chlorophyll and carotenoid) removed by pure ethanol and 50% ethanol at various conditions was correlated to absorbance of protein extracts at 500nm (Fig. 4.3b). When all polyphenols were removed during pre-treatment, the A_{500} of protein extracts was reduced by 59%. Chlorophyll and carotenoids were only detected in pure ethanol extracts, in which some polyphenol was also solubilised. Considering the influence from polyphenol, the influence on color formation from chlorophyll and carotenoid was neglect able.

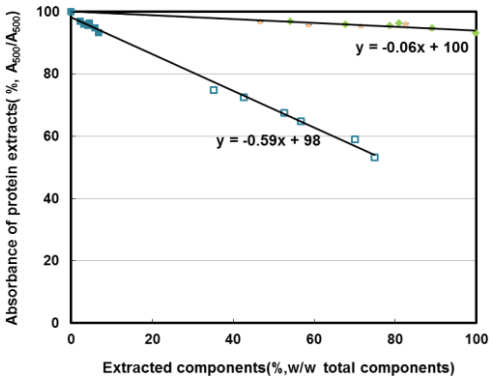

Fig. 4.3b Influence of removed pigments and polyphenols on the color of protein extracts obtained by 0.1 M NaOH with 40 v/w and 95 °C for 2 h. ▲: Carotenoid; ◆: Chlorophyll; ■: Polyphenol extracted by pure ethanol; □: extracted by 50% ethanol.

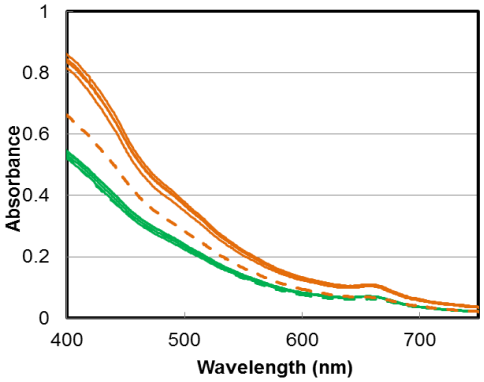

Fig. 4.3c Full wavelength scanning (400-750 nm) of protein extracts with viscozyme pre-treatment (20 times diluted). — —: control with 50% ethanol extraction; ——: viscozyme pre-treatments with different enzyme activities and 50% ethanol extraction; — — : control with water extraction; ——: viscozyme pre-treatments with different activities and water extraction.

When 50% ethanol was used as solvent (Fig. 3c), the absorbance of control coincided with the absorbance of protein extracts with Viscozyme® L in 50% ethanol pre-treatment, demonstrating the released carbohydrates had no influence on color generation. This conclusion was further confirmed experiments with addition of extra sugars in alkaline extraction without

pre-treatments, in which colors of protein extracts also did not increase (results not shown). When water was used as solvent, the absorbance of the control deviated from the absorbance of protein extracts with Viscozyme® L pre-treatment. These phenomenon may suggest that Viscozyme® L also hydrolysed carbohydrates that formed a complex with the more hydrophobic phenolic compounds (Hong et al., 2013). This hydrolysis leads to free phenolic compounds again, with decreased solubility in water and increased solubility in 50% ethanol.

As polyphenol contributed to about 59% of protein color, while carbohydrates and pigments had no contribution, the remaining color contribution was assumed to come from lignin. The influence of lignin on color formation was expected to become clear using H_2O_2 as pre-treatment to remove or degrade lignin. However, Fig. 4.1c and Fig. 4.2b showed that lignin was not removed by H_2O_2. As there was no influence of H_2O_2 on the color formation during the subsequent alkaline protein extraction (data not shown), this indicates that H_2O_2 has no effect on the degradation of lignin or at least does not influences the color formation from lignin in protein extracts.

4.3.4 Influence of pre-treatments on nutritional value of GTR samples and subsequent protein extracts

4.3.4.1 Composition and digestible components

The composition of GTR and protein extracts with and without pre-treatment were determined (Table 4.2) and compared. Ethanol pre-treatment has a small positive influence on dry matter (DM) digestible components, while the combined pre-treatments increased the digestibility of DM considerably. With pre-treatment of Viscozyme® L plus 50% ethanol, the digestible components increased from 200 to 340 g kg^{-1} DM. Likewise, pre-treatment of GTR with H_2O_2 plus ethanol increased digestible components 200 to 360 g kg^{-1} DM. Alkali treatment, however, resulted in protein extracts with a close to full digestibility, independent of pre-treatment. This might be explained by the reduced NSP-content, which was below 300 g kg^{-1} DM in the alkali treated samples, thereby allowing digestive enzymes to solubilize all available nutrients. The protein content of the protein extracts was affected by the method of pre-treatment, and ranged from 450 g

kg^{-1} DM in the control residue to 560 g kg^{-1} DM after pre-treatment with Viscozyme® L plus 50% ethanol.

Table 4.2. Composition and digestibility of GTR samples (g kg^{-1})

	Original GTR	GTR after pre-treatments			Protein extracts (Control)	Protein extracts with pre-treatments		
		50% EtOH	Viscozyme® L + 50% EtOH	H_2O_2+ 50% EtOH		50% EtOH	Viscozyme® L + 50% EtOH	H_2O_2+ 50% EtOH
Dry matter[a]	934±1	909±9	944±1	919±1	892±1	902±1	924±1	913±1
Digestible component[b]	201±3	231±8	339±17	335±59	1000	1000	1000	1000
Ash	41±1	39±3	40±1	35±1	111±1	132±1	77±2	133±8
Crude protein	251±8	274±6	323±1	252±3	451±2	493±1	558±2	436±23
Crude fiber	231±8	273±7	242±3	235±10	9±1	21±4	11±2	18±1
Fat	18±1	13±5	28±1	21±1	17±3	7±1	28±2	33±22
Starch	29±1	52±24	10±1	19±7	7±1	10±1	1±1	19±7
Sugar	48±2	3±1	33±1	20±7	13±1	8±1	9±1	28±21
NSP[c]	550	530	510	570	290	250	250	260

a, Based on sample weight after freeze drying; b, Digestible components were presented as g kg^{-1}; c, Non-Starch Polysaccharides are calculated as dry matter – ash – crude protein – fat – starch – sugar.

The larger scale, at which these experiments were performed, caused some differences with the results in 4.3.1 and 4.3.2. As the shaking efficiency of the water bath is lower than that of the Thermomixer, not all targeted components were removed in the pre-treatment. After alkaline extraction and acid precipitation, protein content in protein products was generally increased. It was mainly due to the complete protein extraction at alkaline conditions (Zhang et al., 2014). Protein can be further purified by acid precipitation, in which most protein can be precipitated while non-protein N containing components, such as caffeine, will not (Tangka, 2003).

4.3.4.2 Amino acids composition

To test the influence of pre-treatments on protein nutritional value, the amino acid contents of GTR and protein extracts with and without pre-treatment were determined (Table 4.3) and compared.

The nutritional value of proteins for monogastric animals and humans is often limited by the amount of essential amino acids, being arginine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Dale et al., 2009b). All three pre-treatments increased the content of total essential amino acid in both GTR samples after pre-treatments and their protein extracts. This is due to the removal of

non-protein N-containing components, such as caffeine, during pre-treatments. Although it was reported that digestibility of biomass can be improved by using alkali or H_2O_2 (Chaudhry, 2000; Mishra et al., 2000), these protein extracts had a lower total essential amino acid content compared to the original GTR samples. This reduction generally resulted from reduced contents of arginine, threonine, and lysine. These amino acids tended to degrade under strong alkaline conditions, which are related to pH, temperature, and reaction time (Hurrell and Finot, 2012).

Table 4.3. Amino acid content in N-containing components (g kg^{-1})

Amino acid	Original GTR	GTR after pre-treatments			Subsequent protein extracts from GTR samples			
		50% EtOH	Viscozyme® L + 50% EtOH	H_2O_2+ 50% EtOH	Control	50% EtOH	Viscozyme® L + 50% EtOH	H_2O_2+ 50% EtOH
His	17±2	18±1	18±2	17±1	23±2	23±1	21±3	19±1
Arg	44±2	40±3	50±2	44±2	8±1	27±1	39±2	27±1
Thr	41±2	42±3	46±1	44±2	15±1	17±1	25±1	18±1
Val	45±2	48±3	51±1	51±2	54±1	57±1	56±1	54±1
Met	14±1	15±1	16±1	14±1	18±1	18±1	14±2	15±1
Try[a]	-	-	-	-	-	-	-	-
Ile	50±3	52±3	56±2	55±2	59±3	63±1	62±1	61±1
Phe	39±2	41±2	43±1	43±2	50±1	54±1	50±1	52±1
Leu	78±4	82±5	86±2	86±3	95±3	102±1	97±2	97±1
Lys	54±7	55±6	56±5	54±2	39±4	43±2	35±6	27±2
Total	381±25	392±27	421±16	408±17	360±15	404±7	409±18	372±8
Asp+Asn	84±5	83±5	93±2	84±3	91±1	89±1	91±1	82±1
Glu+Gln	92±4	93±6	100±2	98±4	106±11	103±3	104±1	99±1
Ser	43±2	45±3	48±1	48±2	21±2	22±1	35±1	25±1
Gly	49±2	52±4	55±1	54±2	62±2	63±1	58±2	57±1
Tyr	29±2	25±1	33±2	29±2	41±1	39±1	40±1	34±1
Ala	51±3	53±3	56±1	56±2	58±2	61±1	59±2	56±1
Pro	43±3	47±3	48±1	48±1	49±1	49±1	51±1	48±1
Cys[a]	-	-	-	-	-	-	-	-
Ornithine	0	0	0	0	5±1	5±1	3±1	5±1
Total	775 ±42	792±51	855±23	826±28	814±25	834±6	840±14	778±2

a, Try and Cys were destroyed during the determination (Meussen et al., 2014)

In protein extracts, addition of Viscozyme® L in 50% ethanol had little influence on the essential amino acid composition compared to the single use of 50% ethanol as pre-treatment. The main differences of essential amino acid contents in these two protein extracts were the contents of threonine, arginine, and lysine, which had higher content with addition of Viscozyme® L. Using Viscozyme® L and 50% ethanol as pre-treatment, less alkali was consumed during alkaline extraction, and so the pH of extracts was higher. This higher pH may have resulted in more sever degradation of amino acids, and therefore reduced nutritional value of protein products. When H_2O_2 was used, almost all amino acids were less compared to those of the control, indicating that H_2O_2 degrades amino acids under alkaline condition. The extent of amino acid degradation due to H_2O_2

depends on H_2O_2 concentration, temperature, and reaction time (Roberts et al., 1989).

Since Kjeldahl determines total N content and protein was calculated using a factor of 6.25, real protein content counted not add up to 100%. The highest real protein content calculated based on amino acid content (855 g kg^{-1} protein) was present in GTR samples pre-treated by Viscozyme® L and 50% ethanol. Using this combined pre-treatment, most non-protein N containing components, such as chlorophyll and caffeine, can be extracted (Castle et al., 2011; Jun, 2009). Assuming for this case that all non-protein N containing components were removed, the amount of non-protein N containing components is approximately 10% of total N and the true N conversion factor that used for GTR is approximately 5.4.

4.4 Conclusions

Ethanol pre-treatment removed polyphenols from GTR, while Viscozyme® L removed carbohydrates, which both led to a high protein yield, protein purity, and less alkali consumption in the subsequent alkaline extraction. The removal of polyphenols decreased 59% of the color components normally generated in alkaline protein extraction, but removal of carbohydrates had no influence. H_2O_2 pre-treatment is not suggested as it didn't release lignin, and it had a negative effect on the alkaline protein extraction with no color change on protein extracts. As less protein was extracted during Viscozyme® L pre-treatment when 50% ethanol was used as solvent, the highest improvement on the protein extraction efficiency of the subsequent alkaline extraction was shown with this combined pre-treatment. Although total essential amino acid contents increased with pre-treatments of Viscozyme® L and/or ethanol, contents of arginine, threonine, and lysine decreased during alkaline protein extraction. An improvement on nutritional value of the final protein extracts can be further improved by an optimization of the integrated processes. It can be concluded that pre-treatment of leaf materials with Viscozyme® L and ethanol, followed by alkali protein extraction, results in protein concentrates (55% of crude protein) that seem to be highly applicable in monogastric diets. The suggested pre-treatment and subsequent alkaline protein extraction can be applied for other leafy biomass, which significantly increases the

protein sources for animal feed.

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CHAPTER 5: INTEGRATION OF PECTIN EXTRACTION FOR GALACTURONIC ACID WITH ALKALINE PROTEIN EXTRACTION FROM GREEN TEA LEAF RESIDUE

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Abstract: Leaf pectin can be used as a feedstock for galacturonic acid (GA) production, but high pectin extraction costs limit economic feasibility. To improve the extraction efficiency, leaf pectin extraction was integrated with an already cost-effective alkaline protein extraction, focusing on high yield of GA without losses of protein. GA extraction efficiencies in NaOH, HCl, phosphate buffer solution, or with Viscozyme® L were determined using green tea residues (GTR) as model material. Most pectin was extracted using Viscozyme® L, mainly due to its cellulase activity. Extraction yielded more than 95% GA with only 5% protein. Alternatively, pectin can be extracted in a weak alkaline solution. Here, pectin yield is dominated by the ratio of extraction volume to biomass weight. The profits of these two integrated processes can be higher than one step protein extraction. The Viscozyme® L integrated process is suitable for GA production for application in chemicals, and may have a profit of 142$/ton GTR when enzyme cost are sufficiently lowered. The profit of the weak alkaline integrated process is estimated at 118$/ton GTR.

5.1 Introduction

Pectin is a family of complex polysaccharides located in the primary plant cell wall and middle lamella (Dashek and Harrison, 2006a; Somerville et al., 2004) that is commonly used as functional ingredient in food industry (Willats et al., 2006). However, leaf pectin has usually been degraded and lost its functionality during plant growth, biomass harvesting, and/or pre-processing before extraction, and may no longer be suitable for food application. Alternatively, leaf pectin can be applied as chemical building block using its predominant components, such as galacturonic acid (GA). GA can be used as the starting material for vitamin C production (Mapson and Isherwood, 1956), and can be chemically transferred into various aromatic compounds under aqueous acidic conditions (Popoff and Theander, 1972). Furthermore, GA can be oxidized to its corresponding aldaric acid (C6-sugar di-acid), which has shown to be an interesting starting material for the production of 2,5-FDCA (Knoop et al., 2013), polyaldaramides and-esters (Lavilla et al., 2012; Lavilla et al., 2011; Muñoz-Guerra, 2012), sequestering agents (Abbadi et al., 1999), and corrosion inhibitors (Koefod, 2007).

However, applying leaf pectin as bulk chemical has a lower value than its application in food, and the production costs are relatively high due to its low content in leaf. To lower extraction costs, pectin extraction can be integrated with an already cost-effective alkaline protein extraction (Zhang et al., 2015; Zhang et al., 2016b). This alkaline protein extraction can be applied with a profit of about 85 €/ton GTR excluding capital and labor cost (Zhang et al., 2014). An integrated biorefinery concept was proposed to further improve the cost-effectiveness of this protein extraction (Zhang et al., 2015). It was suggested that removal of leaf pectin prior to protein extraction can reduce alkali consumption and improve protein quality (Zhang et al., 2016b). Therefore, the next logic step is to consider pectin as a side product of protein extraction.

When integrated with protein extraction under alkaline conditions, pectin extraction should rather focus on GA yield and its influence on the efficiency of alkaline protein extraction rather than on pectin functionalities. Methods that can be used for extracting high yield pectin include use of acid or alkali, and enzymatic methods (Lim et al., 2012;

Renard et al., 1990; Seixas et al.; Sengkhamparn et al., 2010; Shi et al., 1996; Wang et al., 2014; Westereng et al., 2008). Acid is commonly used for pectin extraction, but its integration with alkaline protein extraction will generate large amounts of salts that increase the cost of both pectin and protein extraction. Pectin can be extracted by weak alkaline solution, but the product is rarely used due to the decrease in pectin esterification degree (Jiang et al., 2005), which influences pectin functionality (Assoi et al., 2014). However, this method may be suitable for the proposed integration, since the pH that is required for pectin extraction is lower compared to alkaline extraction, by which pectin product can be extracted separately with no extra salts generated. Enzymes can be used to aid pectin extraction by hydrolyzing pectin into GA (Su et al., 2015) or by hydrolyzing cell wall carbohydrates (Wikiera et al., 2015). Pectate lyase and/or pectinase can be used for hydrolysis of pectin, while galactanase, arabinanase, hemi-celllulase and cellulase are often used for the degradation of cell wall carbohydrates, including rhamnogalacturonan I pectin, hemi-cellulose and cellulose (Taherzadeh and Karimi, 2008). Using an enzyme mixture such as Viscozyme® L, that contains several or even all enzymes mentioned above for cell wall degradation is popular for its high efficiency on hydrolysis (Sari et al., 2015a) and relative low price compared to individual enzymes. Enzyme aided extraction is carried out under either weak acid or alkaline conditions, and may also be suitable for integration with protein extraction.

To determine the most suitable pectin extraction method for integration with protein extraction, green tea residue (GTR) was used as a model material, as it was previously used for a study on alkaline protein extraction (Zhang et al., 2014; Zhang et al., 2015; Zhang et al., 2016b). Pectin extraction yield (represented by GA yield) in NaOH, phosphate buffer solution (PBS), and with Viscozyme® L (containing arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) and its relation to protein yield were first tested using acid or water as controls. Viscozyme® L aided pectin extraction was then optimized. To further investigate what enzymes determine pectin extraction, extraction effects of specific enzymes, including pectinase, arabanase, galactanase, cellulase, and hemicellulase were tested individually. Conditions for weak alkaline pectin extraction were optimized using a uniform experimental design. Economics of

both Viscozyme® L aided or weak alkaline pectin extraction with the integration of alkaline protein extraction were estimated and discussed.

5.2 Materials and methods

5.2.1 Materials

Green tea residue (GTR) is a gift from Damin Company, Fujian Province, China. This residue from tea lemonade production was collected from *Camellia sinensis* trees in Zhejiang province, China, in 2014, and it was sun-dried after soaking green tea leaves in water at 85 °C for 45 min. It contains 25% protein and around 6.7% of GA based on dry matter weight.

Viscozyme® L (Multi-enzyme mixture containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase), pectinase (EC 3.2.1.15), hemi-cellulase (An mixture of glycolytic enzymes containing xylanase, mannanase and other activities), and cellulase (EC 3.2.1.4) were purchased from Sigma, USA. Endo-1,5-α-L-arabinanase (EC 3.2.1.99) and endo-1,4-β-galactanase (EC 3.2.1.89) were purchased from Megazyme, Ireland.

Other chemicals if not stated otherwise were of analytical grade and purchased from Sigma company, the USA.

5.2.2 Pectin extractions

5.2.2.1 Under acid, weak alkaline, or alkaline conditions

Pectin extraction was performed by mixing 200 mg GTR with 4 mL 0.1M HCl, 0.1M PBS (pH 8), or 0.1M NaOH at 60 °C, and then incubated in a thermo-mixer (1000 rpm min^{-1}) for 2 h using water extraction as control. After subsequent centrifugation at 8000 g for 10 min (Sorvall centrifuge, Thermo Fisher Scientific, the USA), supernatants were obtained and stored at 4 °C until further analysis.

5.2.2.2 Viscozyme® L aided pectin extraction

Viscozyme® L with an activity of 2.4 U was added to 200 mg GTR mixed in 4 mL demi-water or 0.02 M pH 4.7 sodium acetate-hydrochloric acid buffer at 30 °C

(Thermomixer, 1000 rpm) for different incubation time (30 min - 28 h). After subsequent centrifugation, supernatants were collected and stored at 4 °C until further analysis.

5.2.2.3 Extraction efficiency of specific enzymes embraced in Viscozyme® L

Arabinanase (4 or 20 U g^{-1} GTR), galactanase (13 or 65 U g^{-1} GTR), cellulase (13 or 26 U g^{-1} GTR) , hemi-cellulase (15 or 30 U g^{-1} GTR), or pectinase (10 or 20 U g^{-1} GTR) were applied to 200 mg GTR mixed in 4 mL 0.02 M pH 4.7 sodium acetate-hydrochloric acid buffer at 30 °C and incubated for 20 h. After centrifugation, supernatants were collected and stored at 4 °C until further analysis.

5.2.2.4 Optimization of weak alkaline pectin extraction

Weak alkaline pectin extraction was optimized using a uniform design (Fang and Lin, 2003). Twenty-four experiments (Table 5.1) were performed to analyze the co-efficiency of five parameters (pH, buffer concentration, temperature, extraction time, and ratio of liquid to solid) and their mutual influences on the yield of GA and the ratio of GA to protein yield. The buffer solution used for pH7 and pH8 was PBS, while for pH9 and PH10 was a sodium-carbonate buffer solution.

Table 5.1. Experimental conditions for weak alkaline pectin extraction

No	Tempera-ture(℃)	Extraction time (h)	V/ W* (mL g^{-1})	pH	[Buffer] (mol L^{-1})	No.	Tempera-ture (℃)	Extraction time (h)	V/ W (mL g^{-1})	pH	[buffer] (mol L^{-1})
1	40	2	40	10	0.08	**13**	40	1	20	7	0.04
2	50	1.5	30	7	0.06	**14**	70	1.5	50	8	0.02
3	30	3	10	8	0.12	**15**	40	0.5	10	10	0.1
4	30	1	60	7	0.04	**16**	70	1	40	8	0.08
5	30	1.5	20	10	0.1	**17**	50	0.5	10	9	0.02
6	60	0.5	10	9	0.06	**18**	60	0.5	60	9	0.08
7	80	2.5	50	9	0.12	**19**	50	2	40	10	0.06
8	30	2.5	50	7	0.1	**20**	50	3	30	8	0.1
9	60	3	20	9	0.02	**21**	60	1	30	10	0.06
10	40	2	60	7	0.12	**22**	70	2.5	40	7	0.08
11	80	2.5	60	8	0.04	**23**	70	2	50	8	0.12
12	80	1.5	30	9	0.02	**24**	80	3	20	10	0.04

*: V/W, Liquid (Volume, mL) to solid GTR (Weight, g) ratio.

5.2.3 Determinations

5.2.3.1 Galacturonic acid

Content of GA was determined by modified carbazole method (Taylor, 1993). Galacturonic acid (Fluka AG, Buchs, Switzerland) was used as a reference in a concentration range from 0 to 50 mg L^{-1}. As GA is the main component of pectin, it represents relative pectin content (or homogalacturonan pectin).

5.2.3.2 Protein content

Protein content was determined with the Dumas combustion method on an NA 2100 nitrogen and protein analyzer (Thermo Quest-CE Instruments, Rodeno, Italy) using methionine as a standard. Although Dumas measures all nitrogen, we assumed that this is all protein and used a conversion factor of 6.25 to calculate protein concentrations. In the enzyme extraction experiments, protein content of samples was subtracted by the amount of protein from the added enzymes.

5.2.3.3 Dry matter

Dry matter weights of samples extracts were determined by analytical balance after oven drying at 60 °C for 48h in glass containers, and corrected for the added chemicals.

5.2.3.4 Xylose, galactose and arabinose, and glucose

Xylose was determined with a D-xylose assay kit (K-XYLOSE, Megazyme, Ireland), galactose and arabinose were determined by L-arabinose & D-galactose kit (K-ARGA, Megazyme, Ireland), and glucose was determined by D-fructose and D-glucose kit (K-FRGLQR, Megazyme, Ireland). These kits determine solubilized sugar content in both mono-sugar and polysaccharide forms. Solubilized hemi-cellulose and cellulose can be presented as xylose content and glucose content, and solubilized rhamnogalacturonan I pectin can be presented by the amount of arabinose and galactose.

5.2.3.5 Polyphenols

Content of tea polyphenols in tea extracts was determined spectrophotometrically (Li et al., 2005; Turkmen et al., 2006). Polyphenols content was calculated assuming polyphenol concentration of 3.914 g L^{-1} leads to an adsorption of 1 at 540 nm.

5.2.4 Result analysis and statistics

Each sample (except from the experiments using uniform design) had duplicates in both extraction and determination, and the standard deviation of the four measurements was calculated by Excel. Experiments in uniform design had no duplicates in extraction, but two determinations were carried for each sample.

For optimization of the weak alkaline pectin extraction, the relation between factors and GA extraction yield as well as relation between factors and values of GA/protein was studied via response surface methodology (Box and Draper, 1987). Response surfaces for extraction yield and GA/protein ratio were obtained using linear regression to fit respective experimental data to second order polynomial functions that in its complete form can be described by the following expression:

$$Y(i) = \beta_0 + \beta_1 F_1(i) + \beta_{11} F_1(i)^2 + \beta_2 F_2(i) + \beta_{12} F_1(i) F_2(i) + \beta_{22} F_2(i)^2 + \cdots + \beta_{45} F_4(i) F_5(i) + \epsilon(i)$$

Where $Y(i)$ is value of protein extraction yield or GA yield/protein yield for i^{th} experiment (factor-level combination), $F_n(i)$ is a value of n^{th} factor for i^{th} experiment, β -coefficients are unknown regression coefficients, $\epsilon(i)$ is the approximation error for i^{th} experiment. Estimated β-coefficients are further used to calculate maximal values of protein extraction yield of GA yield/protein yield. Furthermore, normalized values of estimated β-coefficients (provided in the Appendix) are used as indicators of corresponding factor impacts on GA extraction yield or ratio of GA yield to protein yield.

In this study we used a simplified form of regression that only contained significant parameters (regression coefficients). Parameter significance was determined by fitting the experimental data to the model with ascending number of parameters and calculating the corresponding root mean square error (RMSE). The parameters with the highest contribution to reducing RMSE were step-wise added to model. This was first carried out for all linear effect parameters, including temperature (T), extraction time (t), buffer concentration ([buffer]), pH, and volume to weight ratio (V/W), and resulted in screening out the least significant parameters. Consequently, it was repeated for non-linear and interaction effect parameters taking into account the results of screening.

After parameter screening, the significance of each parameter (P value) was further evaluated. The parameter with highest P value was additional removed when it was higher than 0.05.

5.3 Results and discussion

5.3.1 Comparison of acid, alkaline, weak alkaline, and Viscozyme® L aided pectin extractions

To establish a process with high GA yield and high ratio of GA to protein yield, GA content and protein content of extracts obtained through different methods were determined and plotted in Fig. 5.1. As shown, neither GA nor protein can be efficiently extracted with water, but almost all GA (6.5 % out of 6.7% present in GTR) can be extracted when HCl, NaOH, weak alkaline or Viscozyme® L were used. Protein yield obtained using NaOH solution is about 8% GTR, which is at least 4 times higher than other methods, leading to a low GA/protein ratio. In comparison, the GA/protein ratios obtained by PBS extraction and Viscozyme® L extraction are much higher (2.9 and 3.7 respectively).

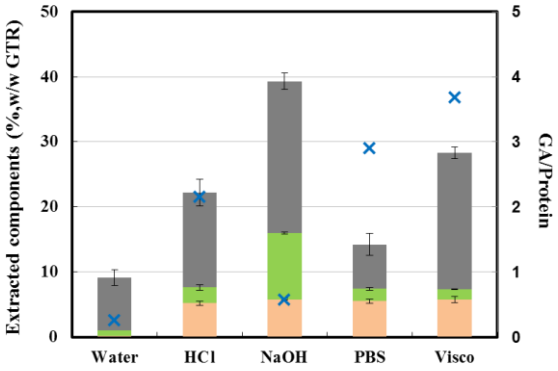

Fig. 5.1 Extracted components from 500 mg GTR by 20 mL water, 0.1 M HCl, 0.1 M NaOH, or 0.1 M pH 8 at 60 ℃ for 2 h, or Viscozyme (30 U g^{-1} GTR, in 10 mL PBS at pH 4.7) at 30 ℃ for 20 h. ■: Galacturonic acid; ■: Protein; ■: Other Components; ×: Ratio of galacturonic acid to protein.

Next to protein and GA, other components are extracted that are of different composition for each method. Using PBS of pH8, polyphenols can be also oxidized and solubilized, while using Viscozyme® L other carbohydrates such as hemi-cellulose and cellulose can be hydrolyzed and released with only little amounts of polyphenol (Zhang et al., 2015; Zhang et al., 2016b).

5.3.2 Is pH control by buffer addition necessary in Viscozyme® L aided pectin extraction?

Viscozyme® L aided pectin extraction is optimal at pH 4.7, for which sodium acetate-hydrochloric acid buffer was used. However, this buffer is mainly used to keep the pH below 7 and the buffer may be not necessary for pectin extraction, as the extract is acidifying during the extraction due to the release of acidic components such as organic acids and pectin.

To test if buffer usage is necessary for Viscozyme® L aided pectin extractions, pectin extraction efficiencies were determined in water or weak acidic conditions. The results on GA yield, protein yield, and their ratio are shown in Fig. 5.2. Pectin yield obtained by Viscozyme® L aided pectin extraction using buffer are generally lower than those without. The lower pectin yield is possibly due to the low solubility of substrate at pH 4.7 compared to that at pH 7 (water). Protein yield was not influenced by the addition of acidic buffer, therefore values of GA/protein obtained at buffer free conditions are generally higher than those with buffer. The highest GA/protein value of 6.8 can be obtained by Viscozyme® L pectin aided extraction at 30 °C for 2h without buffer addition.

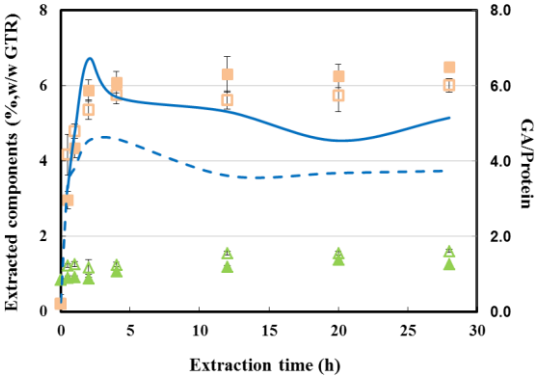

Fig. 5.2 Extracted components (%, w/w GTR) in time from GTR using Viscozyme (30U/g GTR) at 30°C. ■: GA yield without buffer ; □: GA yield with buffer ; ▲: Protein yield without buffer; △: Protein yield with buffer ; ——: GA/protein without buffer; — —:GA/protein with buffer.

5.3.3 Which enzymes in Viscozyme® L determine GA extraction

Viscozyme® L showed high GA extraction with low protein extraction, demonstrating its potential for the integration with the subsequent alkaline protein extraction (Zhang et al., 2016b). To look into the mechanism of enzyme aided pectin extraction, the separate enzymes in Viscozyme® L, including arabinanase, hemi-cellulase, and cellulase on GTR

were tested using extractions with pectinase and galactanase as controls. Compositions of GTR extracts obtained after using specific enzymes are presented in Fig. 5.3.

As presented in Fig. 5.3, cellulase and pectinase extracted the largest amounts of components from GTR, compared to the other enzymes tested. Approximately 33% and 28% dry matter from GTR was extracted respectively, which is similar to Viscozyme® L aided extraction. Other enzymes extracted only minor amounts, suggesting the effects of Viscozyme® L extraction mainly originated from cellulase activity. The highest GA purity, 35%, was obtained by using 10 U g^{-1} GTR pectinase, where almost all GA was extracted with only little cellulose solubilized (Glucose yield). This result illustrates that although hydrolysis of cellulose can lead to high pectin yield, cellulose is not the limiting component for pectin extraction. Addition of Viscozyme® L (12 U g^{-1} GTR) hydrolyzed more components than the addition of 13 U g^{-1} GTR cellulase. This was due to the aid from other enzymes, by which more GA, xylose, arabinose, and galactose were extracted. To further interpret how enzymes work during GTR extraction, values of extracted components are presented in Table 5.2 relative to their concentration in untreated GTR.

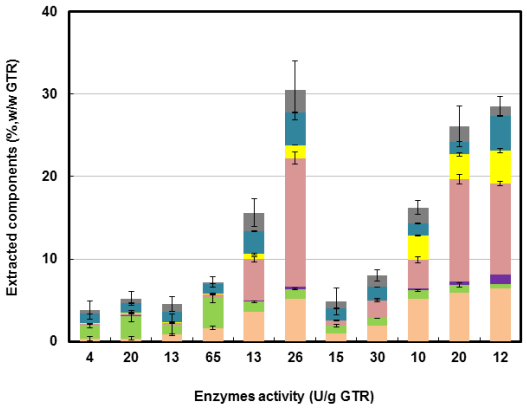

Fig. 5.3 Extracted components from 200 mg GTR by using different enzymes in 4 mL 0.02 M pH 4.7 sodium acetate-hydrochloric acid at 30 ℃ for 20 h. : Galacturonic acid; : Protein; : Xylose; : Glucose; : Arabinose & Galactose; : Polyphenol; : Other Components.

As shown in Table 5.2, using Viscozyme® L, more than 90% of GA was extracted with the other sugar yields are at 50-60%. Arabinanase, galactanase, and hemi-cellulase have no or minor influence on the hydrolysis of side chains of Rhamnogalacturonan I pectin (arabinan and galactanan) and hemi-cellulose when they were used individually. This was probably due to the low substrate accessibility for these enzymes in leaf cell wall (Ding et al., 2012). Cellulase and pectinase not only hydrolyzed cellulose and pectin, but also released the other components. Using 10 U g^{-1} GTR cellulase, cellulose was almost

completely hydrolyzed (96% glucose) resulting in the solubilisation of 77% homogalacturonan pectin (GA content), 25% Rhamnogalacturonan I pectin (arabinose & galactose), and 50% polyphenol. Although cellulose is considered to be the skeleton of plant cell walls (Somerville et al., 2004), its full extraction did not lead to the solubilisation of all components in plant cells. Pectin can be extracted without hydrolysis of cellulose. Using pectinase at 10 U g^{-1} GTR, 77% homogalacturonan pectin (GA content) and 47% Rhamnogalacturonan I pectin were obtained with only minor cellulose released (21% glucose). However, with a further hydrolysis of pectin, large quantities of cellulose were released (76% glucose) suggesting that the hydrolysis and solubility of pectin and cellulose can be both increased by the hydrolysis of only one of these compounds. These results indicate that our enzyme aided extractions are functioning on the primary plant cell wall, which mainly consists of pectin and cellulose that are crosslinked (Ridley et al., 2001). Viscozyme® L, pectinase, and cellulase are recommended for leaf pectin extraction with the integration of alkaline protein extraction.

Table 5.2. Components extracted by enzymes with different activities at pH4.7 and 30℃ for 20h (on the basis of initial content in GTR)

	Activity (U g^{-1} GTR)	Protein (%)	GA (%)	Xylose (%)	Arabinose & Galactose (%)	Glucose (%)	Polyphenol (%)
Initial content[a]	-	24.5±1.2	6.7±0.1	2.1±0.3	6.2±0.3	16.3±1.3	7.8±0.5
Viscozyme® L	12	2.3±1.1	90.8±4.9	55.7±2.4	63.4±4.3	67.7±1.3	53.8±1.2
Arabinanase	4	6.3±2.8	5.4±0.1	1.7±0.5	1.9±0.2	0.8±0.1	14.4±0.3
	20	11.1±1.6	5.7±0.4	1.8±0.2	2.1±0.1	1.6±0.4	14.1±0.6
Galactanase	13	4.8±5.3	12.3±0.2	1.9±0.1	2.2±0.0	1.2±0.3	16.1±0.2
	65	14.9±2.2	24.1±0.2	2.0±0.4	2.2±0.1	2.2±0.1	15.5±0.4
Cellulase	13	4.9±0.3	53.9±1.0	4.2±0.4	10.0±1.8	31.1±1.8	35.8±0.8
	26	5.0±0.6	76.6±2.2	11.8±1.7	24.8±0.5	95.9±4.7	50.4±0.5
Hemi-cellulase	30	4.0±0.5	13.9±0.3	1.7±0.4	0.4±0.0	3.5±0.3	18.9±0.4
	10	3.8±0.1	27.9±0.6	3.1±0.1	0.5±0.0	12.9±1.4	20.6±0.2
Pectinase	20	4.0±0.4	77.4±2.1	13.7±1.3	46.8±0.8	21.1±2.5	18.5±0.4
	65	4.2±1.1	87.4±2.3	20.5±0.4	48.2±3.4	75.7±3.5	19.2±0.1

a: on the basis of GTR dry weight.

5.3.4 Optimization of weak alkaline conditions for GA extraction

GA yield could be described by Equation (1). The statistics of coefficients, including the normalized sensitivity coefficients for this equation can be seen in Appendix, Table A1.

$$\text{GA yield} = -31.2+2.34V/W+168\,[\text{Buffer}] +0.0147T\cdot V/W-0.0261(V/W)^2 \quad \text{Equation (1)}$$

Analysis of normalized coefficients suggests that GA yield was mainly influenced by V/W, as can be seen from the high normalized impact of V/W (see Appendix). This suggests that increasing V/W strongly increases GA yield, however the higher V/W the less pronounced is its effect, also indicated by the negative second order normalized coefficient $\beta_{(V/W)^2\text{-}V/W}$=-24.3. Furthermore, the effect of V/W on GA yield depends on temperature, as indicated by the normalized interaction coefficient $\beta_{T\text{-}V/W}$=21.3. The dominating effect of V/W could be related to the low pectin solubility in water that a higher volume to weight ratio will lead to higher GA yield. At higher V/W ratios, most pectin can be extracted and therefore the second order of V/W was incorporated to model the non-linearity in this part of the graph. Buffer concentration and temperature also influence the solubility of pectin (Stephen and Phillips, 2006; Thakur et al., 1997) and are incorporated in the equation. The dependence of V/W on temperature could furthermore be related to the GA determination procedure, as GA determinations were done after samples were cooled down to room temperature, and part of the pectin might have precipitated. Extraction time and pH have no influence on GA yield, indicating that the minimum extraction time in the studied range was sufficient for pectin extraction and that pH in the range studied (7-10) has no influence on pectin solubility. The results additionally suggest that a large set of conditions exists that ensure 100% GA yield. To further optimize and prevent protein loss during pectin extraction, influence of factors on ratio of GA yield to protein yield were tested.

Ratio of GA to protein yield could be described by Equation (2). The statistics of coefficients, including the normalized sensitivity coefficients for this equation can be seen in Appendix, Table A2.

GA yield/ protein yield = -0.42 + 5.9[Salts] + 0.029V/W + 0.00025T·V/W Equation (2)

Similar to the GA yield, it was found that the ratio of GA to protein yield was also dominated by V/W, and additionally influenced by buffer concentration and temperature. Influence of the three factors (V/W, T, and [Buffer]) on GA to protein yield was only due to the increase of GA yield, and it was therefore concluded that protein yield was not influenced by these factors in the study range. Previous research showed that high

protein yield from leafy biomass correlates to the applied amount of alkali and temperature (Zhang et al., 2014). However, the amount of applied alkali (pH 7-10) in our current experiments is hundred times less than the required amount for high yield protein, 3.2 mmol NaOH g^{-1} GTR (about pH 12.7), leading to very low protein extraction yield. The model estimates that the highest GA to protein yield of 3.3 occurs at 0.12 mol PBS, 60V/W, 80 °C with the minimum extraction time (0.5 h) and neutral pH (pH 7).

To verify the above prediction, GA extraction yield and protein yield obtained at optimal conditions were experimentally determined using 0.09 mol PBS (pH 8), 0.12 mol NaCl, and pure water as control. GA extraction yield obtained by PBS was 6.2% GTR, which is almost 4 times as high as GA yields obtained by water (1.6%GTR) or NaCl (1.6%GTR). The low GA yields in water or NaCl may due to low pH that occurs during extraction. Final pH of PBS pectin extraction was 6.7 while in water or NaCl the final pH was 4.9. At pH above 5, pectin can be degraded as a the result of a β-elimination cleavage of the glycosidic linkage (Stephen and Phillips, 2006). As hydrolysis is needed to facilitate extraction therefore the pH should be above 5 (Stephen and Phillips, 2006; Thakur et al., 1997). The ratio of GA yield to protein yield using 0.09 mol PBS or 0.12 mol PBS was 3.3 and 3.4 respectively, which was close to the predicted results, suggesting the model is able to predict the actual pectin extraction. Previous experiments (Zhang et al., 2016b) with preceding pectin extraction using Viscozyme® L, gave protein yields of 23% GTR and a reduction in alkali consumption with 40%, indicating the benefits of integrating weak alkaline pectin extraction and alkaline protein extraction.

5.3.5 Profit estimation of integrated processes

An optimized one-step alkaline protein extraction based on our previous work has the lowest chemicals and energy cost with highest protein yield among peer studies (Zhang et al., 2014). This process can be improved by an integrated biorefinery concept, in which the extraction of pectin and polyphenol is conducted prior to protein extraction (Zhang et al., 2015). This integration reduces alkali consumption by half and improves protein quality (Zhang et al., 2016b). In this study, pectin was selected as a second product and its extraction was optimized for the highest GA yield and highest GA to

protein yield ratio. Extractions using enzymes or weak alkali were the best candidates for integration with alkaline protein extraction. To further investigate the economic feasibility of the integrated processes, the profits of integrated Viscozyme® L aided or weak alkaline pectin extraction with alkaline protein extraction with the capacity of processing 5,000 ton GTR year^{-1} were estimated using the process and production data as shown in Fig. 5.4a and Fig. 5.4b, and economic data listed in the Appendix (Table A3 and A4). Production cost, capital cost, labor cost, revenue of integrated processes, and their possible profits are listed in Table 5.3.

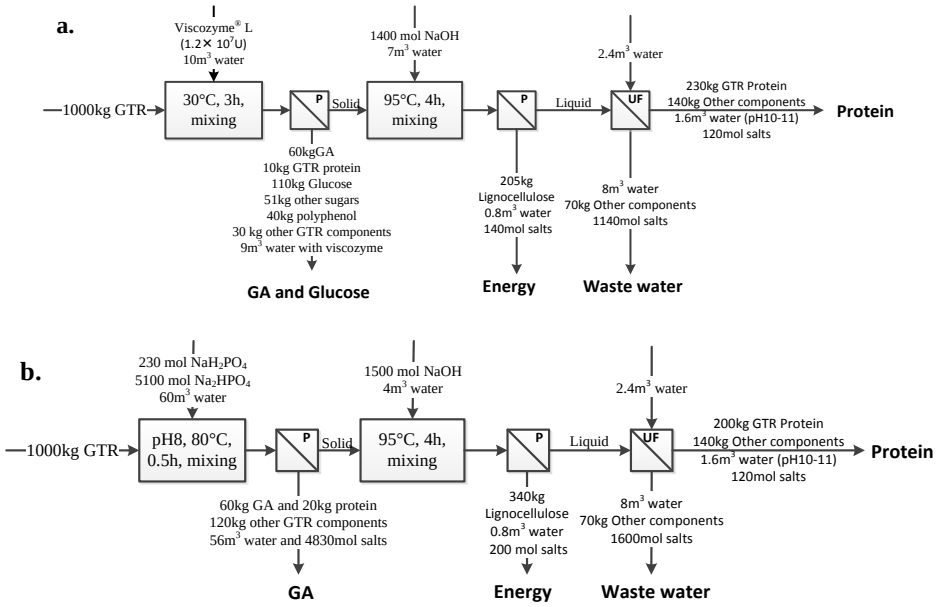

Fig. 5.4 Optimal conditions and products of Viscozyme aided integrated process (a) and weak alkaline integrated process (b). P: press; UF: ultra-filtration.

Viscozyme® L aided pectin extraction integrated with alkaline protein extraction is presented in Fig. 5.4a. Viscozyme® L with 12 million U activity and 10m^3 water is added to one ton GTR, and incubated at 30 °C for 3h. After pressing, 60kg GA, 110kg glucose, 50kg other sugars, and 40kg polyphenols can be obtained with 9m^3 water in the supernatant, while about 680kg GTR press cake is ready for protein extraction. After concentration, GA and sugar can be directly used for chemical conversion through fermentation (Grohmann et al., 1994), but further purification is needed to use

polyphenol as an anti-oxidant in food. Therefore, only the values of GA and glucose are included in the revenue. After adding 1500 mol NaOH and 6 m^3 water to the press cake, alkaline protein extraction will be carried out at 95 °C for 4 h. From this, 230 kg protein, and 205 kg lignocellulose can be obtained via pressing. The cost of this enzyme aided integrated process is 264 \$ ton^{-1} GTR (Table 5.3). Compared to the weak alkaline pectin extraction integrated process, the revenue of enzyme aided integrated process was increased with around 231 \$ ton^{-1} GTR to 286 \$ ton^{-1} due to the added value of glucose. The largest cost were the enzyme cost that accounted for 60 \$ ton^{-1} GTR. The current price of Viscozyme® L could be 15 \$ kg^{-1} protein, but it can be reduced further with the development of enzyme production. Here, an enzyme price of 5\$ kg^{-1} protein was used according to data on optimized cellulase production (Liu et al., 2015). The profit of the Viscozyme® L integrated process is estimated at 142 \$ ton^{-1}, which is higher than that of one-step protein extraction.

Table 5.3. Estimation of cost, revenue and profit of Viscozyme® L aided and weak alkaline pectin extraction integrated processes [a]. (Based on \$ ton^{-1} GTR)

Input (cost)	GA extraction		Protein extraction		**Output (revenue)**	Visco zyme	Weak alkaline
	Visco zyme	Weak alkaline	Visco zyme	Weak alkaline			
GTR	16	16			GA	48	48
Chemical/ Enzyme	60	1282	24	24	Protein	175	171
Water	5	33	5	3	Energy[b]	8	12
Heating	1	25	4	4	Glucose	55	
Waste water[c]	1	8	1	1			
Press-filtration	2	3	1	1			
Ultra-filtration			2	2			
Capital	7	7	7	7			
Labor	4	4	4	4			
Subtotal	96	1378	48	46	**Subtotal**	286	231
					Profit	142	-1193

a, Calculation based on data in Fig. 5.4a and Fig. 5.4b.
b, Energy derived from the combustion.(Robak et al., 2012)
c, Waste water management fee was according to Chinese government policy.

Weak alkaline pectin extraction integrated with alkaline protein extraction is presented in Fig. 5.4b. One thousand kilogram GTR is mixed with 300 mol NaH_2PO_4, 5100 mol Na_2HPO_4, and 60 m^3 water (pH 8, 95 °C, 0.5 h) for pectin extraction. After filtration, 60 kg pectin and 20 kg protein can be obtained with 56 m^3 water and 4800 mol sodium buffer in the supernatant, while about 800 kg GTR press cake is ready for protein extraction. Adding 1500 mol NaOH and 4 m^3 water to the press cake, alkaline protein

extraction will be carried out at 95 °C for 4 h. After filtration, 220 kg protein, and 340 kg lignocellulose can be obtained. In this process, the cost for pectin extraction accumulated to 1378 \$ ton^{-1} GTR as large amounts of water and heating energy were required (Table 5.3). The cost of capital and labor were higher than the enzyme aided integrated process. The large amount of water that is used, increases reactor size and amount of labor needed. The potential revenue of weak alkaline integrated process is only 231 \$ ton^{-1} GTR. Adding the high processing cost makes this process economically not feasible with a loss of 1193 \$ ton^{-1} GTR.

5.3.6 Improvement of weak alkaline pectin extraction integrated processes

The cost-efficiency of the weak alkaline integrated process can be improved using a recycle system as presented in Fig. 5.5. In this system, extraction will start from alkaline protein extraction producing a protein supernatant and a lignocellulosic cake after pressing. The lignocellulose fraction can be sundried and used as fuel for heating energy. The supernatant can be further treated by ultra-filtration obtaining concentrated protein and a residual weak alkaline solution at pH 10-11. The weak alkaline solution can be diluted by fresh water until pH at 9-10 is reached and then used for GA extraction. GA products can be collected using ultrafiltration, and the recycled water can be used for the dilution of the weak alkaline solution obtained from previous alkaline protein extraction. According to Equation (2), using pH 9-10 with 60 V/W at 80 °C for 0.5 h will lead to almost 100% GA extraction with GA to protein ratio of 3. Cost, revenue, and profit of this recycle system are presented in Table 5.4.

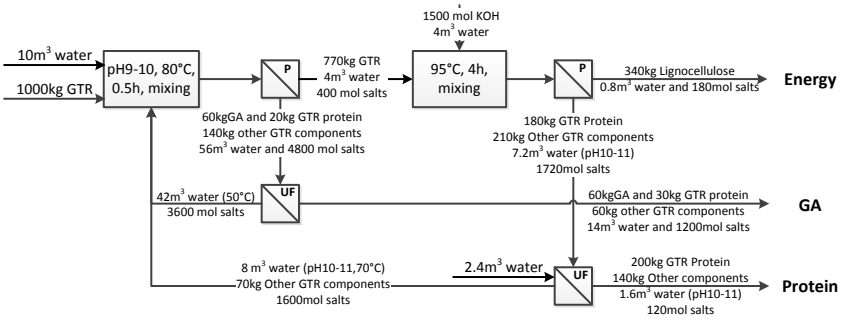

Fig. 5.5 Conditions and possible products in weak alkaline integrated process using a recycle system. P: press; UF: ultra-filtration.

Using this recycle system, the cost-efficiency of the weak alkaline pectin extraction integrated process is improved and a profit of 118$ ton^{-1} GTR can be made. The increase in profit is due to the reduction in chemicals, water, and energy use by using ultrafiltration. At weak alkaline conditions, GA is extracted as a pectin polymer. Further hydrolysis may be needed for subsequent chemical conversion. Alternatively, the weak alkaline extracted pectin can be applied as food pectin or food fiber. The value of pectin (Chang, 2008) can be 10 times higher, which makes this integration more valuable.

Table 5.4. Comparison of one-step protein extraction with weak alkaline pectin extraction integrated recycle system[a] in cost, revenue and profit. (Based on $ ton^{-1} GTR)

Input (cost)	One-step protein extraction	Recycle system		**Output (revenue)**	One-step protein extraction	Recycle system
		GA extraction	Protein extraction			
GTR	16	16		GA		48
Chemical	41		21	Protein	171	163
Water	14	5	2	Energy[b]	12	12
Heating	13	14	3			
Waste water[c]	2	1	1			
Press Filtration	1	3	1			
Ultra-filtration	2	14	2			
Capital	7	7	7			
Labor	4	4	4			
Subtotal	100	64	41	**Subtotal**	183	223
				Profit	83	118

a, Calculation based on Fig. 5.5, which is a stable process after a recycle of second round.
b, Energy derived from the combustion.(Robak et al., 2012)
c, Waste water management fee was calculated according to Chinese policy.

To sum up, the profit of the integrated process using both Viscozyme® L and weak alkaline can be higher than that of the one-step protein extraction. However, additions improvements are needed to fully exploit the potentials that lie in both integrated protocols. The gain in this integrated biorefinery is not only in obtaining multiple products, but also in reducing production cost by introducing new technologies to reduce water, chemicals, energy, labor, and capital input. A simple combination of processes without proper integration will not lead to a profit increase compared to one-step extraction.

5.4 Conclusion

Viscozyme® L aided and weak alkaline pectin extraction can be used to obtain high GA yield while keeping protein yield low. Viscozyme® L aided in pectin extraction by

hydrolyzing cellulose, while weak alkaline degraded and solubilized pectin. Compared to the profit of one step protein extraction (83$ ton^{-1} GTR), the profit of both integrated processes using either Viscozyme® L or weak alkaline can be higher. The Viscozyme® L integrated process is suitable for GA production with application in chemical industry, and may have a profit of 142$ ton^{-1} GTR when enzyme cost are sufficiently low. The profit of the integrated process using weak alkali is estimated at 118$ ton^{-1} GTR, which can be further increased when pectin is applied in food. Both integrated processes may be applied to other leafy biomass, such as Jatropha leaf and grass (Zhang et al., 2014), which significantly expands resources of pectin (or GA) and protein.

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CHAPTER 6: BIOREFINERY SCENARIOS TO REUSE OR RECYCLE SALTS FOR ALKALINE PROTEIN EXTRACTION FROM LEAFY BIOMASS

Submitted as: Zhang, C., Wisse, J., Sanders, J.P.M., Bruins, M.E., 2016. Biorefinery scenarios to reuse or recycle salts for alkaline protein extraction from leafy biomass.

Abstract:

Leaf protein can be extracted cost-efficiently using 0.1M NaOH, but this process is less sustainable due to the generation of large amounts of sodium salts. Two scenarios to reuse or recycle salts for leaf protein extraction using either KOH or $Ca(OH)_2$ were proposed. Protein extraction yields of KOH and $Ca(OH)_2$ were tested on green tea residue (GTR), and the economics of these two scenarios were analyzed. KOH extracted over 90% protein and use of this chemical can be readily integrated with pretreatments that were already designed for NaOH protein extraction. The amount of potassium salts generated by KOH protein extraction from GTR is similar to the demand of K-fertilizer on the tea leaf production field. Profits using KOH are comparable to those with NaOH. $Ca(OH)_2$ extracted less than 50% protein from GTR. This low protein yield may be due to interaction between lignin and the calcium ions, which was further verified by using microalgae, a biomass which contains no lignin. Profits using $Ca(OH)_2$ highly depend on yields of protein products. Only with a protein yield higher than 70%, using $Ca(OH)_2$ can be more profitable than with NaOH. Application of KOH protein extraction and $Ca(OH)_2$ protein extraction on different types of biomass enables commercialization of yet untapped protein resources, which can be beneficial for both environment and economics.

6.1 Introduction

Since the 1960s, leaf proteins have been considered as a new alternative source of protein (Akeson and Stahmann, 1965; Gerloff et al., 1965). Some leaf proteins have been used in food (Ghaly and Alkoaik, 2010), animal feed (Kondo et al., 2004), or when hydrolyzed to amino acids for N-containing bulk chemicals (Sanders et al., 2007). However, application of leaf protein was so far limited by the low cost-efficiency of leaf protein production (Bals and Dale, 2011). Recently, the cost-efficiency of leaf protein extraction was improved by redefining alkaline extraction conditions (Zhang et al., 2014) and by an integration with pre-treatments using Viscozyme® L and ethanol (Zhang et al., 2016a; Zhang et al., 2016b). With these improvements, leaf biorefinery becomes economically feasible. However, the process, is still not very sustainable due to the generation of a large quantity of sodium salts in the alkaline protein extraction and subsequent acidic precipitation. To improve the sustainability of alkaline protein extraction, a suitable alternative should be found. Other alkali sources, such as KOH or $Ca(OH)_2$, can be used instead of NaOH. Salts that are generated from KOH or $Ca(OH)_2$ can be used as fertilizer or can be recycled.

When using KOH for protein extraction, the residual water containing potassium salts can be used as fertilizer turning waste into a product (Hasler et al., 2015). The extra cost associated with the higher price of KOH compared to that of NaOH can be compensated by the value of potassium fertilizer generated at the end of the protein extraction process and by the reduction of waste water management fees. KOH has similar chemical characteristics as NaOH, and therefore a high protein yield is to be expected. The chemical similarity will additionally allow for easy integration with pre-treatments, such as Viscozyme® L aided pectin extraction or weak-alkaline pectin extraction, which were already integrated with protein extraction using NaOH to improve cost-efficiency (Zhang et al., 2016a; Zhang et al., 2016b). Ideally, all the watery potassium salts residues needs to be directly used in local fields as fertilizer. Otherwise, additional processing is needed to reduce the water content of the side stream to lower transportation cost.

Alternatively, leaf protein can be extracted by $Ca(OH)_2$. The residual water and the

calcium salts can be recycled on-site. Pumping CO_2 into the liquid remainder after protein extraction can be used to precipitate the calcium salts as $CaCO_3$. After a subsequent separation, solid residuals, including $CaCO_3$ and waste GTR, can be dried in the sun and subsequently combusted to CaO and CO_2. Heating energy and CaO generated from combustion can be re-used for protein extraction, and produced CO_2 can be re-used for either calcium precipitation again or in green houses. This calcium ion recycle system will improve efficient chemical use. No waste salts are generated and less acid is needed for further protein purification (Baraniak and Baraniak, 1987). In addition, $Ca(OH)_2$ protein extraction can benefit from the lower price of $Ca(OH)_2$ compared to KOH or NaOH. However, using $Ca(OH)_2$ for leaf protein extraction may result in relatively low protein yield (Davison et al., 2005; Holtzapple et al., 2005). This low yield might be due to the low solubility of $Ca(OH)_2$ in water(Bates et al., 1956), which results in a more mild alkaline solution with a relatively low pH compared to NaOH and KOH. Besides, as calcium atoms contain two valence electrons, calcium ions can function as bridges between different chelating molecules (Fontana et al., 1977; Ropers and Leroy, 2008). This chelating effect of calcium ions is widely applied on the coagulation of polymers (Chen et al., 2014; Li et al., 2012). In protein extraction, the bridge function of calcium ions may coagulate leaf components, including polyphenol (Boukhoubza et al., 2009), pectin (Ropers and Leroy, 2008), lignin (Zahrim et al., 2015), and even protein (Marfo and Oke, 1989; Zahrim et al., 2015), and thereby reduce protein extraction yield.

To investigate the possibility of using KOH or $Ca(OH)_2$ for leaf protein extraction, green tea residue (GTR) was used as model material. Protein extraction yields obtained by KOH and $Ca(OH)_2$ were determined using NaOH as a control. To further investigate the influence of calcium ions on protein extraction, GTR extracts, including, polyphenol extracts, pectin extracts, and protein extracts, were analyzed with and without the presence of calcium ions or magnesium ions. To improve $Ca(OH)_2$ protein extraction yield, influence of a combined Viscozyme® L with 50% ethanol pre-treatment (Zhang et al., 2016a; Zhang et al., 2016b) was also analyzed. $Ca(OH)_2$ protein extraction was then further tested using microalgae to confirm some conclusions of the applied techniques. Based on the results, the sustainability and economics using KOH or $Ca(OH)_2$ were

estimated and discussed.

6.2 Materials and methods

6.2.1 Materials

GTR is obtained after hot water extraction of tea leaves product. Carbohydrates and N-containing components account for 31% and 27% of dried GTR. Other components are polyphenol (8%), water (7%), and ash (6%) (Zhang et al., 2015). The residual undetermined part majorly consists of lipid (wax, organic acids) and lignin (Harold N, 1992). Pre-treated GTR was made based on previous work (Zhang et al., 2016b). GTR was treated using Viscozyme® L and 50% ethanol and then freeze dried before further treatments with alkali.

Microalgae (*Nannochloropsis sp.*, CCAP 211/78) were obtained from AlgaePARC, FBR, Wageningen UR. They were grown on natural seawater in a turbidostat set-up, and collected by self-cleaning disc separators (SSD 6-06-007, GEA Westfalia Separator, Germany). The dry matter of the microalgae sample was 11% with a protein content of 37% in dry weight.

6.2.2 Protein extraction yield

6.2.2.1 KOH protein extraction

200 mg (pre-treated) GTR was mixed with 8 mL water and 0.4 mmol or 0.8 mmol KOH, using NaOH as control. The mixtures, were incubated in a thermomixer (VWR International B.V., USA) at 95 °C for 2 h with shaking speed of 1000 rpm min^{-1}. Solid-liquid separation was performed by centrifugation (Sorvall centrifuge, Thermo Fisher Scientific, the USA) at 7000 g and 25 °C for 10 min. Supernatants were collected and stored at 4 °C for further analysis.

6.2.2.2 $Ca(OH)_2$ protein extraction

0.5 g sample was mixed with 20 mL H_2O and 1 or 2 mmol $Ca(OH)_2$ in 50 mL tubes. The mixtures were incubated in a thermomixer at 95 °C for 2 h or 24 h with shaking speed of 1000 rpm min^{-1}. To reach temperatures higher than 100 °C, mixtures were incubated in

75 mL Parr pressure reactors (Parr multiple reactor system series 5000). The reactions were carried out at 150 °C for 2 h (Spekreijse et al., 2012). After pressure release, reactors were allowed to cool down to room temperature. Supernatants were collected by centrifugation, and then stored at 4 °C for further analysis.

6.2.3 Influence of divalent ions on GTR extracts

6.2.3.1 Polyphenol extracts

Most polyphenol can be extracted from GTR using 50% ethanol (Castle et al., 2011; Li et al., 2005; Zhang et al., 2016b). GTR (200 mg) was soaked in 4 mL 50% ethanol or absolute ethanol in 10 mL tubes, followed by incubation in a thermomixer (60 °C, 1000 rpm) for 2 h. After centrifugation, polyphenol extracts with 55% purity on dry weight were collected and stored at 4 °C for further experiments.

6.2.3.2 Pectin extracts

GTR material (2 g) was mixed with 120 mL sodium phosphate buffer (0.02 M, pH 8.2) and incubated in a thermomixer (1000 rpm) at 80 °C for 3 h. After centrifugation, the supernatant was collected and mixed with pure ethanol until the ethanol concentration was around 40% v/v. After keeping the mixture at 4 °C for 30 min, pectin was precipitated and collected by centrifugation. After adding 50 mL demi-water to solubilize the pectin precipitate, the pectin solution was freeze-dried to obtain a powder and then stored at room temperature. The pectin content based on dry weight was 75%. Dried pectin was re-dissolved in demi-water with a final concentration of 5 g L^{-1} right before further experiments.

6.2.3.3 Protein extracts

GTR protein obtained by extraction from GTR with 0.1 M NaOH was purified by acid precipitation (Zhang et al., 2014). Purified protein extract was freeze-dried and then at stored at room temperature. The protein content based on dry weight was about 55%. Dried protein was re-dissolved in demi-water to a concentration of 10 g L^{-1} right before further experiments.

6.2.3.4 Incubation of GTR extracts with different chemicals

4.5 mL of the above three GTR extracts were mixed with 0.5 mL water and 0.25 mmol of $MgCl_2$, $CaCl_2$, $Mg(OH)_2$, $Ca(OH)_2$, or NaOH, and then stirred thoroughly for 3 h at 70 °C. After incubation, the mixtures were stored at 4 °C for 1 h. Images of the mixtures were taken by camera. The supernatants were collected by centrifugation and were ready for further analysis.

6.2.4 Analysis

6.2.4.1 Protein

The protein concentration (g L^{-1}) was determined using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, and The Netherlands). A conversion factor of 5.4 was used to calculate the protein concentration of GTR (Zhang et al., 2016b), while a conversion factor of 4.9 was used to calculate the protein concentration of microalgae (Lourenço et al., 1998). The protein extraction yield was calculated as extracted protein / total protein (TP) * 100%.

6.2.4.2 Polyphenol

Polyphenol content in the tea extracts was determined spectrophotometrically (DU®700, Beckman, USA) (Li et al., 2005; Turkmen et al., 2006). The polyphenol concentration was calculated following the assumption that a concentration of polyphenol of 3.914 g L^{-1} leads to an adsorption of 1 at 540 nm (Li et al., 2005; Turkmen et al., 2006).

6.2.4.3 Galacturonic acid

Pectin content is represented by the amount of galacturonic acid (GA) and was determined spectrophotometrically (DU®700, Beckman, USA) (Taylor, 1993) using galacturonic acid (Fluka AG, Buchs, Switzerland) as a reference in a concentration range of 12.5 to 50 mg L^{-1}.

6.2.4.4 Full wavelength scanning

Supernatants of GTR extracts incubated with different chemicals were scanned spectrophotometrically (DU®700, Beckman, USA) at wavelengths ranging from 200 nm to 850 nm. Samples were diluted when the highest absorbance was higher than 2.5.

6.2.5 Statistics

Each sample (except from the experiments using uniform design) had duplicates in both extraction and determination, and the standard deviation of the four measurements was calculated by Excel.

6.3 Results and discussion

6.3.1 Potassium hydroxide scenario

6.3.1.1 Protein extraction yield

Protein extraction yields obtained using 0.1 M NaOH or KOH on GTR were tested. KOH and NaOH showed similar protein extraction efficiencies. Almost all protein was extracted (>90%). Pre-treatment for polyphenols removal reduced NaOH consumption by 50% in the subsequent protein extraction (Zhang et al., 2016b). This pre-treatment is also applicable for KOH protein extraction. Based on previous studies (Zhang et al., 2016a; Zhang et al., 2016b), the process that combines pectin and protein extraction using alkali, as presented in Fig. 6.1, has been proven to be economically feasible for NaOH and is expected to minimize the generation of potassium salts (Zhang et al., 2016a).

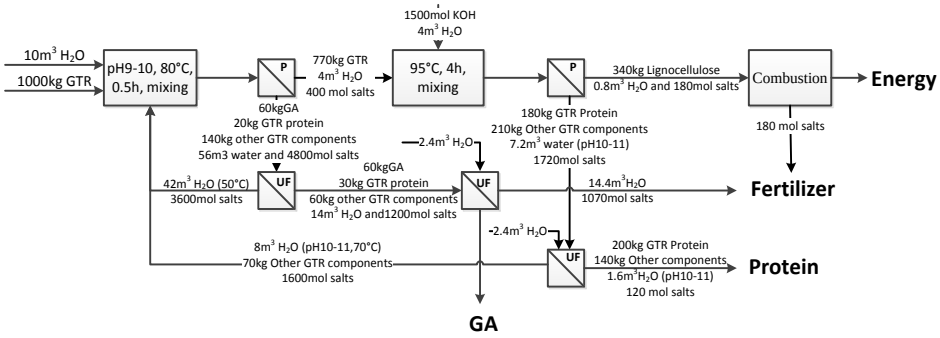

Fig. 6.1 Integrated process of weak alkaline pectin extraction with KOH protein extraction using potassium salts as fertilizer. P: Press; UF: Ultra-filtration.

In the proposed protein extraction system based on KOH (Fig. 6.1), extraction will start with protein extraction producing a protein supernatant and lignocellulose after press filtration. The lignocellulose fraction can be sundried and combusted for heating energy.

The supernatant can be further treated by ultra-filtration obtaining concentrated protein and a residual weak alkaline solution at pH 10-11. The weak alkaline solution can be diluted by fresh water until pH at 9-10 and subsequently used for pectin extraction. Pectin products can be collected using ultrafiltration, and the recycled water can be re-used for the dilution of the weak alkaline solution obtained from previous alkaline protein extraction. The concentrated pectin fraction can be further concentrated by ultrafiltration. This leads to about 1150 mol potassium salts in the residual water. Combined with the potassium salts from combustion, the total amount of potassium salts is 1250 mol.

6.3.1.2 Demands of potassium fertilizer in tea farm

As mentioned, all the potassium salts waste should be directly used on local fields as fertilizer. It implies that the amount of potassium salts generated from KOH protein extraction should be lower than the required amount of K fertilizer for tea fields. Approximately 480 mol (19 kg) K in aboveground parts was required to produce 1 ton dried tea leaf product without considering any losses by leaching and fixation in the soils. As the K fertilizer–use efficiency is 35-50%, the amount of required K fertilizer for tea field could be 960-1370 mol per ton dried tea product (Ruan et al., 2013). After hot water extraction of 1 ton of tea product for tea drinks, most potassium and approximately 40% of dry matter, mainly containing catechins (Harbowy; and Balentine, 1997; Harold N, 1992), are extracted as tea while 0.6 ton GTR remained with little potassium. According to Fig. 6.1, approximately 750 mol potassium salts can be recovered from processing 0.6 ton GTR. The amount of potassium recovered from protein production is lower than the demand of potassium fertilizer in the tea farm for production of the same amount of tea leaves. As waste water is best used directly onto the field without long-distance transportation, small scale production would be suitable for this application (Bruins and Sanders, 2012). Based on the process presented in Fig. 6.1, production costs, revenues, and profits of using either KOH or NaOH were estimated and listed in Table 6.1. The specifics of the economic estimation can be seen in Appendix (Table A3 and A4).

As illustrated in Table 6.1, the profit from processing the GTR only slightly increased from 103 \$ ton^{-1} GTR to 105 \$ ton^{-1} when using KOH instead of NaOH. Using KOH, costs of chemicals increased from 21 to 84 \$ ton^{-1} due to the higher price of KOH, while ultrafiltration costs increased due to the larger processing volume for the isolation of potassium salts from pectin extract. The economic benefit in the KOH scenario is due to the decreased cost of waste water management fees and the increased revenue on fertilizer. Compared to using NaOH, less than 10% of waste water is generated in KOH scenario, and thus the costs for waste water management reduce to only 1 \$ ton^{-1} GTR in both pectin extraction and protein extraction steps. Using potassium salts as fertilizer, an extra revenue of 55 \$ ton^{-1} GTR is expected (Clarke, 2015). Overall, a similar profit is expected for the KOH scenario compared to that using NaOH. In addition, the KOH scenario has lower environmental impact as it reduces the demand of commercial muriate of potassium (Clarke, 2015) and therefore improves the life cycle of potassium fertilizer by reducing CO_2 emissions during conventional potassium production from parent rock materials (Hasler et al., 2015).

Table 6.1 Cost, revenue, and profit of integrated weak alkaline pectin extraction with alkaline protein extraction using NaOH or KOH [a]. (Based on \$ ton^{-1} feedstock)

Input (cost)	NaOH	KOH	**Output (Revenue)**	NaOH	KOH
GTR	16	16	GA	48	48
Weak alkaline pectin extraction	55	48	Protein	163	163
Chemical	21	84	Energy[c]	12	12
Water	3	3	Fertilizer		55
Heating	3	3			
Waste water[b]	8	1			
Press Filtration	1	1			
Ultra-filtration	2	6			
Capital	7	7			
Labor	4	4			
Subtotal	120	173	**Subtotal**	223	278
			Profit	103	105

a, Calculation based on data in Fig. 6.1.
b, Waste water management fee was calculated based on American standard (Waterworld, 2014)..
c, Energy derived from the combustion.(Robak et al., 2012)

6.3.2 Calcium hydroxide scenario of recycling calcium ions

6.3.2.1 Protein extraction yield

To test $Ca(OH)_2$ protein extraction efficiency, protein yields were determined by

applying different amounts of $Ca(OH)_2$ or $Ca(OH)_2$ with NaOH in 20 mL water on 0.5 g GTR over time using NaOH as control. The results of these experiments are presented in Fig. 6.2. Compared to protein yields that were obtained by NaOH, protein yields obtained by $Ca(OH)_2$ were generally lower. The highest protein yield was 47%, when applying 2mmol $Ca(OH)_2$ on 0.5 g GTR at 95 °C for 24 h or at 150 °C for 2 h. This yield was only half of the protein yield when applying 0.1 M NaOH under the same conditions (Zhang et al., 2014).

Low protein yield from $Ca(OH)_2$ protein extraction may be due to chelating effect of calcium ions with GTR components (Eliaz et al., 2006; Harbowy; and Balentine, 1997) and/or insufficient alkali as a result of low $Ca(OH)_2$ solubility (Bates et al., 1956). However, the latter can be excluded from the causes to low protein yield. As can be seen from Fig. 6.2, protein yield obtained from 1mmol NaOH (45%) was higher than that obtained from a mixture of 0.5 mmol $Ca(OH)_2$ and 1mmol NaOH (30%). The addition of $Ca(OH)_2$ did not aid the NaOH protein extraction, but even had a negative effect, which demonstrates that the low protein yield using $Ca(OH)_2$ is not resulted from the insufficient amount of solubilized $Ca(OH)_2$.

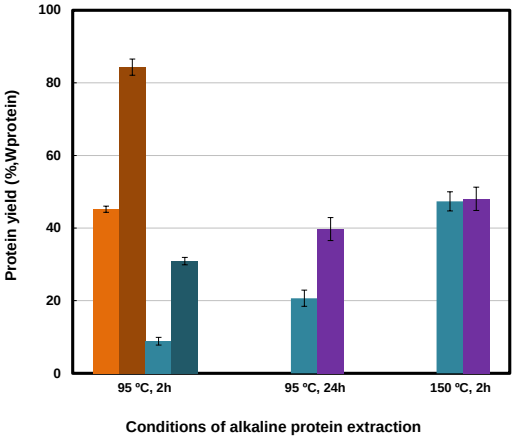

Fig. 6.2 Protein yield (%, $W_{protein}$) obtained by adding alkali to 0.5 g GTR with 20 mL H_2O under different conditions. ■: 1 mmol NaOH; ■:2 mmol NaOH; ■: 1 mmol $Ca(OH)_2$; ■: 2 mmol $Ca(OH)_2$; ■: 0.5 mmol $Ca(OH)_2$ + 1 mmol NaOH.

6.3.2.2 Chelating effect of GTR polyphenol, pectin, and protein with divalent ions

The chelating effect of GTR components with calcium ions could be the reason for a low protein yield obtained by $Ca(OH)_2$ protein extraction. To determine which GTR component hinders $Ca(OH)_2$ protein extraction, chelating effects of divalent ions with polyphenol, pectin, or protein extracts from GTR were tested. The images and

absorbance (from 340 nm to 700 nm) of these three extracts incubated with different ions are presented in Fig. 6.3a, Fig. 6.3b, and Fig. 6.3c.

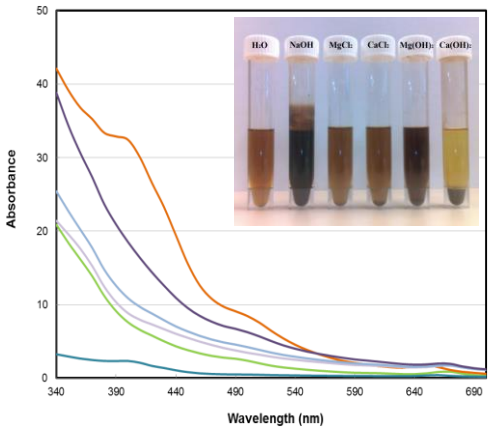

Fig. 6.3a Absorbance variation (within wavelength of 340-700 nm) of 4.5 mL polyphenol extracts treated by 0.5 mL 0.1 M different chemicals at 70 °C for 3 h. ——: H_2O; ——: NaOH; ——: $MgCl_2$; ——: $CaCl_2$; ——: $Mg(OH)_2$; ——: $Ca(OH)_2$.

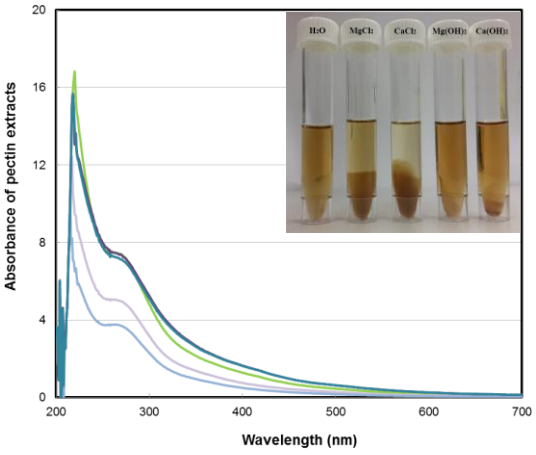

Fig. 6.3b Absorbance variation (within wavelength of 200-700 nm) of 4.5 mL polyphenol extracts treated by 0.5 mL 0.1 M different chemicals at 70 °C for 3 h. ——: H_2O; ——: $MgCl_2$; ——: $CaCl_2$; ——: $Mg(OH)_2$; ——: $Ca(OH)_2$.

Polyphenols (Fig. 6.3a) only coagulated when treated by $Ca(OH)_2$. Approximately 95% of total polyphenols were precipitated. The color of the polyphenol extract was brownish when treated with $Mg(OH)_2$ or NaOH. This browning process may be due to the oxidization of polyphenols under alkaline conditions, and thereby contributing to the absorbance peak at 410 nm. The oxidization speed of polyphenols is positively related to pH, and therefore resulting in a stronger browning with NaOH than with $Mg(OH)_2$ (Couzinet-Mossion et al., 2010; Vieira and Fatibello-Filho, 1999). Owing to the chelating effect of calcium ions with components that potentially cause browning, such as pigments and polyphenols, $Ca(OH)_2$ extracted protein is either white or yellowish showing a better visual appearance than that of NaOH extracted protein.

In the presence of divalent ions, GTR pectins were more prone to coagulation under neutral conditions (Fig. 6.3b). Approximately 60% pectin or 85% pectin was detected in

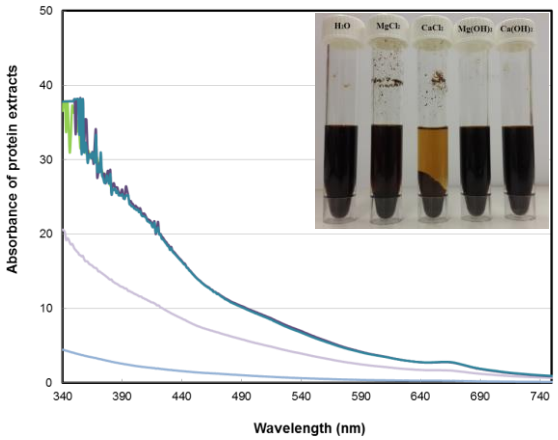

Fig. 6.3c Absorbance variation (within wavelength of 340-700 nm) of 4.5 mL polyphenol extracts treated by 0.5 mL 0.1 M different chemicals at 70 °C for 3 h. ——: H_2O; ——: $MgCl_2$; ——: $CaCl_2$; ——: $Mg(OH)_2$; ——: $Ca(OH)_2$.

the precipitates when pectin extracts were treated by $MgCl_2$ or $CaCl_2$ respectively. Pectin precipitates were jelly, which was probably due to the rigid networks of homogalacturonan pectin solution that are created in the presence of divalent ions under neutral conditions (Fissore et al., 2012; Willats et al., 2006). In comparison, no pectin was precipitated under alkaline conditions with $Ca(OH)_2$ or $Mg(OH)_2$. Absorbance of pectin extracts treated by $Ca(OH)_2$ and $Mg(OH)_2$ coincided with the control suggesting no chelation occurred.

GTR Protein behaved similarly as GTR pectin and was also prone to coagulation under neutral conditions. Similar absorbance spectrum lines for different experimental conditions show the same pattern (Fig. 6.3c). The chelating effects of calcium ions with GTR components are greater than those of magnesium ions indicating a better stability of the formed calcium chelate.

Generally, chelating effects of polyphenol, pectin, or protein with calcium ions did not hinder $Ca(OH)_2$ protein extraction. GTR pectin and protein obtained under alkaline conditions can be fully solubilized in $Ca(OH)_2$ solution (Fig. 6.3b and Fig. 6.3c). Polyphenol may influence $Ca(OH)_2$ protein extraction because of complexation of polyphenol with protein (Jervis and Pierpoint, 1989), but this possibility can be excluded as the possible polyphenol-protein complex extracted by NaOH can be fully solubilized in $Ca(OH)_2$ solution (Dashek and Harrison, 2006b).

6.3.2.3 Influence of (hemi-) cellulose and lignin on $Ca(OH)_2$ protein extraction

Due to the difficulties in the extraction of GTR lignocellulose, its chelating effect with

calcium ions cannot be tested directly as was done for other components. Therefore, indirect methods were applied. A Viscozyme® L and ethanol combined pre-treatment, which removes almost all cellulose, polyphenol, and pectin, as well as 50% hemi-cellulose(Zhang et al., 2016a; Zhang et al., 2016b), was used to analyze the influence of cellulose on $Ca(OH)_2$ protein extraction. Microalgae (*Nannochloropsis sp.*) with no lignin content, was introduced as a control feedstock to analyze the influence of lignin on $Ca(OH)_2$ protein extraction. All results are presented in Fig. 6.4.

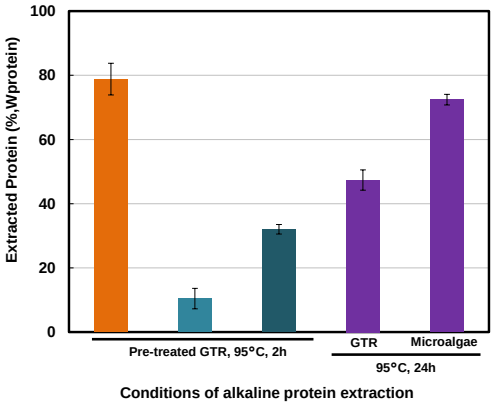

Fig. 6.4 Protein yield (%, $W_{protein}$) obtained by adding different amount and types of alkali to 0.5 g (pre-treated) GTR or microalgae (on dry weight basis) with 20 mL H_2O under different conditions. ■: 1mmol NaOH; ■: 1mmol $Ca(OH)_2$; ■: 2 mmol $Ca(OH)_2$; ■:0.5mmol $Ca(OH)_2$ + 1 mmol NaOH.

Pre-treatment with Viscozyme® L and ethanol increase the protein yield of subsequent 1 mmol NaOH protein extraction from 45% (Fig. 6.2) to about 80% (Fig. 6.4). However, this pre-treatment did not aid the efficiency of $Ca(OH)_2$ protein extraction. As presented in Fig. 6.4, only respectively 11% or 34% protein from pre-treated GTR was extracted with 1 mmol $Ca(OH)_2$ or a mixture of 0.5 mmol $Ca(OH)_2$ with 1 mmol NaOH. Compared to the protein yields obtained under the same conditions from original GTR (8% and 32%, see Fig. 6.2), no significant improvements were made by the pre-treatment. The similarity of protein yield obtained from original GTR and pre-treated GTR indicates that cellulose is not the reason of low protein yield in $Ca(OH)_2$ protein extraction. It is also likely that hemi-cellulose did not cause the low protein yield, as removal of half the hemi-cellulose did not increase protein yield.

When microalgae were used as feedstock, more than 70% protein could be extracted by $Ca(OH)_2$, which is lower compared to using NaOH (90%), but nevertheless better than many other methods for algal protein extraction. The still relatively high protein yield obtained by $Ca(OH)_2$ may be due to the absence of lignin, which indicates that lignin might be a hindrance to $Ca(OH)_2$ extraction for most other lignin-containing materials.

This was further confirmed for other non-lignin containing biomass. When using $Ca(OH)_2$, high protein yields can be obtained from plant seeds with no lignin content, such as dehulled soy bean (Cogan et al., 1967; Smith and Circle, 1938) .

The mechanism behind lignin hindrance on $Ca(OH)_2$ aided protein extraction is not elucidated yet. The interference of lignin with protein extraction may result from the formation of lignin-protein complexes during alkaline protein extraction (Whitmore, 1982). However, in our research this possibility was excluded as the potential lignin-protein complex could be solubilized in $Ca(OH)_2$ solution (see 6.3.2.2). Another explanation for low protein yield can be densification of the secondary plant cell wall, where most of the lignin is located. In the presence of calcium ions, lignin can be coagulated and condensed (Zahrim et al., 2015), thereby increasing the rigidity of secondary plant cell wall, reducing its permeability (Dashek and Harrison, 2006b) for protein.

6.3.2.4 Economic analysis for calcium scenario

Based upon obtained experimental results a protein extraction process with $Ca(OH)_2$ was proposed and is presented in Fig. 6.5. Weak alkaline pectin extraction by calcium hydroxide is not feasible as pectin can be precipitated by calcium ions at a pH close to neutral conditions (Zhang et al., 2016a). Instead, Viscozyme® L aided pectin extraction was chosen for integration with $Ca(OH)_2$ alkaline protein extraction. Viscozyme® L with 12 million U activity and 10 m^3 water is added to one ton GTR, and incubated at 30 °C for 3 h. After filtration, 60 kg GA, 110 kg glucose, 50 kg other sugars, and 40 kg polyphenols can be obtained with 9 m^3 water in the supernatant, while about 680 kg GTR press cake is ready for protein extraction. GA and sugar can be directly used for production of alcohols or organic acids by fermentation (Grohmann et al., 1994), but further purification is needed when polyphenol will be used as anti-oxidant in food. Thus, only the values of GA and glucose are included in the revenue. A system as described in the introduction was applied to recycle calcium hydroxide. Production cost, revenue, and profit of the Viscozyme® L aided pectin extraction integrated process with alkaline protein extraction using $Ca(OH)_2$ were estimated and listed in Table 6.2. Further

specifications can be found in the Appendix (Table A3 and A4).

As most calcium ions and water can be recycled and reused, this process has relatively low costs and can be environmentally friendly (Fig.6.5). The price of $Ca(OH)_2$ is only 70 \$ ton^{-1}, and with a recycling recovery of about 85%, the estimated chemical cost for $Ca(OH)_2$ protein extraction is only 2 \$ ton^{-1} GTR (Table 6.2). After CO_2 treatment, the supernatant of the protein extract can be further treated by ultrafiltration to obtain protein product and about 8 m^3 water. Approximately 7 m^3 water can be reused in the protein extraction step while 1 m^3 water can be reused in ultrafiltration. Because of the recycle, costs of water and waste water management fees are both reduced to 1 \$ ton^{-1}. For the reuse of $Ca(OH)_2$, 178 kJ of energy is required to convert 1 mol $CaCO_3$ into CaO and CO_2 (Lin et al., 2011). A biomass boiler is needed and therefore expenses for heating, capital, and labor increased. Generally, the total cost for processing one ton GTR using calcium hydroxide is 19 \$ ton^{-1} less than the scenario using NaOH.

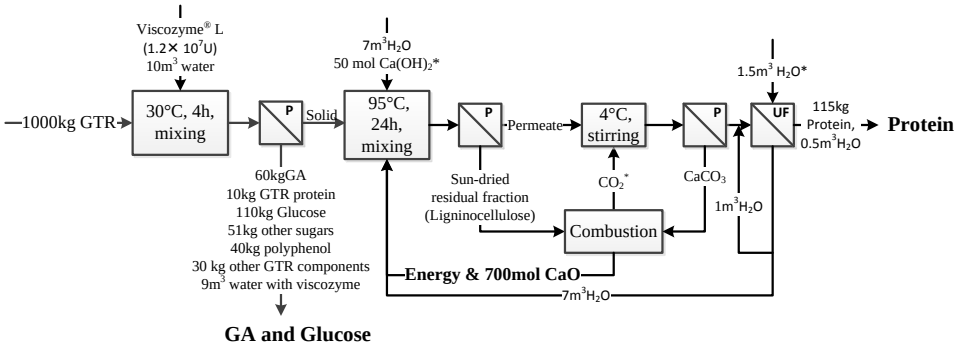

Fig. 6.5 Process of $Ca(OH)_2$ protein extraction using a water and Ca recycle system with a Viscozyme® L pre-treatment. P: press; UF: ultrafiltration; *: 750 mol $Ca(OH)_2$ and 2.5 m^3 water are needed in the first round.

As the pretreatment steps suggested for the $Ca(OH)_2$ process and for the NaOH process are the same, the profit of the total $Ca(OH)_2$ protein extraction process highly depends on the revenue of extracted protein. The revenue on a protein extraction process is mainly determined by the total yield of protein because protein prices for feed are almost linearly related to the protein content, regardless of its original sources (Teekens et al., 2016). Processing 1 ton GTR with NaOH, 400 kg protein product with protein content of 55% was produced, whereas 190 kg protein product with protein content of 60% was

produced using $Ca(OH)_2$. The lower protein yield when applying $Ca(OH)_2$ protein extraction results in a lower total profit (80 \$ ton^{-1} GTR) compared to the process with NaOH as reagent (128 \$ ton^{-1} GTR). Using microalgae instead of GTR, 500kg protein product with protein content of 60% was produced when NaOH was used, whereas 400 kg protein product with protein content of 65% was produced using $Ca(OH)_2$. For microalgae, the protein revenue in the $Ca(OH)_2$ scenario is only about 11 \$ lower compared to NaOH protein extraction. However, due to the low production cost, the profit of processing microalgae using $Ca(OH)_2$ can be slightly higher than that using NaOH. These results suggest that only when protein yields are higher than approximately 70%, $Ca(OH)_2$ based protein extraction can have a higher profit compared to the process using NaOH.

Table 6.2 Cost, revenue, and profit of $Ca(OH)_2$ protein extraction using a recycle system with a Viscozyme® L pre-treatment[a] (based on \$ ton^{-1} feedstock).

Input (cost)	NaOH	$Ca(OH)_2$	**Output (Revenue)**	NaOH	$Ca(OH)_2$
GTR	16	16	GA	48	48
Viscozyme Pectin extraction	80	80	Protein	171 (233*)	94 (222*)
Chemical	24	2	Glucose	55	55
Water	8	1	Energy	8	8
Heating	4	13			
Waste water[b]	8	1			
Press Filtration	1	1			
Ultra-filtration	2	2			
Capital	7	13			
Labor	4	6			
Subtotal	154	135	**Subtotal**	282	215
			Profit	128	80

a, Calculation based on data presented in Fig. 6.5.
b, Waste water management fee was estimated based on American standard (Waterworld, 2014).
*, Protein revenue was calculated as 570 kg protein products with 53% protein content in NaOH protein extraction or 390 kg protein products with 65% protein content in $Ca(OH)_2$ protein extraction from microalgae.

6.4 Conclusion

Waste salts generated from alkaline protein extraction can be either reused or recycled if KOH or $Ca(OH)_2$ is used. KOH has similar functions as NaOH, yielding more than 90% protein from GTR. When NaOH is replaced by KOH, the increased costs for chemicals and ultra-filtration can be covered by the added value of applying residual potassium ions as fertilizer and by the reduction of waste water management fees. The profit of the KOH aided protein extraction process is similar or can be 2 \$ ton^{-1} GTR higher compared to using NaOH. The other scenario uses $Ca(OH)_2$, resulting in an extraction

efficiency of less than 50% protein from GTR. The low yield might be due to the hindrance of lignin. More than 70% protein can be extracted with $Ca(OH)_2$ from microalgae which naturally have no lignin content. The economic advantage of the $Ca(OH)_2$ scenario highly depends on the protein yield. Only when protein yield was higher than 70%, $Ca(OH)_2$ aided protein extraction showed higher profit compared to using NaOH. Application of KOH protein extraction and $Ca(OH)_2$ protein extraction on different types of biomass enables commercialization of yet untapped protein resources, which can be beneficial for the environment and economically attractive.

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CHAPTER 7: GENERAL DISCUSSION

KEY TO "TWICE THE FOOD PRODUCTION AT HALF THE ECOLOGICAL FOOTPRINT BY 2050"

7

7.1 Introduction

Biorefinery of leafy biomass could be the key to "Twice the food production at half the ecological footprint by 2050". However, the challenges of leaf biorefinery mentioned in Chapter 1 were not completely solved by the research that is described in this thesis. More effort should be made in all aspects of leaf biorefinery development, within the domains of research, transition development, industry, and society. As shown in Fig. 7.1, each of these domains includes several subjects, and the development of these subjects is critical to the success of leaf biorefinery.

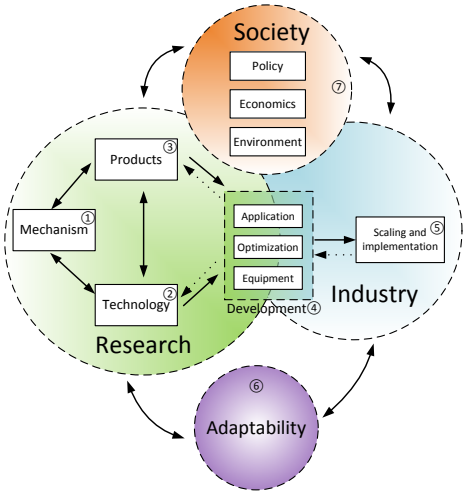


Fig. 7.1 Aspects of leaf biorefinery development.

The main challenge in leaf biorefinery is to improve its cost-effectiveness, which can either be done by improving the efficiency of production or by increasing the value of products (Chapter 1). The current focus of leaf biorefinery development is in the research domain, including the subjects of mechanism study, technology development, and product development (Fig. 7.1). These three subjects are mutually influenced, constituting a circle in the scientific research domain. This circle often begins with a breakthrough in technology, followed by progress in product quantity and quality. During development of new technologies, product quality or cost-price is often too low to immediately meet market demands. This is why an improvement of the product or further investigation into the mechanism of the new technology is necessary. When the mechanism is fully understood, the technology can be developed further or replaced by another new technology, starting a new circle. The main focus of previous chapters was on the development of technologies and on protein quality accompanied by a discussion on mechanisms. To start a new research circle, this chapter will begin with an overview of the mechanism of alkaline protein extraction (Section 2), and then propose possible

improvements to upgrade technologies (Section 3).

To practically apply new technologies to leaf biorefinery, a transition from research to industrial production is critical. This transition includes issues concerning improvements for product application, process optimization, and equipment selection. Development of process optimization and equipment selection is the key to lowering production cost, which was discussed in Chapters 5 and 6. Development of product applications is key to upgrading product value. Although upgrading product was rarely mentioned in previous chapters, it could be even more important than the other two subjects. For instance, upgrading the application of protein from inclusion in animal feed to inclusion in human food, increases the market value of the protein product from roughly 360 \$ ton^{-1} (WorldBank, 2012) to 2000 \$ ton^{-1} (Barb, 2011); upgrading application of pectin from bulk chemicals to use as food fibre or even in food jelly increases the market value of leaf pectin from 800 \$ ton^{-1} to 4000 -10000 \$ ton^{-1} (Chang, 2008). The possibilities for upgrading products obtained from leaf biorefinery are presented as the main focus of this chapter in Section 4.

Development of the industry and society domains is not the focus of this thesis, but they are essential to production of commercial products. Scaling and implementation of leaf biorefinery should be conducted with regard to the local situation, including the production chain and logistics, for which both large-scale production and small-scale production should be considered (Bruins and Sanders, 2012). “To double the amount of food production”, leaf biorefinery technologies should furthermore be applicable to many species other than GTR. For a factory, the applicability of technologies for different types of leaves reduce the risk of raw material dependence. In addition, although few investigations were made in this thesis, impacts from society, which includes issues on the environment and governmental policy, could be the most important factor to halve “the ecological footprint”. In this chapter, information on industrial scaling and implementation, adaptability of technologies and processes, and impact of society is discussed in Section 5.

7.2 Mechanism of alkaline protein extraction

To clarify the mechanism of alkaline protein extraction from leafy biomass, the structure and composition of leaf tissues, and the location of organelles with high protein content should be better understood. Based on our previous studies, the mechanism of alkaline protein extraction was hypothesised by simplified models of leaf tissues, cell structure, primary cell wall, and secondary cell wall. This hypothesized mechanism may be further used for the evaluation of other extraction technologies and offers a basis to improve the efficiency of protein extraction.

7.2.1 Structure of leaf tissues and their compositions

As mentioned in Chapter 1, most proteins are located in organelles in mesophyll tissues protected by the epidermis. Mesophyll tissues and the epidermis are adhered by the lamella layer that includes a large quantity of pectin (Dashek and Harrison, 2006b) (see Fig. 7.2a (Zephyris, 2011)). The epidermis, consisting of 95% keratinocytes (McGrath, 2004), protects cells from mechanical damage from the environment. It is the first shield against protein extraction. A close-up view of mesophyll tissues, as shown in Fig. 7.2b (Stern, 1997), illustrates how protein-containing organelles, such as chloroplasts and the plasma membrane, are restricted inside the cell by cell walls. For most leaves, a secondary cell wall between the primary cell wall and plasma membrane is present. It is produced after the primary cell wall is complete (Buchanan et al., 2000). To extract protein from the inside of cells, cell walls must be opened, and therefore, the composition and structure of cell walls should be investigated.

Although cell wall composition and structure may differ according to the plant species, the functions of leaf cell walls are similar, and therefore, a general model of cell walls was proposed, as shown in Fig. 7.2c and Fig. 7.2d. The primary cell wall is freely permeable and permits the passage of small molecules depending on pH. A model structure of the primary plant cell wall was presented by Somerville (see Fig. 7.2c) (Somerville et al., 2004), in which the location of all types of pectin can be seen. Xyloglucan (XG), RGI pectin, and glucuronoarabinoxylan (GAX) form cross-linked nets and stretch to all spaces within the plant cell wall. The secondary plant cell wall mainly

consists of lignin and (hemi-) cellulose that increases the rigidity of the cell and stops cell expansion (Dahl, 30 April 2011) (Fig.7.2d). Compounds in the secondary cell wall are rigid and waterproof, making them stiff and the toughest shield of cell organelles.

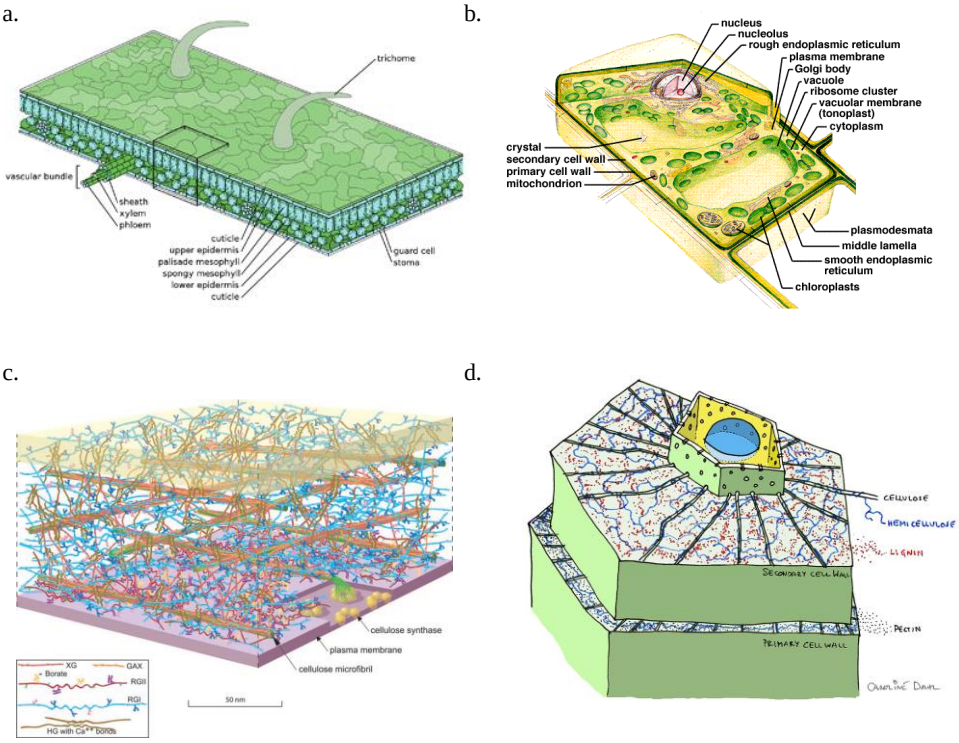


Fig.7.2a, Morphology model of leaf tissues (Zephyris, 2011); b, Morphology model of plant cell (Stern, 1997); c, Scale model of the polysaccharides in the primary cell wall of an *Arabidopsis* leaf (Somerville et al., 2004). XG, xyloglucan; GAX, glucuronoarabinoxylan; RGI, rhamnogalacturonan I; GRII, rhamnogalacturonan II; HG, homogalacturonan; d, Plant cell showing primary and secondary wall (Dahl, 30 April 2011).

7.2.2 What are the limiting factors for leaf protein extraction

In Chapter 2, leaf proteins were fractionated into easy and difficult to extract groups by alkaline extraction. In green tea residue (GTR), approximately 35% was easy extractable protein, in which only 50% of were "true proteins" (consisting of amino acids) (Zhang et al., 2014). These true proteins may consist of glycoprotein from the lamella layer (Dashek and Harrison, 2006a) and proteins released from inside cells caused by cell wall damage from mechanical disruption. The difficult to extract proteins were mainly located inside cells, such as membrane protein and RuBisCO (Feller et al., 2008).

Protein solubility and the barriers of the epidermis and cell wall were the main hindrances to extracting proteins located inside cells.

The low solubility of proteins might have resulted from their location, or/and composition and structure during metabolism, or the extraction process. For instance, membrane proteins have a very low solubility in aqueous buffers (Vertommen et al., 2010). They are more hydrophobic than other proteins and deeply embedded in the membrane lipid core (Feller et al., 2008; Seigneurin-Berny et al., 1999; von Heijne, 1992). Lignin can bind with cell-wall glycoprotein forming a lignin-protein complex (Whitmore, 1982), which is insoluble in water. This complex can both be generated during metabolism via peroxidase catalysis (Whitmore, 1982) and during alkaline protein extraction (tannin-protein) (Van Soest, 1994; Vertommen et al., 2010).

To obtain high protein yields, barriers of the epidermis, primary cell wall, and secondary cell wall must be overcome (Zhang et al., 2015). The leaf epidermis contains cutin and is covered with a cuticle, which is occasionally also covered with wax. The cuticle reduces water loss to the atmosphere, whereas the surface wax acts as a moisture barrier and protects the plant from intense sunlight and wind (Raven et al., 1999). In general, the plant epidermis layer blocks access of chemicals and enzymes to mesophyll tissues, and therefore reduce the efficiency of protein extraction. Primary and secondary cell walls function as sieves with different pore sizes, which are covered by a semi-fluid component. In the primary cell wall, the semi-fluid components may mainly contain HG pectin, whereas in the secondary cell wall, the main semi-fluid component may be RG I pectin. Proteins with large molecular weight located inside cells could not be extracted without the degradation of these cell walls.

7.2.3 Possible mechanism for alkaline protein extraction

Previous research focused on the search for limiting components of alkaline protein extraction. These studies used various types of biomass, for which oil, cellulose, hemi-cellulose, and lignin were estimated as limiting components based on a fixed alkaline extraction protocol using statistical correlations (Sari et al., 2015b). In Chapter 3, the possible limiting components for alkaline protein extraction were determined to be

RG I pectin and/or lignin, for which temperatures higher than 60 °C are needed. In Chapter 5, RG I pectin was hydrolysed by Viscozyme with no protein release, eliminating RG I pectin as the sole limiting component. The other component — lignin was consequently expected to be the key limiting component for alkaline protein extraction.

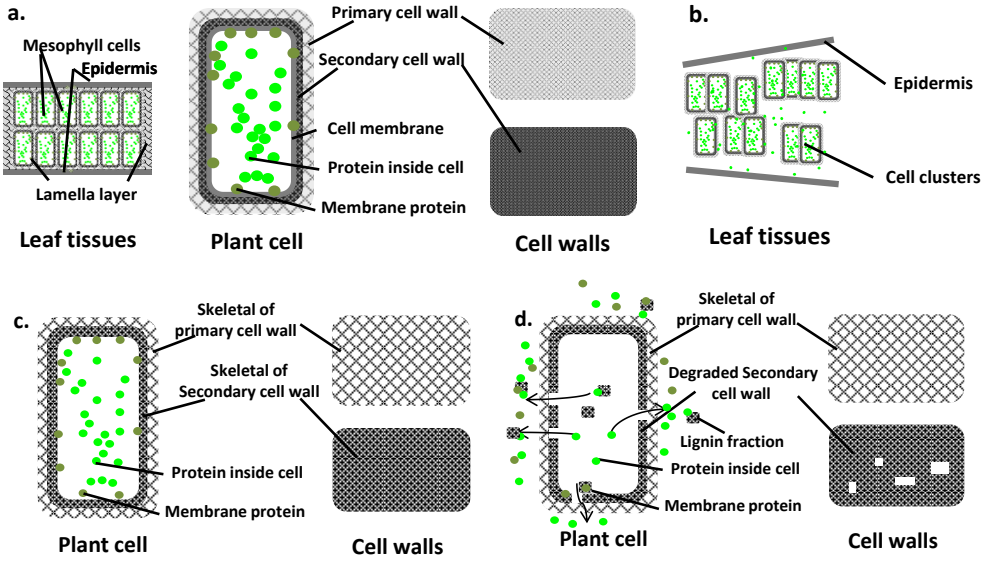


Fig. 7.3 Hypothesized mechanism of alkaline protein extraction from leafy biomass on tissue, cell, and cell wall level. a, simplified models of leaf tissues, cell structure, primary cell wall, and secondary cell wall; b, first phase: solubilisation of lamella layer; c, second phase: solubilisation of lamella components in cell wall and cell membrane; d, third phase: disruption of secondary cell wall.

To describe the mechanism of alkaline protein extraction, simplified models of leaf tissues, cell structure, primary cell wall, and secondary cell wall are presented in Fig. 7.3a according to previous studies by others (Dashek and Harrison, 2006b; Somerville et al., 2004). Based on these models, the mechanism of alkaline protein extraction is hypothesized to have three phases, as illustrated in Fig. 7.3b, c, and d. The first phase is solubilisation of HG pectin in the lamella layer (Fig. 7.3b). In this phase, alkali accesses the lamella layer and solubilizes HG pectin. The most critical factor for solubilizing all pectin is the amount of alkali. With a sufficient amount of epidermis peeled off, the mesophyll tissues will be disintegrated to cell clusters. This phase can obtain easy extractable protein, which constitutes approximately 32% of N-containing components

(22% are true proteins (Zhang et al., 2014)). The second phase is the solubilisation of semi-liquid components that cover the primary and secondary cell wall, for which elevated temperatures (>60 °C) are needed. In this phase, the cellulose structure and lignin structure in the primary cell wall are intact, but the RGI pectin that functions as a scaffold in both two cell walls was degraded (Somerville et al., 2004). With the removal of the outer components, the pores of these two walls are exposed, and proteins with a particle size smaller than the pores can diffuse into the alkaline solution. Noticeably, protein-containing organelles inside the cell may also be disrupted in this phase, whereas before the removal of the cell wall covering components, no alkali could access the inside of cells. The third phase is the disruption of lignin structure (Fig. 7.3d). In this phase, some part of the lignin structure can be hydrolysed and then solubilized in alkaline solution. In addition to relatively high temperature, a solution with a pH higher than 12 is essential for lignin solubilisation (Carvalheiro et al., 2008; Sambusiti et al., 2012). It is not necessary to solubilize all lignin for all proteins to be extracted; only a hole of a sufficient size is needed. During alkaline protein extraction, the second and third phases happen simultaneously, and RGI pectin was extracted in combination with lignin. Not all lignin was extracted when proteins were fully extracted, suggesting most proteins did not bind to lignin before extraction. The lignin-protein complex is only formed after the diffusion of protein and lignin fractions. Developing a method that can hydrolyse lignin structure may obtain high-yield protein in its native form.

7.3 Technology development

Technological developments in all production steps, including extraction, separation, and their related machinery, are critical to improve the cost-efficiency and sustainability of leaf biorefinery. In addition to the presented extraction technologies, other leaf protein extraction technologies can be considered for leaf biorefinery. In this section, some examples on improvement of the protein extraction process, separation technology, and their related machinery are presented.

7.3.1 Evaluation of current leaf protein extraction technologies

Current protein extraction technologies can be roughly divided into two categories:

physical and chemical. Mechanical milling, ultra-sonic, steam explosion, and similar techniques are mainly considered to be methods of physical protein extraction; whereas the use of alkali, acid, enzymes, and similar substances are considered to be methods of chemical protein extraction. Some methods, such as ammonia fibre explosion are considered combined treatments. Fig. 7.4, Fig. 7.5, and Fig. 7.6 illustrate how these methods to extract leaf protein can be analysed using simplified models. The results are summarized in Table. 7.1.

Table 7.1 Mechanism and protein yield of current protein extraction technologies for leaf protein

Protein extraction technology	Selectivity	Influence on three barriers			Current protein yield (%)	Potential protein yield (%)
		Epidermis	Primary cell wall	Secondary cell wall		
Mechanical grinding/press (Baraniak and Baraniak, 1987; González et al., 1988; Telek, 1983)	No	Disrupted and fractionated	Some lamella and cellulose structure	Almost not influence	20-50	40-60
Steam fiber explosion	No	Disrupted and fractionated	Lamella and some cellulose structure	Lamella and some lignin structure	-	50
Ammonia fibre explosion (Bals et al., 2007a)	No	Disrupted and fractionated	Lamella and some cellulose structure	Lamella and lignin structure	70-95	>90
Acid (Sari et al., 2014)	No	Peel off	Lamella and cellulose structure	Lamella	30-50	>90
Carbohydrase (Rosset et al., 2014; Shen et al., 2008)	Yes	Peel off	Lamella and cellulose structure	Lamella	10-30	30-50
Proteinase (Sari et al., 2015a)	Yes	No influence	No influence	No influence	50	50

Mechanical protein extraction is a traditional method that breaks solid materials into smaller pieces by grinding, crushing, or cutting, and has been studied for protein extraction (Baraniak and Baraniak, 1987; González et al., 1988; Telek, 1983). In the example shown in Fig. 7.4, leaf tissues were sheared into small pieces by mechanical devices, which disintegrate the epidermis, lamella, and mesophyll tissues. However, the smallest pieces obtained by most milling machines are only about 0.05 mm (diameter) or 20 times larger than the particle size of a plant cell, which means most plant cells still remain intact. About 30% of total protein can be obtained by milling, which is primarily derived from cells accidentally cut by the mechanical devices. To improve protein extraction efficiency of mechanical milling, the capacity of producing finer particles, smaller than a cell, is critical. However, as finer particles are produced the energy input will be increased exponentially, leading to higher production cost.

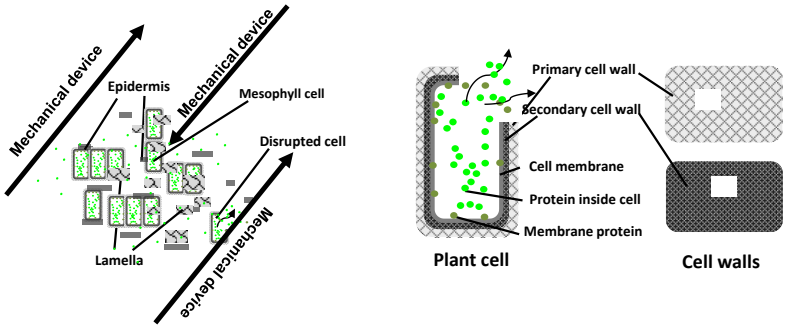


Fig. 7.4 How does milling work on leaf protein extraction on tissue, cell, and cell wall level.

Steam explosion is a thermo-mechanic-chemical pre-treatment, which allows for the breakdown of lignocellulosic structural components by the combined action of heating, formation of organic acids during the process, and shearing forces resulting from the expansion of the moisture (Jacquet et al., 2015). Steam explosion can break down cellulose structure for ethanol production, but it has no influence on lignin structure (Li et al., 2009). One possible mechanism for protein extraction using steam explosion is illustrated in Fig. 7.5. The epidermis, pectin matrix, and cellulose structure can be degraded because of the steam pressure and the high temperature. The pore size of the "lignin sieve" may instantly increase when the explosion is conducted, which may aid in the release of protein. However, the lignin structure can recover, which will retain most proteins inside the cells. To improve protein-extraction yield, ammonia is recommended as a replacement for steam. As lignin structure can be degraded under alkaline conditions; an ammonia fibre explosion (Bals et al., 2007a) will have a better protein extraction yield than steam explosion.

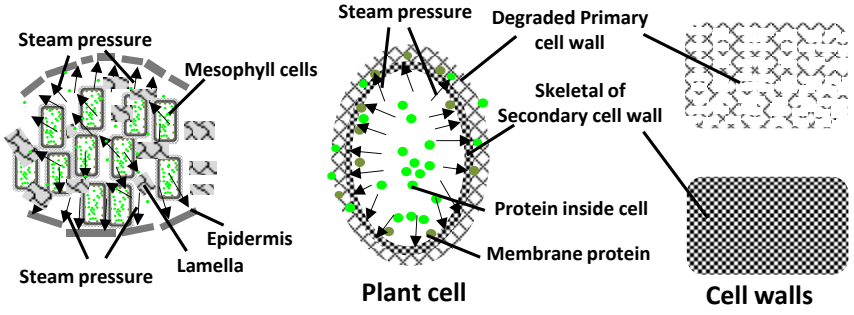


Fig. 7.5 How steam explosion works for leaf protein extraction on tissue, cell, and cell wall level.

Leaf protein can also be extracted by acid, but its mechanism is different from that of alkaline protein extraction. One possible mechanism for acid protein extraction is presented in Fig. 7.6a. Under mild acidic conditions, pectin in the lamella layer can be hydrolysed and solubilised (Ele-Ekouna et al., 2010; Westereng et al., 2008), leading to a detachment of the epidermis and mesophyll tissues. Proteins located inside cells can also coagulate at mild acidic condition, thus increasing their particle sizes and causing deposition inside cells. Using highly acidic conditions, such as 6 M HCl at 110 °C, cellulose structure, as well as proteins will be hydrolysed (Garna et al., 2006; Meussen et al., 2014), and the hydrolysis efficiency will depend on acid concentration, temperature, and time (Sari et al., 2014). After hydrolysis, proteins will be degraded into small peptides or often even amino acids (Dewanji, 1993; Sari et al., 2014). The protein fractions with particle sizes smaller than the pore size of the lignin structure can be extracted. Under acidic conditions however, the lignin fabric coagulates rather than degrades (Horst et al., 2014), and therefore, condenses the secondary cell wall. This coagulation may reduce the pore size of the "lignin sieve", hindering protein extraction.

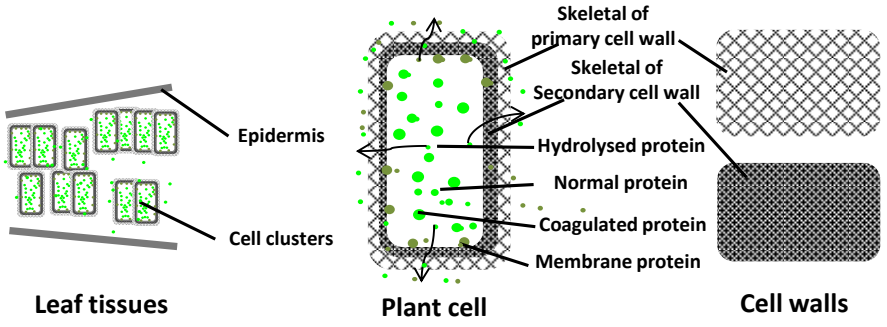


Fig. 7.6a How acid aids leaf protein extraction on tissue, cell, and cell wall level.

This hypothesis was experimentally verified by soaking GTR in 0.1 M HCl with 40 v/w at 40 °C or 95 °C over time. The yields of extracted protein are plotted against extraction time in Fig. 7.6b. The highest protein yield obtained using 0.1 M HCl was around 60%, which is lower than the protein yield obtained using the same amount of alkali. All proteins are expected to extract using HCl when more intense conditions were applied (Dewanji, 1993; Meussen et al., 2014). Similar to alkaline protein extraction, acid

protein extraction can serve as a core process in an integrated biorefinery process. Because of the hydrolysis effect of acid, the focus of the acid extracted protein applications will be in animal feed and/or bulk chemicals rather than in human food.

Enzymes, such as protease and carbohydrase, are also used for protein extraction. With the addition of protease and/or carbohydrase, high-yield protein was obtained from rice bran, lupin, and rapeseed, but enzymes did not perform well in leaf-protein extraction (Sari et al., 2015a; Shen et al., 2008). As protease cannot cross the barriers of secondary cell walls, proteinase is not effective for the extraction of proteins located inside leaf cells (Shen et al., 2008). Carbohydrases cannot degrade the lignin structure in secondary plant cell walls, and therefore, lead to low leaf-protein yields (Rosset et al., 2014; Zhang et al., 2016a; Zhang et al., 2016b). If enzymes targeting the degradation of lignin can be developed, full protein extraction is expected with the least influence on the structure of native proteins.

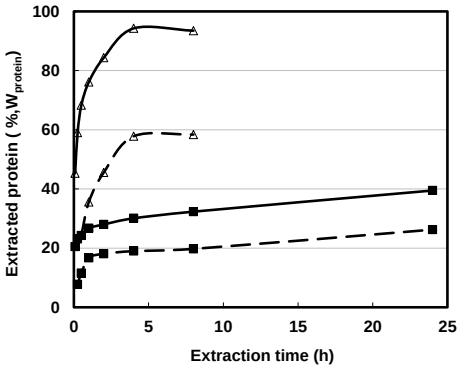


Fig. 7.6b Extracted protein ($W_{Protein}$,%) with 0.1M solution and v/w of 40 mL g^{-1} at different temperatures. ——: NaOH; — —: HCl; △:95 °C; ■:40 °C.

Based on above analysis, ammonia fibre explosion, acid protein extraction, and lignin degrading enzymes may have potential to extract more than 90% protein from leafy biomass.

7.3.2 Separation technologies: ultrafiltration

In leaf biorefinery, the solid-liquid separation technologies, such as press filtration and centrifugation, are commonly used in product recovery (Huang et al., 2008). To increase protein or pectin purity, pectin or protein extracts were first precipitated by ethanol (Wang et al., 2014) or acid (Chiesa and Gnansounou, 2011), and then the pectin or protein precipitations were collected by centrifugation. After product recovery, the supernatants that contain added chemicals, such as ethanol, should be recycled.

Otherwise the excess chemicals will lead to high production cost and also generates environmental problems. A liquid-liquid membrane separation system for ultrafiltration was therefore recommended. Using ultrafiltration, protein or pectin products can be purified and concentrated excluding the addition of flocculants (ethanol or acid), which may be the key technology for future biorefinery.

Ultrafiltration membranes are usually made from polymers, such as polysulfone (Binabaji et al., 2015). Target components can either be retained or passed through the membrane with the solvent, whereas impurities are in the other fraction. The separation is based on the pore size of the membrane, by which components can be separated depending on their molecular weight (Calvo et al., 2015). New membranes, which can separate components by their characteristics, such as polar or anti-polar, have also been developed (Huang et al., 2008).

In our study, the efficacy of ultrafiltration for desalting protein extracts was tested. The practical isolation efficiency of GTR protein extracts (0.1 M NaOH, 40 v/w, 95 °C, 4 h) was determined using membranes with pore sizes of 1k MWCO in a Laboratory Cell CF-1 system (KOCH, USA, See Fig. 7.7). After separation for 4 h, 99% of the proteins with 50% salts were retained in the tank, meaning 50% of salts was removed and protein content was concentrated 2 times. After processing for 4 h, the permeate volume was only half of the initial sample volume, suggesting the capacity of separation is rather low. As most GTR protein molecular weights are higher than 10k Da, a membrane with pore size between slightly higher pore sizes; 1–10k MWCO should be further tested for high separation efficiency with a better processing capacity.

Fig.7.7 Ultrafiltration system, LABCELL CF-1.

7.3.3 Protein extraction: a continuous reactor

In Chapter 5, it was suggested to integrate weak alkaline pectin extraction with protein extraction. However, as large amounts of water are critical for pectin extraction, many reactors with large volumes are needed. The capital and labour costs are therefore high

for both purchasing and operating these reactors. Continuous counter-current extractors were suggested as a replacement for conventional reactors (Davison et al., 2001). Using a continuous extractor, the waste product lignocellulose that is produced after protein extraction, can be removed, combining extraction and separation. Washing and reloading time between batches can be saved in the continuous extraction system. It was reported that the recoveries of soluble components can be higher than 90% with less water usage when a continuous counter-current extractor was applied (Davison et al., 2001). The capacity of a continuous counter-current extractor can be 500-1000 kg raw material per hour using e.g. a CONTEX™ Extractor (Group, 2015), which is 2 times higher than the processing capacity of the reactor stated in Chapters 5.

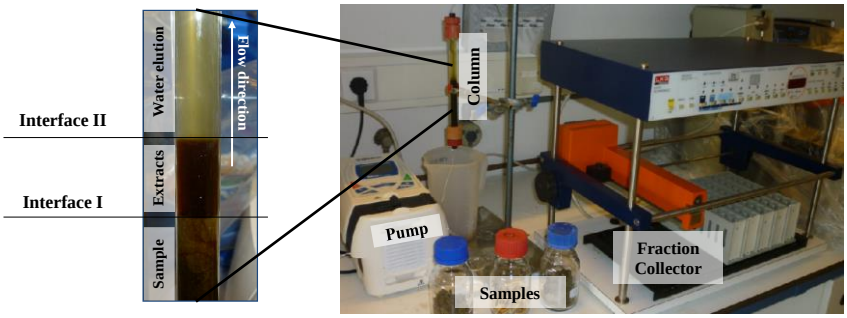


Fig.7.8 Chromatography counter-current extraction system for leaf protein. Left: Status of column when the extraction was running. Right: All units of extraction system.

Continuous separation technologies are not new in industrial-scale production, but application of separation technologies, such as chromatography systems, in the design of continuous reactors for extraction are not yet fully developed. To maximize the efficiency of an extractive process for protein separation, counter current motion of solid and liquid with minimal axial mixing is required (Gordon et al., 1990). Machineries of fixed bed chromatography, rotating column chromatography, and simulated moving bed chromatography systems are already designed (Gordon et al., 1990). These machineries can be also applied to design a continuous counter-current extractor, in which the chromatographic material used for separation can be replaced by raw material for extraction. To verify this possibility, a lab scale counter-current chromatography system, containing a pump, column, and fraction collector was established as presented in

Fig.7.8. The pump was used to control the flow rate of elution, and the fraction collector was used to collect fractions in time. Using the principle of counter-current extraction (Davison et al., 2001; Lestari et al., 2010), the solution used for extraction was pumped into the column from the bottom to the top When the elution went through the sample, two interfaces (between sample and extract, and between extract and water) could be seen in the column as presented in Fig. 7.8. This system was tested using NaOH solutions with concentrations of 0.1 M at 60 °C. Using a flow rate of 0.067 mL min^{-1}, more than 90% of the protein could be extracted with 4 g L^{-1} as the highest protein concentration. The protein content could be improved to 15 g L^{-1} using a lower flow rate or higher temperature.

This system could be further upgraded. Using a jacketed column, temperature of this system can be controlled using a water bath. Two pumps and a solution mixer can be used to create a solvent gradient, enabling leaf protein to be extracted at its highest protein content (Lestari et al., 2010). When several columns using different solutions or extraction conditions are connected, a continuous integrated process for multiple products, as presented in Chapters 4 and 5, can be made. In addition, this designed system can also be applied for analysis when it is connected to a UV detector, pH meter, HPLC, infrared Detector, or mass spectrometry.

7.4 Products

Other than the improvement of production efficiency, upgrading product value is an alternative way to improve the cost-effective of leaf biorefinery. Product value is determined by its applications, which are related to composition, nutritional value, and functionalities. In Chapters 2, 3, and 4, leaf protein was considered for animal feed and leaf pectin for its chemical uses. To improve product value, application of leaf extracts in food or medicine should be considered.

7.4.1 Protein

Two possibilities can be considered to upgrade the value of leaf protein: 1, increase the nutritional value; 2, use leaf protein for its functionalities.

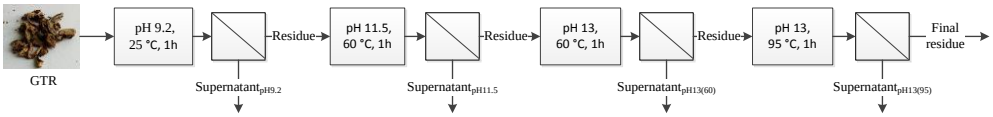
When applying leaf protein in feed, the protein pricing is mainly determined by its nutritional value. The nutritional value of proteins for monogastric animals and humans is often limited to the amount of essential amino acids, being arginine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Dale et al., 2009b). Among these essential amino acids, lysine, threonine, and tryptophan, are limiting amino acids for growing pig, whereas methionine, lysine, and threonine are limiting amino acids for broiler (Teekens et al., 2015; Toride, 2004). This demonstrates that not all amino acids contribute to the value of animal feed, and therefore isolation of non-essential amino acids does not improve protein nutritional value (Sanders et al., 2007). These non-essential amino acids can possibly be better applied in other applications like for chemicals, such as glutamic acid for synthesis of succinonitrile (Lammens et al., 2011), which can possibly increase the total GTR protein revenue from 170 \$ ton^{-1} GTR to 510 \$ ton^{-1} GTR (Sanders et al., 2007; Zhang et al., 2016a).

Protein products can have higher market values when used for their functionalities, as e.g. gelatine has a market price of 6600 \$ ton^{-1} (Chang, 2008). Alkaline extracted protein is expected to have lower value because it is denatured, modified, and/or degraded (Bals and Dale, 2011) during extraction. However, during processing and storage of GTR protein extracts, the foaming and gelling properties of extracts were described, which indicated the protein product obtained by alkaline extraction could still be used in food. Also, only very limited protein hydrolysis occurred during alkaline protein extraction of GTR (Chapter 3). Processing at 95 °C reduced the free $-NH_2$ content of protein, which may have been caused by the reaction of $-NH_2$ with other components, such as polyphenol (Ozdal et al., 2013). To clarify alkaline protein extraction, SDS-PAGE was used to analyse GTR protein extracts obtained by sequential alkaline treatments as shown in Fig. 7.9a, with results presented in Fig. 7.9b.

The smear in all lanes of the SDS-PAGE suggested that proteins had been modified. At pH 9.2, some clear bands could be seen with molecular weights that ranged between 38–98k Da. This fraction was previously described as easy to extract protein. When more severe conditions were applied (Lanes 3 and 4), more protein could be extracted (mainly difficultly extracted protein) with no clear bands presented, but the proteins were

primarily in two molecular weight ranges: 62–98k Da and 6–17k Da. Using NaOH with pH 13 at 95 °C, almost all protein could be extracted. Although the whole lane was covered by blue, proteins were still mainly concentrated in the areas of the two molecular weight ranges mentioned above. This phenomenon demonstrates that molecular weight distribution of proteins extracted at pH 13 and 95 °C is similar to those at pH 11.5 at 60 °C, which may be considered mild conditions. In addition, no band could be seen at the bottom of the gel in Lane 2 compared to those in other lanes, suggesting it is not necessary to hydrolyse leaf proteins when server conditions were applied. Compared to previous SDS-PAGE results (Shen et al., 2008) of GTR protein extracts, our alkaline protein extracts were definitely modified. These modifications may be from the reaction between pectin or polyphonic compounds with protein (Ahmed et al., 1995; Ishii et al., 2008). Nevertheless, the question remains if the modified protein always has worse functionalities than the native protein?

a.



b.

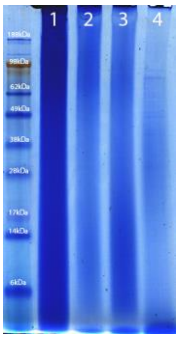


Fig. 7.9a: Protein extracts obtained sequentially by different alkaline conditions from GTR; b: SDS-PAGE results of protein extracts obtained from Fig. 7.9a. 1, Supernatant$_{pH13(95)}$; 2, Supernatant$_{pH13(60)}$; 3, Supernatant $_{pH11.5}$; 4, Supernatant$_{pH9.2}$ with 2 dilution.

Protein samples can have better functionalities when mixed with other components or are modified. Protein-pectin complexes were found to have better emulsifying and gelling properties than the single components (Bueno et al., 2009; Neirynck et al., 2007; Zhang and Vardhanabhuti, 2014). Although protein-phenolic complexes have lower solubility and digestibility, they might have better thermal stability and antioxidant capacities (Ozdal et al., 2013). In addition, proteins may have better functionalities when they were modified by chemical, enzymatic, or physical techniques (Kester and

Richardson, 1984). Generally, it is possible to apply modified protein in food for their functionalities, which could be an important topic for leaf biorefinery research.

7.4.2 Pectin

In Chapter 5, the possibility of using pectin for its galacturonic acid as a secondary product from GTR was discussed. It was suggested that the application of pectin in food was the key for enhanced economics of the weak alkaline pectin extraction integrated process. Leaf pectin can be applied in food for its gelling properties (Chen et al., 2014; Singthong et al., 2005). It was however expected that the degree of esterification of leaf pectin would be reduced in alkaline conditions (Jiang et al., 2005), which influences pectin functionality (Assoi et al., 2014).

However, it was also found in Chapter 5 that pectin can be extracted in water, as long as the pH of the extract is higher than 5, where pectin can be degraded as a the result of a β-elimination cleavage of the glycosidic linkage (Stephen and Phillips, 2006). This reaction only occurs at glycosidic bonds adjacent to an esterified carboxyl group (Stephen and Phillips, 2006; Thakur et al., 1997), and thus the backbone of pectin was retained. The weak alkali was adding to neutralize the extract, which pH was reduced because of the extraction of acidic components, such as organic acid and pectin. With proper control, it is possible to obtain food-grade pectin from GTR. If the pectin extracted by weak alkali can be applied in food, revenue from the weak alkaline pectin extraction integrated process will increase with 550 \$ ton^{-1} GTR, leading to a profit of more than 600 \$ ton^{-1} GTR.

The gelling properties of leaf pectin were not determined, but from the appearance of pectin gel obtained from GTR, its potential could be estimated. When pure ethanol was added to GTR alkaline protein extract (Fig. 7.10 right) until a final ethanol concentration of 40%, GTR pectin gelled and precipitated. GTR pectin gel could be re-dissolved by adding water, and the gel was

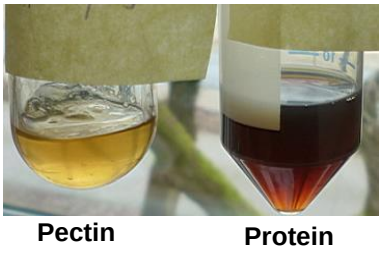


Fig. 7.10 Pectin gel (left) obtained from alkaline protein extract (right) by adding ethanol to 40% (v/v).

colourless after several cycles of precipitation and dissolving (Fig. 7.10 left).

7.4.3 Other products

Protein, pectin, (hemi-) cellulose, and lignin are the four major components in leaves (Chapter 1). In this thesis, protein and pectin are proposed as the main products while (hemi-) cellulose and lignin were suggested to be combusted for energy. However, (hemi-) cellulose and lignin may have better applications, such as making paper or board (Anwar et al., 2014; Pothiraj et al., 2006). Adding alkali treated grass residue at 15% in the feedstock while making board, improved both the strength and cracking resistance of the product. These improvements may have resulted from the smaller particle size of the fibers in alkali treated grass, due to the hydrolysis of lignocellulose at alkaline conditions. In addition, alkali treated leaf residues can be used as substrate for producing second generation ethanol. Due to the degradation of cellulose and hemi-cellulose, the efficiencies of enzymatic hydrolysis of xylan to xylose and glucan to glucose can be improved from 20% to 80% and from 37% to 92%, leading to an improvement of total conversion from 30% to 55% (Maas, 2008).

Some plants contain special components, such as polyphenols, which may have even higher value than protein and pectin. The values and contents of leaf polyphenols highly depend on the leaf specie and the growth period of leaf. For example, only the young shoots of tea trees can be used to produce green tea, mainly containing catechins and flavonols (Harbowy; and Balentine, 1997), while the old tea leaves are usually abandoned. Other leaf species, such as olive leaf (Lalas et al., 2011) and Hibiscus sabdariffa leaf (Zhen et al., 2016), also contain large quantities of polyphenols with high anti-oxidizing activity. These polyphenol may also be applied in food or medicine.

7.5 Outlook

7.5.1 Economic feasible and scaling of leaf biorefinery

Chapters 2-5 discussed the economic feasibility of leaf biorefinery based on lab-scale research. To transfer lab research to practical production, the production process should be optimized according to the local situation, suitable equipment should be found and the

product should meet the market requirements. For years, large-scale production was considered as cost-effective owing to relatively low capital, labour, and production cost for per unit product (Alchian and Demsetz, 1972). However, it was recently found that small-scale production may have specific advantages in the biorefinery of many biomass types. Small-scale production can be beneficial not only socially and ecologically, but also economically, due to its high efficiency in the local re-use of materials, including water, minerals, organic matter, CO2, and heat (Bruins and Sanders, 2012).

Biorefinery of leafy biomass may be more suitable to implement as a small-scale production because the benefits of large-scale production may not compensate cost for transportation. Assuming that fresh leafy biomass (FLB) contains 80% water, 6% carbohydrates, and 3% protein, and travels an average distance of 100 km from the field to the factory, the transportation cost can be estimated as 12.7 \$ ton^{-1} FLB by truck or 2.8 \$ ton^{-1} FLB by rail in 2015 according to U.S. National Transportation Statistics (Nguyen et al., 2015). The waste water produced by the factory will be about 12 times the dried biomass (0.2 ton). To send back the salts as fertilizers to the field, the transportation costs can be 29.5 \$ ton^{-1}, 6.6 \$ ton^{-1} FLB by rail, or 3.3 \$ ton^{-1} FLB by pipeline when water is not removed. These costs are higher than the value salts used as fertilizer (about 6 \$ ton^{-1} FLB according to Chapter 6). This is why, on a large scale, water is removed prior to transportation of the salt by-product. Using small-scale production in a mobile system (Bruins and Sanders, 2012), production could be done next to the field where the biomass grows. According to the fertilizer requirement of the fields, the potassium scenario (Chapter 6) or ammonia fibre explosion (Bals et al., 2007a) could be selected. Waste water containing potassium or ammonium salts could be send back to the field as fertilizer immediately, whereas only the dry or concentrated products will be transported. The total transportation fee is then only on products, which is approximately 10% of 1ton FLB that the costs would be reduced to 1.3 \$ ton^{-1} FLB by truck and 0.3 \$ ton^{-1} by train.

With proper design, the cost reduction in transportation could be higher than the production increment at small scale compare to that at large scale. Small scale production may have higher efficiency in the usage of water, minerals, organic matter,

CO_2, and heat. The more efficient use of these materials can lead to a lower "life cycle cost" (Hasler et al., 2015; Huppes et al., 2004), which is normally unseen when environmental cost is not included in the cost estimation (Huppes et al., 2004).

7.5.2 Twice the food production

In this thesis, we developed biorefinery technologies using GTR as starting material. However, the annual world tea production is only about 4 million tons of which only 25% can be recycled. Even through turning all tea residues to food production, it is not possible to meet the target "twice the food production". The developed technologies are however expected to be applicable to other raw materials, specifically other leaf species. The suggested protocols in this thesis are universally applicable in raw materials other than GTR. An example was shown in Chapter 2, where alkaline protein extraction could be universally applied on oolong tea leaf residue, barley straw, jatropha leaf and grass. Furthermore, its application to sugarcane leaf, mango leaf, duckweed, and microalgae was verified. The applicability of extracted protein may be better than for the leaf protein inside leaves.

Table 7.2 Degradation of plant toxins in 0.1M NaOH as function of temperature and time

	25 °C			60 °C			95 °C		
	15min	2h	6h	15min	2h	6h	15min	2h	6h
Atropine	★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★
Scopolamine	★★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★
Aconitine	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★	★★★★
Ergotamine	☆	☆	★	★	★★★★	★★★★	★★★★	★★★★	★★★★
Anagyrine	☆	☆	☆	☆	☆	☆	☆	☆	☆
Coniine	☆	☆	☆	☆	☆	☆	★	★★	★★
Sparteine	☆	☆	☆	☆	★	★	★	★	★
Colchiceine	☆	☆	☆	☆	★	★	☆	★	★
Digoxin	☆	☆	☆	☆	★★	★★★★	★★★★	★★★★	★★★★
Digitoxin	☆	☆	☆	☆	★★	★★★	★★★	★★★★	★★★★
Convallotoxin	★	★	★	★	★★	★★★	★★★★	★★★★	★★★★
Alpha-solanine	★	★	★	★	★★	★★★	★	★★	★★★

☆: Stable; ★:some degradation (5-25%); ★★:moderate degradation (30-50%); ★★★:strong degradation (55-80%);★★★★:complete degradation.

Some leafy biomasses are not suitable to be used for animal feed or human food due to

contamination with weed plants containing toxins (De Nicola et al., 2011; Jakabová et al., 2012). This is especially true for leaves harvested close to the ground, like e.g. grass and sugar beet leaves. Fortunately, some toxins can be degraded when the optimal alkaline conditions for leaf protein extraction are applied. The effect of alkaline conditions, applied during leaf protein extraction, on the degradation of several toxins in solution was tested, and the results are listed in Table 7.2.

Under alkaline conditions (Czauderna and Kowalczyk, 2007; Theodorou et al., 2007), ester-type alkaloids toxins, such as atropine, scopolamine, aconitine, ergotamine, can be completely degraded by saponification of the ester groups. Using 0.1M alkaline at 95 °C, these toxins were destroyed within 15mins. The effect of alkali on the degradation of cardiac glycosides, including digoxin, digitoxin, convallotoxin, and alpha-solanine was temperature and time dependent. This was related to the presence of the glycosidic linkage at the terminal reducing unit, which requested more severe alkaline conditions for its degradation (Ballou and Melville, 1954). Alkaline conditions had minor effect on the degradation of non-ester type alkaloids, including anagyrine, coniine, sparteine, and colchiceine, even at 95 °C. To use biomass that contains these components in animal feed, pre-treatments, such as ethanol extraction (Couch, 1939; Cromwell, 1956), that may be used for the removal of these components should be developed. Removal of plant toxins requires further investigation to verify the applicability of proteinous leaf extracts for animal feed and food, which is critical for upgrading the value of leaf products. When all available leafy biomass is used, it can be foreseen that the food production can be doubled with no extra farming.

7.5.3 Half at the ecological footprint

In addition to economic drivers, environmental issues could be a reason for the development of leaf biorefinery, which is critical to halve the ecological footprint. When potassium hydroxide or calcium hydroxide is used in protein extraction, almost no waste is generated (Chapter 6). Using small scale production, leaf biorefinery can have the least influence on environment due to its high efficiency of materials usage. In addition, leaf biorefinery can be conducted on local crops. With proper integration, the total waste

can be minimized, which also minimizes the “ecological footprint”.

Development of leaf biorefinery technology can reduce the influence of human farming on the environment. For undeveloped countries that required a food production boost, food production can be doubled with no extra farming. Obtaining products from unused biomass and using them in food or animal feed can reduce the demand for crop production, and therefore, reduce the requirements of farmland, alleviating the stress of land degradation. For developed countries that have enough food supply, agricultural fields can be halved and therefore halve the “ecological footprint”.

As a new technology, leaf biorefinery not yet be attractive economically, but it reduces environmental cost. However, the benefit of environmental cost is often unseen until it is paid for by nature. Development of leaf biorefinery requests support from government in early stage. Research can be accelerated when the government is willing to issue research projects to develop biorefinery technology. The US and EU have invested much money in biorefinery research, with a lot of success. The Brazilian government-based policies on biofuel research and provision of subsidies for biofuel production led to a boost of biofuel production in Brazil. Brazil is now one of the largest biofuel producing countries in the world. With the depletion of fossil fuel resources and the increasing demand for food, biorefinery technologies have been gaining increasing attention all over the world, indicating a brilliant future.

7.6 Concluding remark

The aim of this study was to develop new processes and applications to optimally utilize all components, particularly protein, of leafy biomass in the feed and/or food industry. In this thesis, we developed a cost-effective alkaline protein extraction process and its related integration process for GTR. To improve sustainability, process potassium hydroxide and calcium hydroxide scenarios were suggested to replace sodium hydroxide, by which all waste salts can be either applied as fertilizer or recycled. Lignin and its structure in the secondary cell wall are the primary limiting factors in leaf protein extraction, and development of more mild methods for lignin degradation may further improve extraction efficiency with higher product quality. The future focus on leaf

biorefinery should be on food application of plant products, including protein and pectin, which may further improve its economics. Leaf biorefinery produces food with no extra farming and less environmental cost, which can be the answer to "Twice the food production at half the ecological footprint by 2050".

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APPENDIX

Table A1. Statistics of regression coefficients for protein yield approximation model.

	Coefficients	Normalised Coefficients	Standard Error	t Stat	P-value
Intercept	-31.2	59.7	11.7	-2.669	0.015
V/W	2.35	36.5	0.764	3.069	0.006
[Buffer]	168	3.5	73.1	2.291	0.034
T·V/W	0.015	-24.3	0.004	4.169	0.001
$(V/W)^2$	-0.026	21.3	0.010	-2.644	0.016

Table A2. Statistics of regression coefficients for GA/protein approximation model.

	Coefficients	Normalised Coefficients	Standard Error	t Stat	P-value
Intercept	-0.42	1.46	0.17	-2.5	0.021
C	5.9	0.19	1.8	3.3	0.0032
V/W	0.029	0.13	0.006	4.8	0.00011
T·V/W	0.00025	0.31	8.4E-05	2.9	0.0082

Table A3. Specifics and prices of chemicals and energy.

Chemicals /enzyme	Price ($ ton^{-1})	specific	Ref.
GTR	16	250g protein kg^{-1} GTR	(Yu, 2010)
Coal	54.9	30 MJ kg^{-1} (Ulrich and Vasudevan, 2006)	(Stevens, 2015)
Glucose	500		(Mudi, 2015b)
Pectin	11000	Use average price of 5$ lb^{-1}	(ICIS, 2008)
Galacturonic acid	800	Use for fermentation, similar as the price of Gluconic acid	(ICIS, 2008)
Protein	350	45% purity	(Mudi, 2015a)
Water	0.95	1080 $ for the first 300,000 gallons, and then 3.57 $ for each additional 1,000 gallons	(Food & Water Watch, 2010)
Waste water management fee	0.53	$2.06 (ranging from $1.23 to $3.42 per 1000gallon)	(water, 2011)
KOH	1000	Flake, 88-92% 400-lb. dms., c.l., works	(ICIS, 2008)
$Ca(OH)_2$	70	Chemical pebble (quicklime), hydrated bulk, c.l., f.o.b. works	(ICIS, 2008)
NaOH	400	Caustic soda	(ICIS, 2008)
Viscozyme	15000 / 5000	1000 UFBG g^{-1} product (Equivalent to industrial cellulase)	(Alibaba, 2015; Liu et al., 2015)

To process 5,000 ton GTR within 300 days per year, processing capacity should be higher than 17 ton GTR per day. The processing capacity of the reactor for protein

extraction is about 2 ton GTR per batch, leading to a necessity of 3 reactors with 3 batches per day. The number of reactors required for pectin extraction would be the same as that for protein extraction. In Viscozyme treatment, the V/W is 10 with a processing time of 3 h, which is similar as that of protein extraction with 8 V/W and 4 h. In weak alkaline pectin extraction, although the volume of reactor is much larger when V/W of 60 is used, its processing time is only 0.5 h. It means that within the processing time of protein extraction, 6 batches of pectin extraction can be done. Therefore, in both two integrated processes, processing capacity of pectin extraction and protein extraction are similar.

Table A4 Specifics of utilities and labor cost

Utilities	Price ($)	specific	Ref.
Reactors	113300 piece^{-1}	Volume of 30 m^3 (80000 gallon), jacketed and agitated.	(Matches, 2014)
Solution heater	9200 piece^{-1}	Capacity of 1M BTU hr^{-1} (1055 MJ hr^{-1}). Each reactor will install with one solution heater	(Matches, 2014)
Biomass boiler	5749100 piece^{-1}	100000 Ib hr^{-1}, stoker coal fired, 150 psi Sat.	(Matches, 2014)
Ultra-filtration	0.25 ton^{-1} liquid	Including capital and production cost. Cost based on the volume of solution	(Drouiche et al., 2001)
Press	4 ton^{-1} Solid	Capital and production costs are calculated based on the weight of dried matter with the use of only press utility as shown in the reference.	(Tripathi et al., 1998)
Labor	9000 year^{-1} person^{-1}	8 hour day^{-1}, 270 day year^{-1}. Each person supervised one production line per day.	According to Chinese labor cost

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SUMMARY

With the rapidly growing world population and improving living standards, food demand is increased with a simultaneous desire for less human impact on the environment, such that "Twice the food production at half the ecological footprint" could be the EU goal for 2050. In fact, a boost in food demand is mainly required in developing countries, where the farmlands are limited and/or they are of poor quality. Rather than improving crop-production yield, developing biorefinery technology with unused biomass, such as leaves, in developing countries may be the key to fulfil the food demand.

Four major components, protein, pectin, lignin, and (hemi-) cellulose, account for more than 70% of the materials in leaves in almost all species. Among these components, protein and pectin can be used in food and animal feed, and they are key components for supplementing food production. However, the production and application of leaf products is limited for four reasons: unstable raw materials, complex components, rigid plant cell walls, and underdeveloped leaf logistics and economics. The limitations cause low pectin and protein yields, and low cost-efficiency in current extraction technologies, including mechanical milling, chemical extraction (acid and alkaline), solvent extraction, and ammonia protein extraction. Development of an integrated process for multiple products might be a good option for leaf biorefinery, but the compatibilities of these processes were unknown.

The aim of this study was to develop new processes and applications that optimally utilize all components, particularly protein, of leafy biomass in the feed and or food industry using green tea residues as a starting material. The method should also be applicable to other leafy biomass. The research started from the development of alkaline protein extraction technology as presented in Chapter 2. We found that in alkaline protein extraction, temperature, NaOH amount, and extraction time are the parameters determining protein yield, while pH and volume of extraction liquid are critical parameters for production cost. After optimization, more than 90% of leaf protein could be extracted at a cost of 102 € ton^{-1} protein by single step alkaline extraction. The

extracted protein nutritional value was comparable to soybean meal and this technique can be adapted to various tapes of leafy biomass. Main drawback of this technique is the overuse of alkali, generation of salts, and the destruction of key amino acids, such as lysine, during the extraction. We tried to overcome its drawbacks by developing integrated process with a recycle for chemicals.

Chapter 3, 4, 5, and 6 refer to the integrated biorefinery. For a better design, we investigated how the alkali aided protein extraction (Chapter 3), and proved that alkaline protein extraction was not facilitated by increased solubility or hydrolysis of protein, but positively correlated to leaf tissue disruption. HG pectin, RGII pectin, polyphenols, and organic acids can be extracted before protein. Protein extraction can then be followed by the extraction of cellulose and hemi-cellulose. RGI pectin and lignin yield were both linearly correlated to protein yield, which indicated that they are likely to be the key limitation to leaf protein extraction. Based on the above findings, an integrated biorefinery that combined protein extraction with a pre-treatment was proposed. In Chapter 4, ethanol, viscozyme, and H_2O_2 were selected for pre-treatments targeting on the removal of polyphenols and pigments, carbohydrates, and lignin accordingly. Ethanol and viscozyme could extract their targeting components efficiently while H_2O_2 could bleach GTR with no lignin extracted. The best pre-treatment was the combination of viscozyme and 50% ethanol extraction, which not only reduced the use of alkali by 50%, but also improved protein content and its nutritional value. As pectin can be applied for food or chemicals, enzyme and PBS buffer were investigated for pectin extraction (Chapter 5). Both enzyme and PBS buffer extraction could not only extract high yield HG pectin (predominated by galacturonic acid) with no protein extraction, but also reduced alkali usage in subsequent protein extraction. Pectin obtained using PBS buffer could be present in its native form, which can be precipitated by 40% ethanol. Buffer is suggested to extract pectin when pectin is to be used in food. Otherwise, hydrolyzed pectin that mainly contains galacturonic acid can be converted to other useful chemicals. For this the enzymatic methods, such as using Viscozyme® L, are recommended.

Alkali usage was further optimized. It was found that by using potassium hydroxide, the

protein extraction efficiency was similar to that using sodium hydroxide. The waste water, mainly containing potassium salts, can then be used as fertilizer. This technique is highly depending on the location of factories, which should be built close to the field. Alternatively, calcium hydroxide can be used. As calcium salts can be precipitated by CO_2 and calcium hydroxide can be regenerated through burning of the precipitate, this scheme is sustainable and adaptable to most situations. However, as calcium also precipitated pectin, ploy phenols, and even proteins, the protein yield is relatively low. Although a pre-treatment can improve extraction efficiency of calcium hydroxide, economic results suggested that a pre-treatment is not necessary unless the products obtained by pre-treatment have an attractive market value.

In Chapter 7, we extend our knowledge on leaf biorefinery with some additional experiments and literature. Simplified models of leaf tissues and cell walls were proposed and used to explain the mechanism of alkaline protein extraction. The models were also used to explain other mechanisms for protein extraction; mechanical milling, steam explosion, acid, and enzyme aided extraction. The possible improvements of leaf biorefinery economics were illustrated either by reducing production cost, by e.g. using counter current extraction or ultrafiltration, or by upgrading product value by applying protein and pectin in food. The processes recommended in this thesis show an excellent prospective, in which they are applicable to other leaf biomass and suitable for small-scale production.

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CURRICULUM VITAE

Chen Zhang was born on September 26, 1982, in Quanzhou city, China. There he graduated from Quanzhou No. 5 middle school, after which he continued his bachelor and master in Fuzhou University, Fuzhou City. His master thesis "Anti-irradiation Capacity of Fusion protein PTD-SOD with membrane transduction" was granted as the "Excellent Master Thesis of Fuzhou University in 2007".

Chen started his research career as a researcher in Institute of Biotechnology, Fuzhou University, where he continued to work after his MSc. In the meantime, he was practicing as a manager of Cell Laboratory and leader of student research group. In 2010, Chen decided to start his PhD with his personal finance in Wageningen UR under the supervision of Prof. Dr. Johan P. M. Sanders and Dr. ir. Marieke E. Bruins. Within two years, Chen made great progress in alkaline protein extraction technology. Based on these results, a research grants was obtained from STW, which guaranteed a three-year further finance to his research. Chen returns to China after PhD study, where he can be reached at zhangchenfj@sina.com or 44407227@qq.com.

LIST OF PUBLICATIONS

Articles:

1. Zhang C, Sanders JPM, and Bruins ME, Critical parameters in cost-effective alkaline extraction for high protein yield from leaves. *Biomass Bioenergy*, 67, 466-472 (2014).

2. Zhang C, Sanders JPM, Xiao TT, and Bruins ME, How does alkali aid protein extraction in green tea leaf residue: A basis for integrated biorefinery of leaves. *PLoS One*, 10, 1-14 (2015).

3. Zhang C, Bozileva E, Klis Fvd, Dong Y, Sanders JPM, and Bruins ME, Integration of pectin extraction for galacturonic acid with alkaline protein extraction from green tea leaf residue. *Ind. Crops Prod*. submitted (2016).

4. Zhang C, van Krimpen MM, Sanders JPM, and Bruins ME, Effect of pre-treatment on the efficiency of alkaline protein extraction from green tea residue and on the feed quality of its protein product. *Anim Feed Sci Technol*, submitted (2016).

5. Zhang C, Bruins ME, Yang ZQ, Liu ST, and Rao PF, A new formula to calculate activity of superoxide dismutase in indirect assays, *Anal. Biochem.* inpress (2016).

Patent:

EU Application No. 12185420.2. PCT/NL2013/050676. Subject：A process for isolating proteins from solid protein –containing biomass selected from vegetable biomass, algae, seaweed and combinations thereof.

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline-specific courses & conferences

- Food & Biorefinery enzymology, VLAG, Wageningen, the Netherlands, 2011;
- Biorefinery productions: Renewable resources for the bulk chemical industry, WUR/ORC-90306, Wageningen, the Netherlands, 2011;
- Biorefinery for biomolecules, VLAG, Wageningen, the Netherlands, 2012;
- Sustainability analysis in food and bio-based production, VLAG, Wageningen, the Netherlands, 2013;
- Biorefinery for Food, Fuel and Materials, VLAG, Wageningen, the Netherlands, 2013;
- 15th European Congress on Biotechnology, Istanbul, Turkey, 2013;
- Industrial Food Proteins VLAG, Wageningen, the Netherlands, 2013;
- The 8th Tea Science Symposium Across the Taiwan Straits, Taiwan, China,2014
- NPS14 conference 'Fundamentally Innovative', Utrecht, the Netherlands, 2014;
- Advanced Chemistry Course: Scattering techniques, VLAG, Wageningen, the Netherlands, 2014;
- Advanced Chemistry Graduate Course, VLAG, Wageningen, the Netherlands, 2015.

General courses

- PhD competence assessment, WGS, Wageningen, the Netherlands, 2010;
- Academic writing II, WGS, Wageningen, the Netherlands, 2011;
- Information literacy PhD including Endnote introduction, Library, Wageningen, the Netherlands, 2011;
- Scientific writing, WGS, Wageningen, the Netherlands, 2011;
- Writing Grant Proposals, WGS, Wageningen, the Netherlands, 2011;
- VLAG PhD week, VLAG, Baarlo, Wageningen, the Netherlands, 2011;
- Project and time management, WGS, Wageningen, the Netherlands, 2013;
- Mobilising your scientific network, WGS, Wageningen, the Netherlands, 2013;
- Techniques for writing and presenting a scientific paper, WGS, Wageningen, the Netherlands, 2014;
- WGS PhD Workshop Carousel, WGS, Wageningen, the Netherlands, 2014.

Optional activities

- PhD study trip, BCT, Brazil, 2013
- PhD study trip, BCT, China, 2015

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