Specific conversion of amino acids as a means for their separation

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Specific conversion of amino acids as a means for their separation

Yinglai Teng

Thesis

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Table of Contents

List of abbreviations

Amino acids

AA	Amino acid
Ala	L-Alanine
Arg	L-Arginine
Arg·HCl	L-Arginine monohydrochloride
Arg·2HCl	L-Arginine dihydrochloride
Asn	L-Asparagine
Asp	L-Aspartic acid
Cys	L-Cysteine
GABA	γ-Aminobutyric acid
Gln	L-Glutamine
Glu	L-Glutamic acid
Gly	L-Glycine
His	L-Histidine
Ile	L-Isoleucine
Leu	L-Leucine
Lys	L-Lysine
Lys·HCl	L-Lysine monohydrochloride
Lys·2HCl	L-Lysine dihydrochloride
Met	L-Methionine
pGlu	L-Pyroglutamic acid
Phe	L-Phenylalanine
Pro	L-Proline
Ser	L-Serine
Thr	L-Threonine
Trp	L-Tryptophan
Tyr	L-Tyrosine

Val	L-Valine

Chemicals and materials

BDA	1,4-Butanediamine
DDGS	Dried distillers grains and solubles
FUM	Fumaric acid
MAE	Maleic acid
PDA	1,5-Pentanediamine
PLP	Pyridoxal 5'-phosphate
PLP·H_2O	Pyridoxal 5'-phosphate monohydrate

Enzymes

ADC	L-Aspartate α-decarboxylase
GAD	L-Glutamate α-decarboxylase
IGAD	Immobilized GAD (by entrapment in calcium alginate beads)
ILDC	Immobilized L-lysine decarboxylase (by entrapment in calcium alginate beads)
LDC	L-Lysine decarboxylase
ODC	L-Ornithine decarboxylase
PAL	L-Phenylalanine ammonia-lyase
PDC	L-Phenylalanine decarboxylase
SDC	L-Serine decarboxylase
TDC	L-Tyrosine decarboxylase
VDC	L-Valine decarboxylase

Others terms

AEM	Anion exchange membranes
BtU	British thermal unit
CEM	Cation exchange membranes

ED	Electrodialysis
EIA	The U.S. Energy Information Administration
E stream	Electrode stream
EU	European Union
GHG	Greenhouse gases
IEA	International Energy Agency
IFPRI	International Food Policy Research Institute
IP	Isoelectric point
ISPR	*in-situ* product formation and removal
MMM	Mixed matrix membrane
OECD	Organization for Economic Co-operation and Development
SMB	Simulated moving bed chromatography
U-HPLC	Ultra high pressure liquid chromatography
EDAA	Electrodialysis separation of amino acids

Chapter 1

General Introduction

1.1 Introduction

The current dependency of our society for chemicals and fuels on fossil based resources (oil, gas, coal) causes great uncertainties for the future. The fossil sources are diminishing, unequally distributed, prices are fluctuating – but overall increasing, concern about their (negative) environmental impacts and geopolitical issues have arisen. This required development of replacements for fossil feedstocks to achieve more carbon neutral and sustainable production approaches.[1] Biomass and thus biorefinery technology is one of the potential solutions to secure future supply of chemicals and fuels and is therefore receiving more and more attention.[2] This chapter will provide background information about replacing fossil fuels with biomass, the concept of biorefinery, the application of biomass in the production of chemicals, and the possibility of using amino acids (AAs) as a feedstock for chemical industry.

1.2 Drawbacks of fossil fuels

Currently, fossil fuels gas, coal and especially oil (petroleum) are dominantly used to meet our societies need for energy carriers (fuels) and chemicals as feedstock for making materials.[3] For example, according to BP,[4] in 2010 the production of oil, coal and gas in 2010 was approximately 3900, 3700 and 2900 million tons of oil equivalent respectively. However, the use of fossil resources has many drawbacks as briefly addressed in the previous paragraph. Here I will focus in some detail on 1) depletion of resource, 2) insecure supply and 3) CO_2 emission.

1.2.1 Depletion of limited reserves

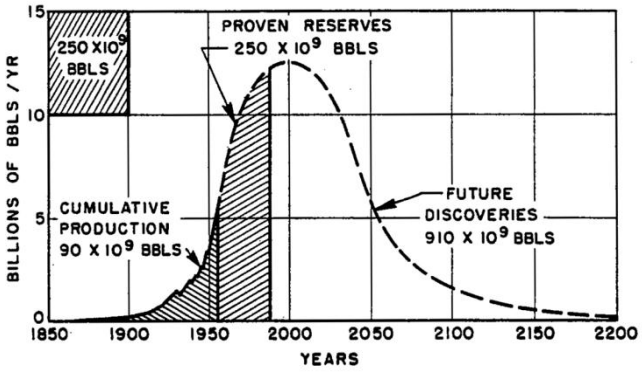
Fossil fuels are formed from fossilized plants and animals over millions of years.[5] Due to that long time scale fossil fuels are regarded as non-sustainable. Therefore their use will lead to an inevitable depletion. The peak oil theory developed by M. K. Hubbert is often used to discuss the timeframe of depletion.[5,6] Hubbert showed that the extraction rate of a finite resources will first exponentially increase and after passing through a maximum (Hubbert peak) decrease with time (Fig. 1.1A).[3,6] As a result after passing the maximum reserves would run out if the need is not

decreased or the supply increased.[5] As predicted by Hubbert, the peak oil of U.S. would be reached around 1970 as compared to 2000 for the world production.[5] The US prediction has been proven, and nowadays the peak oil theory is the most widely useful model for the oil depletion.[7]

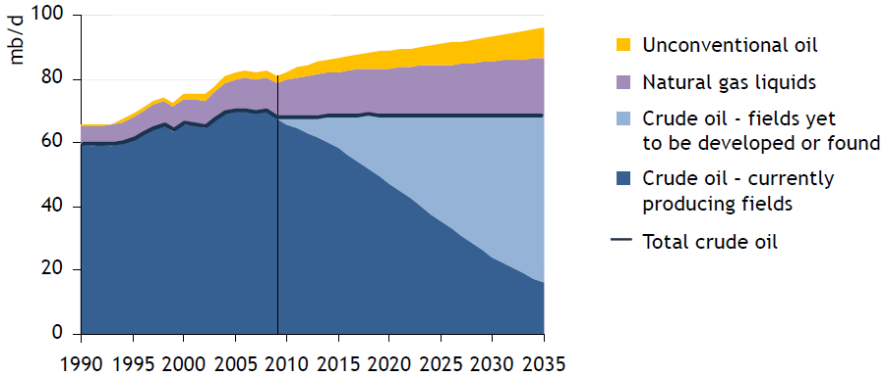
Hubbert estimated that the peak of world oil production would occur around 2000 (Fig. 1.1 A).[5] However, according to the International Energy Agency (IEA), the peak of world oil production was delayed to 2006.[8] This could be due to several reasons related to both changes in production and consumption rates which will be discussed now.

Firstly, more oil fields have been found in the recent years thus increasing the reserves. For example, the proven oil reserves in 1990, 2000 and 2010 were about 1000, 1100 and 1400 thousand million barrels respectively,[4] while oil production in those years was 24, 28 and 31 thousand million barrels respectively.[9] Secondly, more advanced technologies were used in the oil extraction process thus increasing the production. For example, after 2010 deep water drilling gained more attention to meet the oil demand.[10] Finally, more alternatives for oil have been developed thus decreasing the oil use. These include unconventional oil (*e.g.* oil shale, extra heavy oil and bitumen[11]) and hydrocarbons produced from natural gas (*e.g.* via Fischer Tropsch synthesis) the so called natural gas liquids.[8] The latter is estimated to reach a globe output of 11 million barrels in 2030.[10]

Recently, the extraction of shale gas in U.S. resulted in an oversupply of natural gas, leading to a decline of the U.S. gas prices and related oil prices (shale gas revolution).[12] The production of shale gas is considered to delay the depletion of oil and is therefore in competition with other options to decrease fossil resource use *e.g.*, the use of biomass.[12,13] Based on these developments, IEA have estimated that from 2009 to 2035, the world total oil production could even increase at a slow rate (Fig. 1.1 B).[8]



A



B

Fig. 1.1 A. Prediction of world crude-oil production originally in Hubbert's paper.[5] **B.** World oil production by type as presented by IEA in the World Energy Outlook 2010 – presentation to the press.[8]

The depletion of oil is also related to the demand of energy *i.e.* the rate of consumption of fossil sources. The U.S. Energy Information Administration (EIA) estimated that from 2008 to 2035 the world energy consumption would increase by 1.6% per year, and oil based liquid fuels would remain the largest product group in that respect but the oil share is expected to slightly reduce due to the possible increase in oil price.[14] The expected changes in energy use vary geographically. The energy consumption of non-OECD (Organization for Economic Co-operation and Development) countries already exceed that of OECD countries in 2008 (*ca.* 260 *vs.* 240 quadrillion Btu, or *ca.* 270 *vs.* 250 exajoule), and the difference is expected to further

increase.[14] The projected energy consumption of non-OECD countries in Asia, especially China and India, will be the largest contributors due to the expected growth of population and economy.[14] This is a trend also predicted by many other international organizations and energy companies, such as IEA,[8,15] ExxonMobil,[10] BP,[16] and Shell.[17]

Though exact timescales are difficult to establish, at which rate fossil source will deplete, as discussed above, it is clear that the reserves will diminish with time. Therefore, to meet the need of energy and materials in the future, it is necessary to shift from fossil fuel to other resources.

1.2.2 Insecure supply

Industrial investments and thus advancements are based on expected return of investment.[18] Costs of feedstock can be an important issue in that respect. To make sound business models to justify an investment the price of the feedstocks has to be predicted with certain accuracy. However for fossil based feedstocks, and especially for oil, this is challenging if not impossible.

Oil prices have fluctuated heavily as a result of its insecure supply.[19] Fig. 1.2 displays the world crude oil price in recent decades. Overall the price is increasing but with significant fluctuations, especially after year 2000.[20] The figure clearly shows that major global events are directly followed by a response in the oil price. A recent example is the war in Libya which led to a peak in the oil price in 2011.[21]

In addition to regional events, also the financial crisis adds to the fluctuations of oil price. Typically, such crisis reduces the demand of energy and fuel in a short period and hence leads to temporary drop in the crude oil price.[22] For instance, throughout 2008 the oil price dropped steeply from nearly 140 $/barrel to below 40 $/barrel. It is believed that the main reason for this decrease is the recession of the U.S. economy which cut down the consumption of oil.[23]

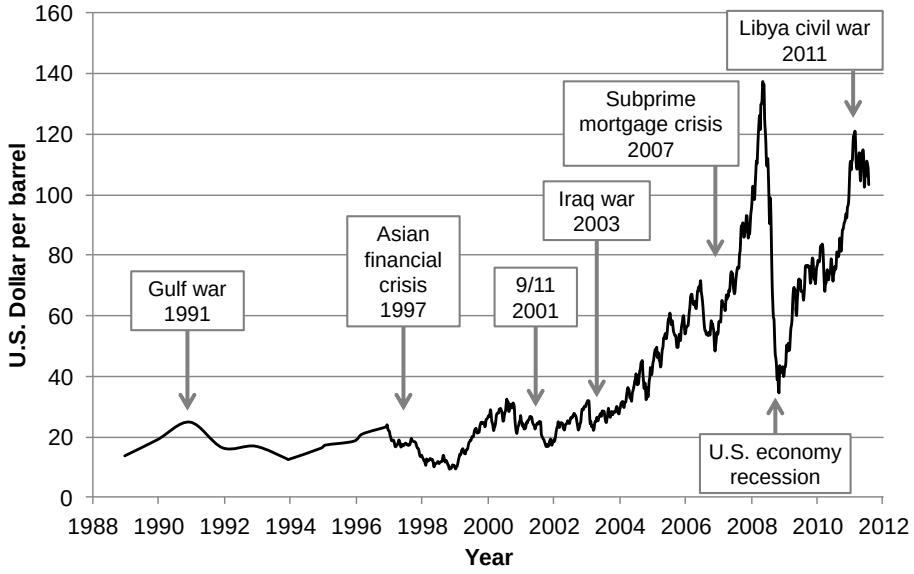


Fig. 1.2 World crude oil price from 1989 to 2011 according to the data from EIA,[20] and some related international events during this period.[24]

1.2.3 CO_2 emission

The use of fossil fuels has many negative side effects such as air pollution, oil spillage and global warming.[26,27] Global warming is now generally considered to be related to the emission of greenhouse gases (GHG), such as CH_4, N_2O, O_3, water vapor and CO_2.[26,28] As a result of globe warming climate changes and increasing sea levels are claimed.[27] Nevertheless some doubt still exists about the link between GHG emission, global warming and the negative environmental impacts.[29,30]

Combustion of fossil fuels is considered to be the main source of the anthropogenic CO_2 emission.[14] Fig. 1.3 shows the share of the three fossil sources (oil, gas and coal) as function of time in the world wide CO_2 emissions. Clearly in the period 1971 to 2009 oil is the predominant source of the CO_2.

More recently some changes took place in the origin of the CO_2 emissions.[25] In 2009 the CO_2 emissions decreased slightly. The share from coal increased as developing countries such as China and India used more coal to meet their growing need of energy.[25] This increase as the result of coal use is however counteracted by an increase in shale gas use in the U.S. as

replacement for oil which overall results in slightly lower CO_2 emissions worldwide.[12] Overall trends and predictions, however, indicate a still increasing trend in CO_2 emissions with coal and oil being the major contributors.[16,25]

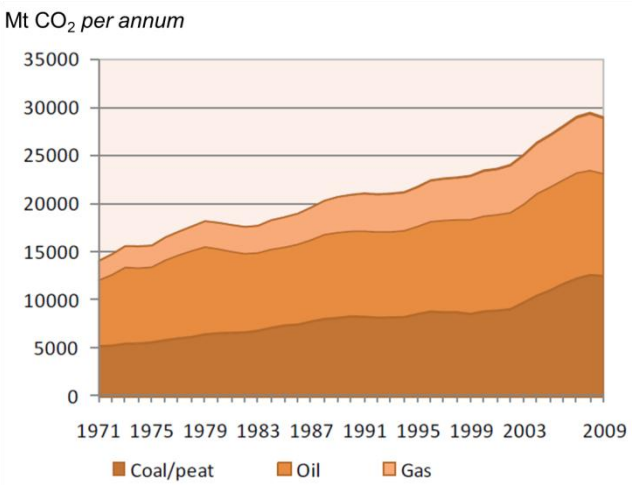


Fig. 1.3 Annual global CO_2 emissions (in million tons, Mt) by combustion of fossil fuels from 1971 to 2009 as published by IEA.[25]

Many countries have signed the Kyoto Protocol that entered into force in 2005 to reduce the CO_2 production the GHG emissions.[28] Carbon tax, an environmental tax on the emission of CO_2, was introduced by governments to slow down the process of global warming by CO_2 emissions. A recent example of legislation in that respect is the carbon tax rules implemented by the European Union on airlines.[32] Although the effect of carbon tax is debated,[33] the trend is that more countries will adopt it. Therefore ways for industry to reduce CO_2 emissions are of eminent importance.

1.3 Biomass and biorefinery

1.3.1 Biomass as an alternative to fossil fuels

After having discussed the disadvantages and challenges related to fossil resources I will now turn to review the same issue, as discussed above, for one of the promising alternatives *i.e.*

biomass. According to the EIA, biomass is an organic non-fossil material of biological origin mostly originating from plants.[34] Historically, biomass has been widely used for food, heat and as a construction material. Currently, due to the drawbacks of fossil fuels, biomass is used to (partly) replace fossil fuels for energy purposes and as starting material for materials and chemicals.[1]

Energy security. Biomass is a renewable resource and therefore cannot be depleted with time.[1] However, the amount of land on our planet is limited and our energy demand enormous. For example, currently in the world about 87 million barrels (14 million m^3) of oil are produced per day and also consumed, with about 68 million barrels (11 million m^3) used for energy.[9] When biomass is used for fuel only a small part of the energy required can be satisfied (*ca.* 1.9 million barrels or 300 thousand m^3 of biofuels per day).[9]

Though biomass can only satisfy part of our energy needs, it has one large advantage over fossil sources, *i.e.* it is available at many places on earth, in contrast to coal, gas and oil, thus decreasing our dependency on a limited number of locations.[35] For industrial countries that are dependent on oil imports, such as European Union (EU) countries and the U.S.,[36] this is very interesting due to the benefits in reducing the impact of price fluctuation and ensuring energy security.[19]

Reduction of GHG emission. Biomass is considered to be close to carbon neutral. The CO_2 produced and released to the atmosphere by the use of biomass originated from the atmosphere as well to grow the biomass, so the net amount of carbon in the atmosphere is not changing.[37] Although in the energy sector, many alternatives for oil have been developed such as nuclear, wind, solar and hydro, biomass based energy carriers result in a much lower GHG emission per unit of energy.[38] Moreover, of these alternatives only biomass produces a liquid fuel which is easy to store and transport.[35] As estimated by the European Union, by 2030 up to one quarter of its transportation fuels could be replaced by biofuels.[39]

Though biomass based fuels are more beneficial from a GHG emission point of view, some discussion exists on the exact beneficial effect of the use of biomass.[40] An important basis for this discussion is that the production of the biomass is energy intensive which results in the need of additional fuel and thus CO_2 emissions. Considering the fossil energy input to obtain bioethanol from corn (cultivation, processing and distillation *etc.*), the use of corn bioethanol in the U.S.

only provides a 12% reduction in the emission of GHG.[42] This indicates that bioethanol prepared from corn starch is not a good replacement of oil.

Development of rural economy. As stated above, one of the advantages of biomass is the wide availability across the globe. This allows for local conversion of the biomass which can help to develop the economy in rural areas.

Biobased processes often use bacteria, fungi and enzymes which require low heat transfer, and reduce the capital investment required to do this. Thus the process can be operated at a smaller-scale. Since small-scale processes can have simpler and less expensive process technologies, the entry barrier to run those processes will be low enough to benefit rural areas with little infrastructure, allowing the biomass to be processed in the production area.[43] Also, biomass production is related to agriculture and forestry which is usually carried out in rural areas. Therefore rural economies can benefit from both the production and processing of biomass. For example, in Brazil bioethanol production from cane sugar in the poorest regions provided employment to the poor and deprived to gain a basic income.[19] In other countries, the biomass raw materials can be different depending on the climate and land conditions.[44-46]

Reliable carbon source. Currently, the main application of biomass is in the energy sector, but for the chemical industry, biomass can be an attractive feedstock as it is the only reliable carbon source besides fossil fuels.[24]. The use of biomass instead of fossil fuels provides the opportunities for chemical industry to reduce the reliance of fossil fuels and avoid the impact caused by the fluctuation of oil price. Thus, biomass is becoming more and more important for chemical industry.[1]

1.3.2 Worries on the competition with food

As described above, replacing fossil fuels with biomass is the current trend in the energy, materials and chemical sectors.[1-3] This leads to concerns on the risk of using too much farmland and crops to produce biofuels instead of using them for food.[47-49] This debate is international and is known as the "food *vs.* fuel" discussion.[48] This discussion is only relevant as long as the edible part of biomass is used as the raw material.[44,48] As a result, China has forbidden the use of grains

such as rice, corn and wheat to produce bioethanol,[50] and India banned the use of edible oil seeds to produce biodiesel.[51]

To answer if the production of biofuels competes with the need of food, it is necessary to know the causes of the hunger problem. As concluded by International Food Policy Research Institute (IFPRI), from 2001 to 2011 global hunger has actually declined.[52] Consider the fact that during the same period of time the global production of biofuels was more than doubled,[9] it can be seen that at present the production of biofuels was not directly linked with the hunger problem. Nevertheless the increases in food prices are considered to be related to the higher cost of biofuels however no agreement is currently achieved on the exact nature of this relation.[53]

According to the EU, the present world biofuel production only occupies less than 3% of the global cropland, and in EU only *ca.* 2% of agricultural land is used for biofuels, so the impact of biofuel production on the global production of food is limited.[54] According to IFPRI, the increase and volatility of food prices include climate related reasons which led to poor harvests, and increased volume of trading in commodity futures markets.[52] In recent years, developing countries such as India and China are consuming more animal products hence the demand of animal feed is also growing, which also contribute to a price increase.[55] The improvement of the diet in those countries is also considered to push up food prices.[55] Based on these facts, it can be concluded that the present production of biofuels should not be blamed for the food shortage.

To overcome the food *vs.* fuel discussion two options are available *i.e.* more efficient use of the available land and (or) make use of the non-edible part of the biomass. When the area productivity of food can be increased, then there will be more food available which leaves more space for non-food applications. In a land which lacks water and nutrition conditions to grow traditional food crops (*e.g.* wheat, corn and rice), it can be useful to grow plants that can be used for non-food applications for examples jatropha.[45,46] Alternatively, microalgae can be grown for biofuels.[56] Microalgae can be grown in industrial, municipal, agricultural wastewater and seawater. This overcomes the drawbacks of competing with the need of water for drinking and food but also provides a sort of water treatment.[57]

A better solution to avoid competing with food is use the non-food (part) of biomass. These biomass feedstocks can be the side products from food production, such as waste cooking oil

which can be used to prepare biodiesel.[58] In addition second generation biofuels can be produced fro, lignocellulosic biomass which does not conflict with the need of food.[59] Thus, second generation biofuels could be the trend for the future biofuel production.

When non-edible parts of biomass are used for production of fuels and chemicals they need to be separated from the edible parts. This asks for a biorefinery approach which will be discussed in the next section.[2]

1.3.3 Biorefinery

Biomass, also the non-edible part, usually has complex components with different potential applications. These components include proteins, lipids, lignin and carbohydrates (cellulose, starch and sugars). If these components can be separated efficiently, value can be created from these parts of the biomass. This separation process is called biorefinery and converts biomass to a range of marketable products and energy.[19] Biorefinery is often compared with petroleum refinery, but the raw material to be refined is biomass not oil and gas. The concepts are different (Fig. 1.4). In petroleum refinery, fossil resources are cracked into low molecular weight hydrocarbon building blocks at high temperatures. These building blocks are then functionalized by reacting with extra chemicals (*e.g.* ammonia), forming the final product.[1] Such a route is very energy intensive. In the biorefinery approach, many functional groups that are needed by petrochemicals (*e.g.* O- N- and S-containing groups) can be found in the biomass fractions. These groups can be preserved when converted to chemical products. Thus, biorefinery and conversion to products can be performed at much lower reaction temperature using techniques such as fermentation and enzymatic conversions. This provides opportunities to save process energy and extra chemicals required to functionalize the base petrochemicals.[2]

A typical example of a biorefinery process is the processing of sugar cane. First the sugar cane is crushed to yield a juice which contains the sugar. The remaining product is called bagasse which contains mainly cellulose and hemicellulose. The sugar in the juice can be used for food applications and the latter can be used for the second generation bioethanol production.[60]

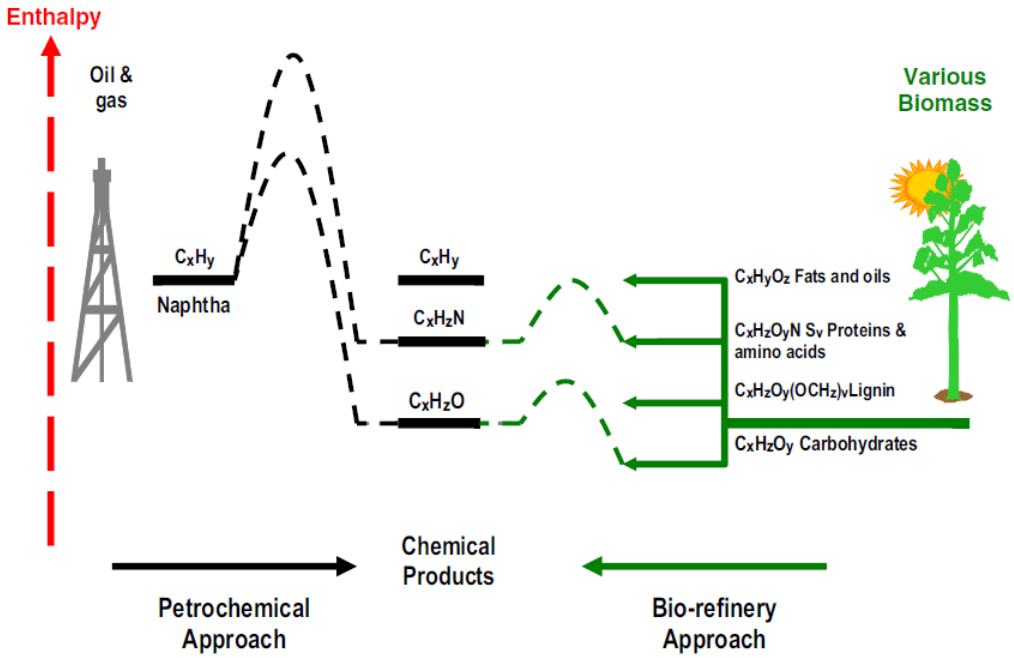


Fig. 1.4 The concept of biorefinery in comparison of petroleum refinery in case of producing chemical products. The figure was originally published in Macromolecular Bioscience.[2]

As mentioned in the previous section, biomass can be an attractive feedstock for the chemical industry as it is the only reliable carbon source besides fossil resources.[24] Next to that the molecules present in the biomass contain a significant number of functional groups. For example, amino acids (AAs) that can be derived from proteins have amine and carboxyl groups, carbohydrates contain hydroxyl group, and lignin contain aromatic groups.

This introduces the advantage, compared to the use of fossil sources which mainly contain C-C and C-H bonds, that the functionalities are intrinsically present thus potentially omitting a number of conversion steps. In many cases the starting material may be converted to a product under ambient conditions *e.g.* the decarboxylation of amino acids to amines. This avoids the use of harsh, energy intensive processes and extra reagents (*e.g.* use of high temperatures and ammonia) currently used to prepare amines from petrochemical routes. As well as this, the calorific value of both the biobased starting material and product are similar (Fig. 1.4).[2] Therefore

the use and dissipation of large amounts of energy may be limited. In the case of the decarboxylation of an amino acid, the driving force for the reaction is the elimination of CO_2. Therefor the entropy will increase and this will lead to a $-\Delta G$. Therefore some energy loss occurs. However, this is much lower compared to the energy losses eschewed in petrochemical processes.

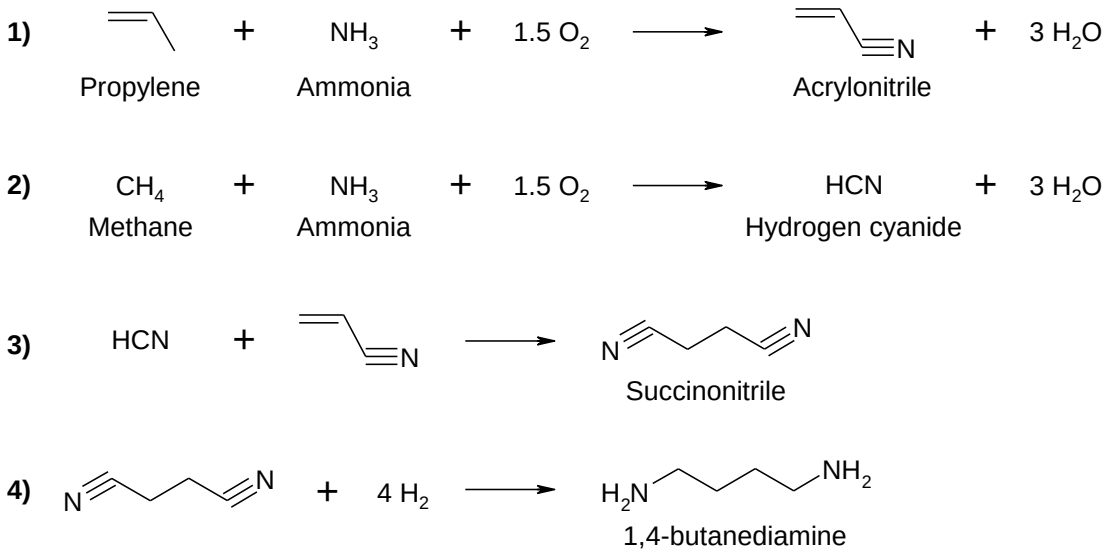
Due to the possibility to eliminate processes and reagents as well as reduce energy use, opportunities to produce current chemical products under less intensive capital conditions may arise.

1.4 AAs as a feedstock for chemical production – an example

When producing nitrogen containing chemicals, AAs from biomass proteins could be considered due to their functionality and large availabilities.[1]

Fig. 1.5 compares the petrochemical and biobased routes to prepare 1,4-butanediamine (BDA). It has been reported that BDA can be used to prepare nylon-4,6.[1] In the petrochemical route a number of steps that requires the use of hydrocarbons such as methane and propylene are required as well as other reagents such as ammonia and involves the production of acrylonitrile and hydrogen cyanide. The production of both acrylonitrile and hydrogen cyanide is energy intensive and the products that are formed are highly toxic.[64,65] For example hydrogen cyanide is produced at temperatures in excess of 1000^{o}C. In the biobased route, BDA is prepared from arginine *via* ornithine.[66,67] Here the production of (and use of) hazardous materials under harsh conditions can be avoided. This represents tremendous savings both in terms of fossil feedstock and energy use, but also the elimination of extra requirements which surround the use of hazardous materials.[68]

Petrochemical Route



Biobased Route

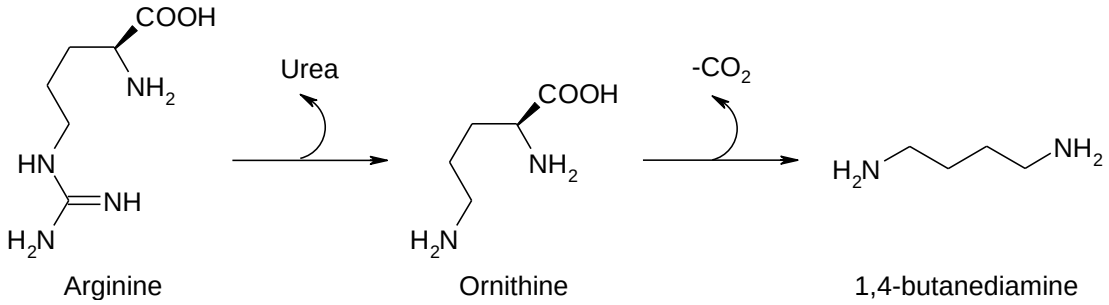
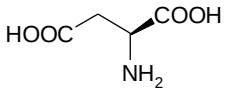
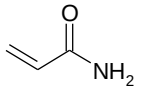
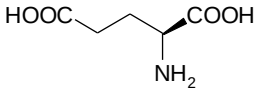
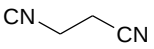
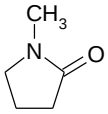
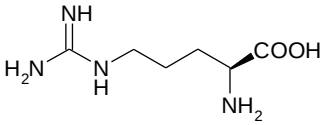
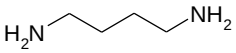
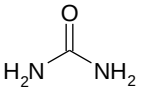
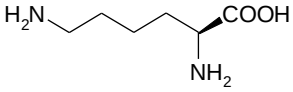
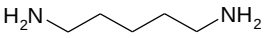
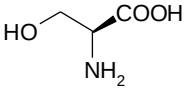
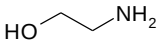
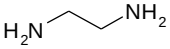
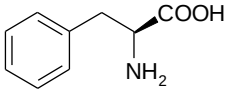
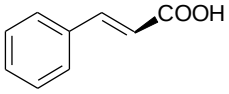
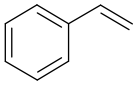
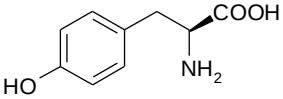
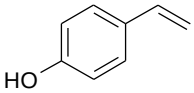
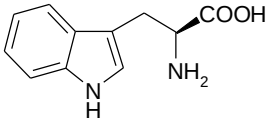
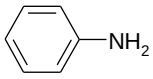


Fig. 1.5 Comparison of petrochemical and biobased route to BDA.[1]

1.4.1 Functionality of AAs

As previously mentioned, preparing chemicals from AAs the functionalities provide the advantage of saving energy and extra chemicals. The bulk chemicals that can be synthesized from amino acids are listed in Table 1.1.

Table 1.1 Bulk chemicals that can be produced from AAs.

Amino Acids (Abbr.)	Bulk chemicals can be produced[1]	
 L-Aspartic acid (Asp)	 Acrylonitrile[69]	 Acrylamide[69]
 L-Glutamic acid (Glu)	 Succinonitrile[70]	 NMP[71]
 L-Arginine (Arg)	 1,4-butanediamine[66,67]	 Urea[66]
 L-Lysine (Lys)	 1,5-pentanediamine[72]	
 L-Serine (Ser)	 Ethanolamine[73]	 Ethylenediamine[1]
 L-Phenylalanine (Phe)	 *trans*-Cinnamic acid[74]	 Styrene[75]
 L-Tyrosine (Tyr)	 4-Vinylphenol[76,77]	
 L-Tryptophan (Try)	 Aniline[78]	

The chemicals that can be prepared from an AA depend on the functionalities in the AA. These functional groups include carboxyl, hydroxyl, amine and phenyl groups.[1] AAs are the building blocks of proteins. As the name implies, each AA has a primary amine group and a carboxyl group on α carbon. The primary amine group makes AAs interesting feedstocks for the production of nitrogen containing chemicals. However, other functional groups in the side chain are also important. For example, acidic AAs (Asp and Glu) have a carboxyl group; basic AAs (Arg and Lys) have a primary amine group; aromatic AAs (Phe, Tyr and Trp) have a phenyl group, and Ser and Thr have a hydroxyl group. All these functionalities provide the possibilities to convert AAs into the corresponding chemical products.

It can be seen from Table 1.1 that the carboxyl group usually is not present in the final chemical products; phenyl and primary amine groups are the mostly preserved. This suggests that decarboxylation of AAs plays an important role in the conversion of AAs to bulk chemicals.

Also, the carboxyl group has a certain charge depending on the pH. Decarboxylation of an AA modifies charge behavior and may lead to the separation of the product by electrodialysis (ED).[72] This will be explained further in section 1.6.

1.4.2 Availability of biomass derived AAs

As biomass derived AAs can be used as a feedstock for producing bulk chemicals, it is important to investigate their availability with respect to it expected use. A small amount of free form AAs can be found in some biomass sources. For instance, Protamylasse™ is a waste stream from the potato starch processing industry that contains large amounts of AAs, with an annual production at about 100,000 tons).[1] Proteins are widely present in nature.[79] For example, proteins can be obtained from fresh cassava leaves, with a potential worldwide availability to be about 16 million tons per year.[79] Since proteins can be relatively easy to be hydrolyzed to AAs, proteins are the preferred source for AA.[1] Preferably, these proteins are obtained from residual streams of agro-food processes so as to minimize cost and avoid issues relating to competition with feed/food.

In general, there are three major sources of residual proteins: 1) by-product from biofuel production, 2) agricultural waste streams, and 3) food waste streams. Biofuel by-products that

contain protein include dried distillers grains and solubles (DDGS) (*ca.* 30% protein by dry weight) from bioethanol production and oilseed cake (*ca.* 40% protein by dry weight) from biodiesel production.[79] When a volume of 10% of the transportation fuels is replaced by biofuels as is projected for 2020, every year 100 million tons of protein will become available as a by-product. Thus approximately 5 million tons of each AA will become available annually.[1] Some deviation from this value occurs as some amino acids are more abundant in the protein (*e.g.* Glu).[79] As a comparison, the annual market for Lys is less than 1 million tons.[80] If the Lys derived from the by-products of biofuels production is used to satisfy market demand, there is still a large amount of Lys remaining for other uses. Similar conclusions can also be made for other AAs. Thus it can be safely summarized that the use of residual protein from biofuel production will not compete with the need of food or animal feed. In addition proteins are also available from agricultural waste streams, such as crop leaves. On a dry weight basis, grasses which are representative of herbaceous biomass contain around 10% protein on average.[81] Switchgrass (*Panicum vergatum*) is such a forage crop in North American. It is particularly interesting because it is potentially a major player to provide the cellulosic feedstock for bioethanol production.[82] An analysis indicated that the U.S. agricultural lands could produce about 53 million tons of dry switchgrass every year.[83] Assuming that 10% of it can be proteins, this amount of switchgrass would potentially provide about 5 million tons of proteins. Switchgrass also brings benefits by giving high yield, reducing soil erosion, requiring less irrigation water, fertilizer and pesticide compared with many other energy crops (*e.g.* corn).[84]

Other potential sources of proteins from agricultural rest streams are animal slaughter wastes, such as feather, blood, meat and bone meal.[85] For example, globally the poultry feather meal available could provide about 21 million tons of protein each year.[79] Waste streams generated from the food industry are also possible AA sources. For example, brewer's spent grain is the major byproduct from the beer brewing industry and contains >20% of protein on dry weight basis.[86] In Brazil alone, about 1.7 million tons of wet spent grain (*ca.* 340 thousand tons of dry spent grain) are produced every year.[87]

After these proteins sources are obtained, proteins need to be extracted in a proper way. Typically, this is achieved by extraction using an acid or base and ethanol, depending on the

protein sources and AA composition.[88] The following step is hydrolysis which converts proteins into a mixture of AAs. Traditionally, protein hydrolysis was carried out under extreme acidic (*e.g.* 6 M of HCl) or alkaline pH (*e.g.* 4.2 M of NaOH) for a considerable amount of time (1-70 h) at high temperature (*e.g.* 110-165 °C) to obtain complete hydrolysis.[89] However, the stability of AAs varies as function of the hydrolysis method. In acid hydrolysis, asparagine and glutamine are converted into Asp and Glu; Ser, threonine and Tyr are partially decomposed; cysteine and methionine are partially oxidized into cysteic acid and methionine sulfoxide or methionine sulfone and tryptophan are completely decomposed.[89] In alkaline hydrolysis tryptophan is preserved, but Ser, threonine, Arg and cysteine are destroyed and the others are racemized.[90] Racemization of AAs is a problem when further conversion of the AA to a chemical product by enzymes is desired. Enzymatic conversions generally convert only substrates with an L-configuration, thus racemization would render half of the AA unconverted.[69,72]

Recently, enzymatic hydrolysis of proteins was also carried out by using combination of proteases.[91] Compared with chemical hydrolysis, it employs milder reaction conditions (*e.g.* at 50 °C) preserves AAs that are otherwise destroyed, and does not induce racemization during digestion.[90] These advantages make this a promising method in the future.

1.5 Separation of AAs

When used to produce chemicals, AAs are converted into different products, and at present most of these conversions reported start with a single AA.[69-71] However, protein hydrolysis derives a mixture of AAs, thus separation is essential. This can be achieved by different methods of which the most important *i.e.*, pH-depending crystallization, chromatography (ion exchange and size exclusion), reactive extraction, and ED will be shortly discussed now.

1.5.1 pH-dependent crystallization

The solubility of an AA is pH-dependent and reaches a minimum when the pH of the solution is close to the isoelectric point (IP) of the AA.[92,93] Thus it is possible to separate some of the AAs by their different solubility at a given pH. An early example of this method is the separation of

Glu from hydrolyzed wheat gluten which was used for the first industrial production of monosodium glutamic acid in 1908.[94] In this method, wheat gluten was used as the protein source for its high total content of glutamate after acid hydrolysis. The solution of the AAs mixture obtained was concentrated followed by a crystallization of glutamic acid hydrochloride below pH 0.45. Glu was the only AA salt in the hydrolysate.[94] A second crystallization at pH 3.2 resulted in the desired purified Glu crystals.

Crystallization of AAs as a purification method does not need complex equipment. In addition it only needs simple acids and bases to tune the pH. However, a limitation is that it is only applicable for some combination of AAs, as many AAs have very similar IP and solubility. For example the case described above for Glu was only successful because the amount of Asp (IP = 2.8) was almost an order of magnitude lower (5% Asp, 35% Glu of all AA).[79] If the amount of Asp and Glu are comparable, other means of separation have to be used.[95] Also, the growth of crystals can take up to a week which limits the production rate and increases the capital cost.[94]

1.5.2 Reactive extraction

Reactive extraction is an extraction method which uses the reaction between an extractant and a solute to extract the latter. The reaction between the solute to be extracted and the extractant can take place on the interface of the solute phase (usually organic phase) and extractant phase (usually aqueous phase) (Fig. 1.6 A), or it happens in the extractant phase when the extracted solute diffuses into the extractant phase (Fig. 1.6 B). In both cases the reaction product is limited in the extractant phase.[96] Blaga *et al.* reported a method to separate AAs mixture from fermentation or protein hydrolysis.[97] In this method, the extractant, di-(2-ethylhexyl) phosphoric acid, only reacts with the cationic forms of AAs. Thus by tuning the pH, AAs are separated into acidic, basic and neutral AAs. Verkuijl reported the chiral separation of D- and L-tryptophan using reactive extraction.[98] The stereoselectivity was achieved as the chiral extractant used only binds to D-tryptophan.

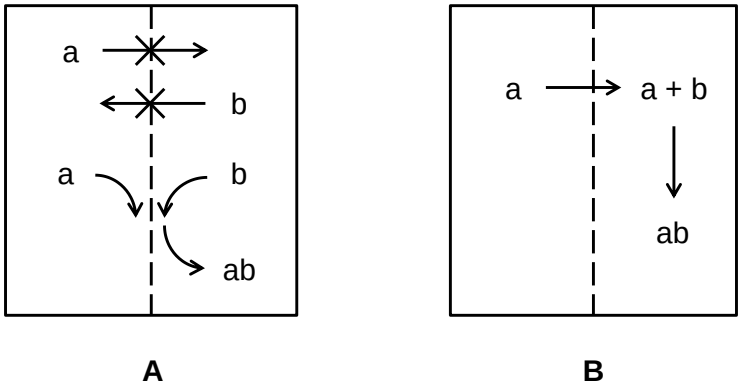


Fig. 1.6 The principle of reactive extraction when **A)** the reaction happens on the interface; **B)** the reaction happens in the extractant phase. *a*: the compound to be extracted; *b*. the extractant. The original figure was published by Steensma *et al*.[96]

From the principle of reactive extraction, it can be concluded that the AA extracted depends crucially on the nature of the extractant. Thus, by a carefully choice of the extractant all AAs can be separated. However, the cost of the extractant and the reagent needed to recover the product should not be ignored.

1.5.3 Chromatography

Ion-exchange and size exclusion chromatography are two methods that can be used to separate AAs. The former is suitable for both analytical purpose and industrial purification of AAs.[99] In this method, the mixture of AA passes through a chromatography column, allowing different compounds to bind to the charged stationary phase according to different ionic interaction. In purifying AAs produced from fermentation, this method is often applied.[100] It is also applicable to separate AAs from biomass. For example, Das *et al.* reported a method to produce Glu from palm waste hydrolysate. A cation-exchange resin was used to purify Glu.[101] To a lesser extent, size exclusion chromatography is also applied to fractionate AAs. In this method, the stationary phase, usually a neutral polymeric resin, traps small molecules to a larger extent then large molecules, therefore molecules with large sized will elute faster from the column. Thang and

Novalin developed such a method to isolate small organic molecules in grass silage juice. This method can also be used to separate AAs.[102]

The main advantage of chromatography is that it can give AAs with very high purity, and thus it is dominantly used for analysis of amino acids.[99] However, its main limitation lies in cost (and scale). For ion-exchange chromatography, beside the cost of the column, the chemical cost to regenerate the column (usually by strong acid or base) is also considerable.

For both methods, the load of the column limits its scale. Semi-continuous chromatography has been developed and has been applied to separate AAs, but it is still limited to pilot-scale.[103] Thus it is necessary to find other methods for continuous and large scale separation of AAs for the production of bulk chemical.

1.5.4 Electrodialysis separation

Electrodialysis (ED) is an electro-membrane separation method that uses an electrical potential difference between membranes as the driving force to extract ions from the solution.[104] It is a continuous separation process that can be carried out on large scale.[105] An existing application is the desalination of seawater or brackish water.[105,106] ED has also been used in the separation of chemicals such as organic acids.[107]

A typical example of an ED stack is shown in Fig. 1.7. It is composed of electrodes and ion exchange membranes. The ion exchange membranes are usually made of polymeric materials and can be divided into two classes *i.e.*, anion exchange membranes (AEM) and cation exchange membranes (CEM). AEMs contain positively charged groups (*e.g.* quaternary ammonium groups) therefore it only allows negatively charged ions to pass through. In contrast, CEMs contains negatively charged groups (*e.g.* sulfonic acid groups) therefore it only allows positively charged ions to pass through. As summarized by Krol,[108] an ion exchange membrane should have a high permselectivity (being highly permeable to counter ions but impermeable to co-ions), a low electrical resistance, a good mechanical property (mechanically strong and less swelling or shrinking in different solution), and a high chemical stability (over a wide pH range, resistant to oxidizing, and no cleavage of ionic groups from the membrane).

The principle of ED separation is illustrated in Fig. 1.7. The feed solution contains ions that flows into the ED cells. Under an electrical potential difference, cations (*e.g.* Na^+, Ca^{2+}) move towards cathode and can pass through the cation exchange membranes but are prevented from further transportation through the AEMs. By contrast, anions (*e.g.* Cl^-, SO_4^{2-}) move towards anode and can pass through the anion exchange membranes but cannot pass the CEMs. As a net result, ions are removed from the dilute streams and accumulated in concentrate streams. Around the electrode, there is a flow of electrode stream (E stream) whose ions are also transported. The use of E streams is essential to allow current to be transported across the stacks and to maintain the stack solutions to be electrically neutral. Typically, Na_2SO_4 solution (0.5 M) was used as the electrode stream.[104] In the setup shown in Fig. 1.7, the feed stream passes through the ED stack only once. Alternatively, the concentrate streams and dilute streams can be circulated back to the original cells to enhance the separation efficiency.

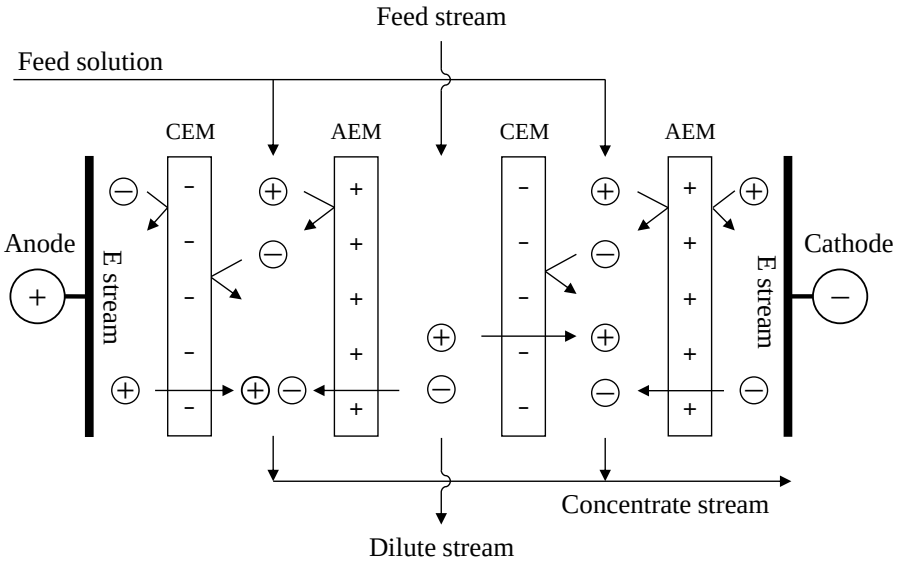


Fig. 1.7 The principle of ED separation. AEM: anion exchange membrane; CEM: cation exchange membrane; E stream: electrode stream.

Since AAs are zwitterionic molecules whose charge depends on the pH of the solution, they can also be separated by ED.[109,110] Fig. 1.8 shows a typical ED process to separate AA mixtures

into a few classes. At pH 6, AAs can be divided into three classes *i.e.* acidic AAs are deprotonated therefore bear a negative net charge; basic AAs are protonated and bear a positive net charge; the rest are neutral AAs with almost zero net charge. In the applied electric field, acidic AAs can go through the anion exchange membrane and basic AAs can go through the cation exchange membrane. Thus, the mixture of 20 AAs can be separated into three streams.

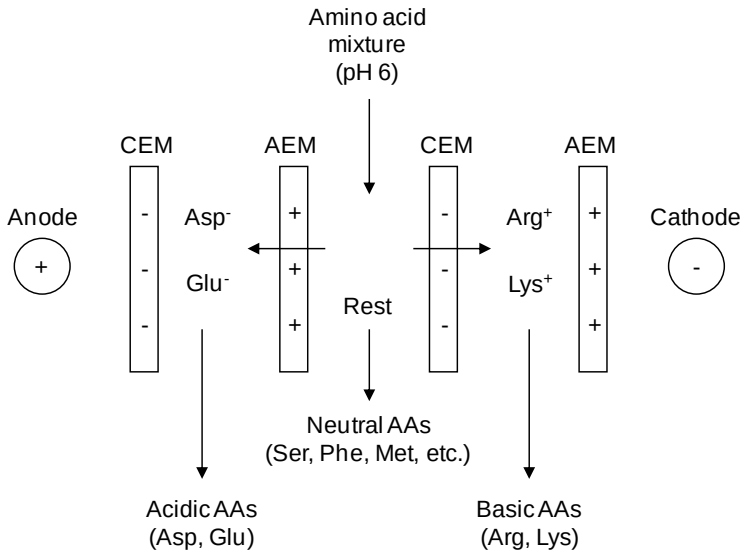


Fig. 1.8 ED separation of AAs from protein hydrolysis into acidic, basic and neutral streams.[109,110] CEM: cation exchange membrane; AEM: anion exchange membrane.

As summarized by Novalin *et al.*, ED is a superior method compared to ion exchange chromatography since it consumes less energy and produces less waste water.[111] Therefore ED is the method of choice in this research. However, as ED separate ions based on their charge difference, the charge similarities of AAs inside the three streams greatly limits the further separation of them by ED. To aid the further separation of AAs within each of the three streams, one solution is to carry out **conversions that change the charge behavior of the AA for further separation.** These conversions are the **main topic** of this thesis.

1.6 AA conversions

1.6.1 Criteria for choosing conversion methods

First of all, the conversion of AAs should lead to compounds with different charge behavior. However at a given pH the AA can only be separated in three classes (Fig 1.7). Within these classes the charge behavior is similar thus further separation is cumbersome. However when the charge behavior of the AA is modified, further separation should become possible. Thus the further conversion should target on the charged groups of AAs. These include the primary amine groups and carboxylic groups. To achieve that, reactions such as deamination and decarboxylation of AAs are very interesting.[69-74]

Secondly, the conversion of AAs should be specific. In this case, enzymatic reactions which usually have high specificity are very suitable. In contrast, specific chemical conversions are more difficult to achieve, as AAs with similar charge behavior usually have similar chemical structure which makes them display similar chemical properties.

Thirdly, the conversion of AAs should lead to useful products or intermediates. For example, those products listed in Table 1.1 are all very interesting. This gives a chance to integrate the chemical production process with the ED separation of those products. Thus, an integrated *in-situ* product formation and removal (ISPR) process can be created which saves process steps and energy input.[112]

In the end, practical issues such as solubility and the release of by-product should be considered. In the aforementioned deamination and decarboxylation, the ammonia and carbon dioxide formed can be easily released from the reaction mixture. So these reactions are very suitable to be used in this research and in a future industrial application.

1.6.2 An example of applying the criteria to choose conversion method

In this example, the AAs to be separated are Arg and Lys. Both of them are basic AAs which end up in one stream after electrodialytic separation of a hydrolyzed protein (*e.g.* hydrolysates of poultry feathers).[109,110] To find the best reaction to aid the following ED separation of them, the criteria listed in section 1.6.1 are applied. Fig. 1.9 shows the potential reactions that convert a

basic AA into useful chemical products. The first reaction is the decarboxylation which changes the charge behavior of Lys which is specific as the enzyme lysine decarboxylase (LDC) is used for the conversion.[72] The second reaction converts Lys to α-amino-β-caprolactam.[113] It also changes the charge behavior of Lys, but in the same temperature range used for that conversion the hydrolysis of Arg can also take place, therefore it is not specific. The third reaction converts Arg which forms L-ornithine which is still a basic AA,[66,67] therefore it cannot aid the separation. To conclude, the first conversion, the enzymatic decarboxylation of Lys is the most promising candidate to aid the ED separation of the basic AAs Lys and Arg.

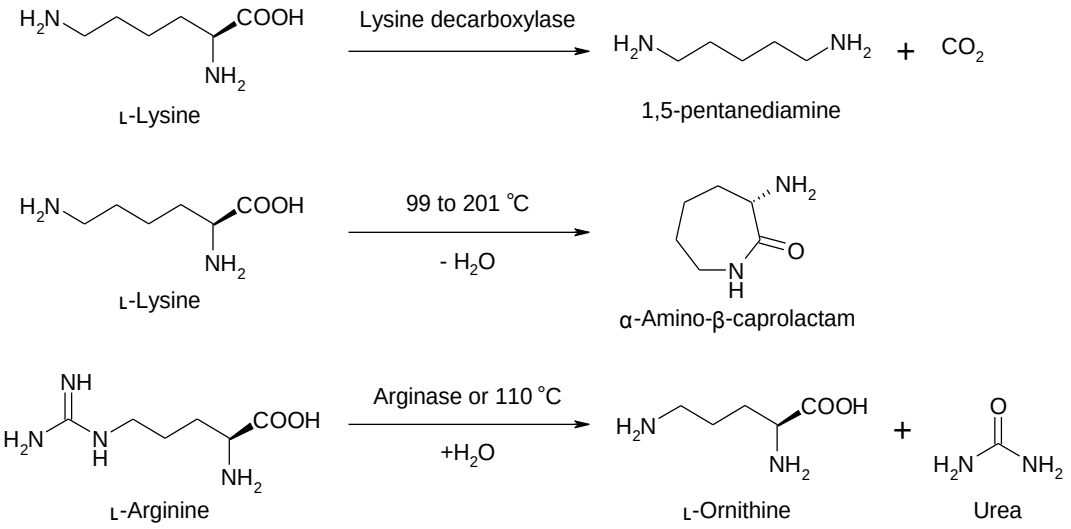


Fig. 1.9 Reactions that converts basic AAs into useful chemicals.

1.7 Aim of this research

The goal of this work is to develop conversion methods for AAs with similar charge behavior to aid their separation (or products thereof) by ED. These AAs are obtained from a proposed initial ED separation of an AA mixture to separate the AAs into three classes *i.e.*, acidic, neutral and basic AAs. The main driver is the development of new conversion methods of AAs to produce sustainably bulk chemicals which uses biomass rather than fossil fuels as the starting materials (Fig. 1.10).

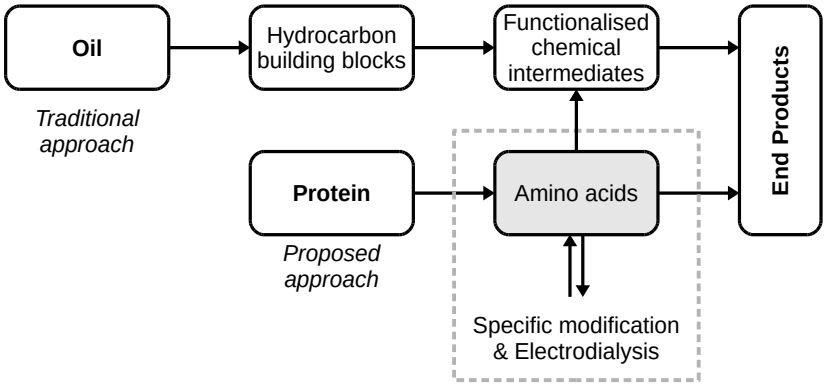


Fig. 1.10 New route for chemical production based on AA's from natural protein as the feedstock.

1.8 Outline of this thesis

Chapter 2 is about the specific conversion of Lys present in a mixture which also contains Arg as a means to aid the ED separation of the two AAs. LDC was used to specifically convert Lys into 1,5-pentanediamine while Arg remains unchanged. The reaction kinetics, enzyme inhibition by products, immobilization in calcium alginate and the operational stability under different reaction temperatures were studied and discussed. A process was designed to integrate the enzymatic conversion with the ED separation of basic AAs form a protein hydrolyzate. Based on experimental results, the costs of this process were also estimated.

Chapter 3 describes the chemical conversion of Glu to L-pyroglutamic acid (pGlu) as a means to separate acidic AAs (Asp was used as second AA) using solubility differences in water. The conversion was carried out under both aqueous conditions and melt conditions (Glu was in melt state). Under aqueous conditions, the reaction was carried out in D_2O, ^{1}H-NMR could be used to follow the course of the reaction. Reaction conditions studied include time, temperature, effect of Asp and the ratio of Glu to D_2O to determine the optimal conditions for the separation of Glu from Asp. Under melt conditions, the optimal conditions of converting Glu alone according to literature were applied for the separation. The reaction of recovering Glu from separated pGlu

was also studied. The separation yield and purity results were compared to find the best conditions to apply the conversion.

Chapter 4 describes the enzymatic conversion of Glu to γ-aminobutyric acid as a means to aid the separate of acidic AAs. The enzyme used was L-glutamate α-decarboxylase. The enzyme kinetics, immobilization in calcium alginate, and the effect of Asp on the operational stability of native and immobilized enzyme were studied at 30 °C. A column reactor packed with immobilized glutamate decarboxylase was used to study the performance of the enzyme in order to be integrated with the following ED separation of GABA from the residual acidic AAs.

Chapter 5 deals with the separation of neutral AAs. A mixture of L-serine, L-phenylalanine and L-methionine were used to simulate a mixture of neutral AAs. The reaction of L-serine decarboxylase and the L-phenylalanine ammonia-lyase were employed to specifically convert their native substrate into ethanolamine and *trans*-cinnamic acid. These products will be separated from neutral AAs and go to different streams in the following ED separation. It is expected that the rate of formation is similar. Therefore fluctuations in pH should not occur and further pH control is not required. The enzyme preparation, kinetics and the effect of other AAs and reaction products were studied.

Chapter 6 is the general discussion which concludes the results of chapter 2, 3, 4 and 5 in this thesis. The integration of specific conversion of AA with ED separation was discussed. Based on that, a new route for producing chemicals from protein obtained from biomass was given. The impact of such a route for the chemical industry is discussed and suggestions for further research are discussed.

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Chapter 2

The use of L-lysine decarboxylase as a means to separate amino acids by electrodialysis

This Chapter is based on

Teng Y, Scott EL, van Zeeland ANT and Sanders JPM

The use of L-lysine decarboxylase as a means to separate amino acids by electrodialysis

Green Chem **13**:624-630 (2011)

Abstract

Amino acids (AAs) are interesting materials as feedstocks for the chemical industry as they contain chemical functionalities similar to conventional petrochemicals. This offers the possibility to circumvent process steps, energy and reagents. AAs can be obtained by the hydrolysis of potentially inexpensive voluminous protein streams derived from biofuel production. However, isolation of the preferred AA is required in order to carry out further transformation into the desired product. Theoretically separation may be achieved using electrodialysis (ED). To increase efficiency, specific modification to a product of industrial interest and removes charged groups of AAs with similar isoelectric points is required. Here, the reaction of L-lysine decarboxylase (LDC) was studied as a means to specifically convert L-lysine (Lys) to 1,5-pentanediamine (PDA) in the presence of Arg to produce products with different charge thus allowing isolation of products by ED. Immobilization of LDC in calcium alginate enhanced the operational stability and conversion in mixtures of amino acids was highly specific. At 30 °C the presence of L-arginine (Arg) had little effect of the activity of the enzyme although inhibition by the product PDA could be observed. Volumetric productivity was calculated and raw material and transformation costs were estimated for a potential process using a mixture of Arg and Lys.

2.1 Introduction

Diminishing reserves, environmental impacts, fluctuating prices and geopolitical problems are forcing people to find alternatives to fossil resource.[1] The application of biomass and the associated biorefining is gaining more interest.[2] For example, The European Union has estimated that by 2030 up to one quarter of its transport fuel could be replaced by biofuels.[3] However, most research focuses on bioenergy and biofuels but the application of biomass to produce industrial chemicals has received less attention.

The traditional route to produce chemicals is heavily dependent on fossil resources as the carbon and energy source. The crude oil products are cracked into hydrocarbon building blocks and functionalized into end products by reaction with other chemicals such as ammonia or chlorine. Thus the production route is very energy intensive. However, many products derived from biomass, such as AAs, carbohydrates and lignin contain the functional groups needed by the chemical industry.[4,5] If chemicals could be synthesized from biomass fractions, the carbon content together with the process energy and the extra chemicals required to functionalize the base petrochemicals might be reduced. For example, carbohydrates can be fermented to bioethanol and dehydrated to ethylene, while lignin is a potentially interesting source to make aromatic compounds.[5] Currently the use of AAs as a raw material for the production of chemicals is less well studied. This is surprising given that they contain functional groups, such as primary amine and carboxylic acid groups, which makes them similar to many chemicals. For example, Asp can be converted to *β*–alanine that could be an intermediate for producing acrylamide or acrylonitrile,[6] Glu can be converted to *γ*-aminobutyric acid which can further be converted to *N*-methylpyrrolidone.[7,8] Other examples of amino acid conversions are also known.[9] Potentially AAs can be obtained by the hydrolysis of proteins from a number of agriculture waste streams or the rest streams of biofuel production, therefore the raw material will be potentially inexpensive and sufficiently available.[1]

The hydrolysis of bio-derived proteins would results in a mixture of up to 20 AA species. As previously mentioned some have the potential value to be converted into chemicals, while others such as methionine may be more useful as animal feed.[10] To make the best use of protein, or

amino acid rest streams, it is necessary to separate them in order to make them applicable for their different applications. A promising technology to achieve this is electrodialysis (ED), which removes ions from a dilute solution through ion-exchange membranes to another concentrate solution by applying an electrical potential as the driving force. In principle, it could separate the protein hydrolysate into acidic, basic and neutral AA streams according to their charge behavior.[11,12] However, a further ED separation within these streams is challenging due to the similarity of their isoelectric points (IPs). To overcome this specific modification of the charge behavior of one of these molecules is required. This may be done by removing charged groups such as the carboxylic acid group. In this case, enzymatic modification may be considered. If such a modification directly leads to an end product, the production of a chemical can be coupled with the separation process. Therefore a sustainable chemical production and separation route may be achieved (Fig. 2.1).

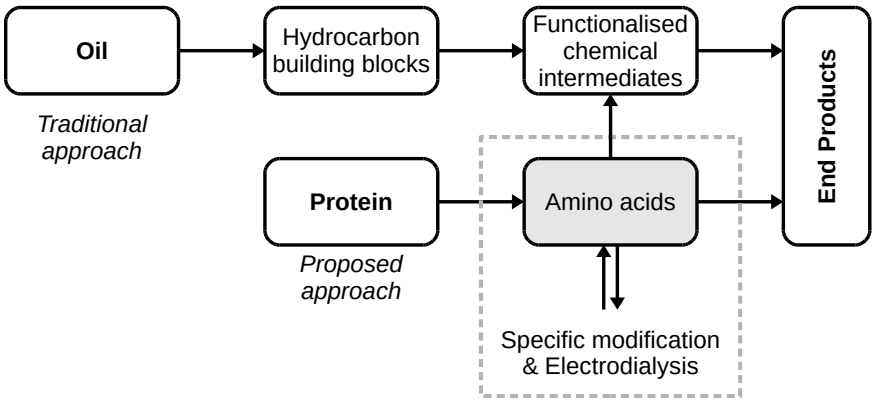

Fig. 2.1 New route for chemical production based on AAs from natural protein as the feedstock.

Here we focus on the basic AA stream containing Arg and Lys (IP at pH 10.76 and pH 9.74 respectively) obtained from a potential ED process. Both of them are interesting feedstocks and thus it would be advantageous if they (or products thereof) could be separated from one another. Arg could be a raw material for the production of 1,4-butanediamine (BDA) and urea, which have application in the plastics and fertilizer industries.[9,13] Lys can be used to prepare a monomer

for the synthesis of nylon-6 or used to make other aliphatic α,ω-diamines for nylon such as PDA.[14,15] To tackle the separation, specific modification to a product of interest, coupled with change in charge behavior, should be attempted. In this case, the enzyme LDC was applied. LDC specifically converts Lys to PDA and CO_2.[16] To the best of our knowledge, no literature has reported that the enzyme has activity to Arg. After reaction, a mixture of Arg (potentially with some unreacted Lys) and the product PDA are obtained. At a pH > 11, Arg (and residual Lys) are negatively changed while the PDA is neutral, thus allowing further separation by ED. The potential ED separation and reaction configuration is given in Fig. 2.2. The pH of the AA mixture obtained from protein hydrolysis is altered to *ca.* pH 6 so the basic and acidic AAs can be separated from the neutral amino acids. Lys and Arg are isolated in the ED stacks, which is connected to a reactor, with a constant volume. As the enzymatic conversion of Lys to PDA proceeds, Lys and Arg are also being continuously transported through the cation exchange membrane. As a net result, PDA and Arg are concentrated while Lys concentration is constant. This is comparable to a fed batch reaction which has (solid) substrate added into the reactor.

In order to achieve optimal operating conditions of the enzyme, and therefore have maximum conversion of Lys, a number of parameters (such as pH, time and temperature) were studied as well as the conditions to improve the operational stability of the process (enzyme immobilization). To simulate the possibility of carrying out a specific modification in a substrate solution of Lys and Arg fed with a stream of Lys and Arg a fed batch method was investigated. Finally, the process costs were estimated and a possible production route discussed.

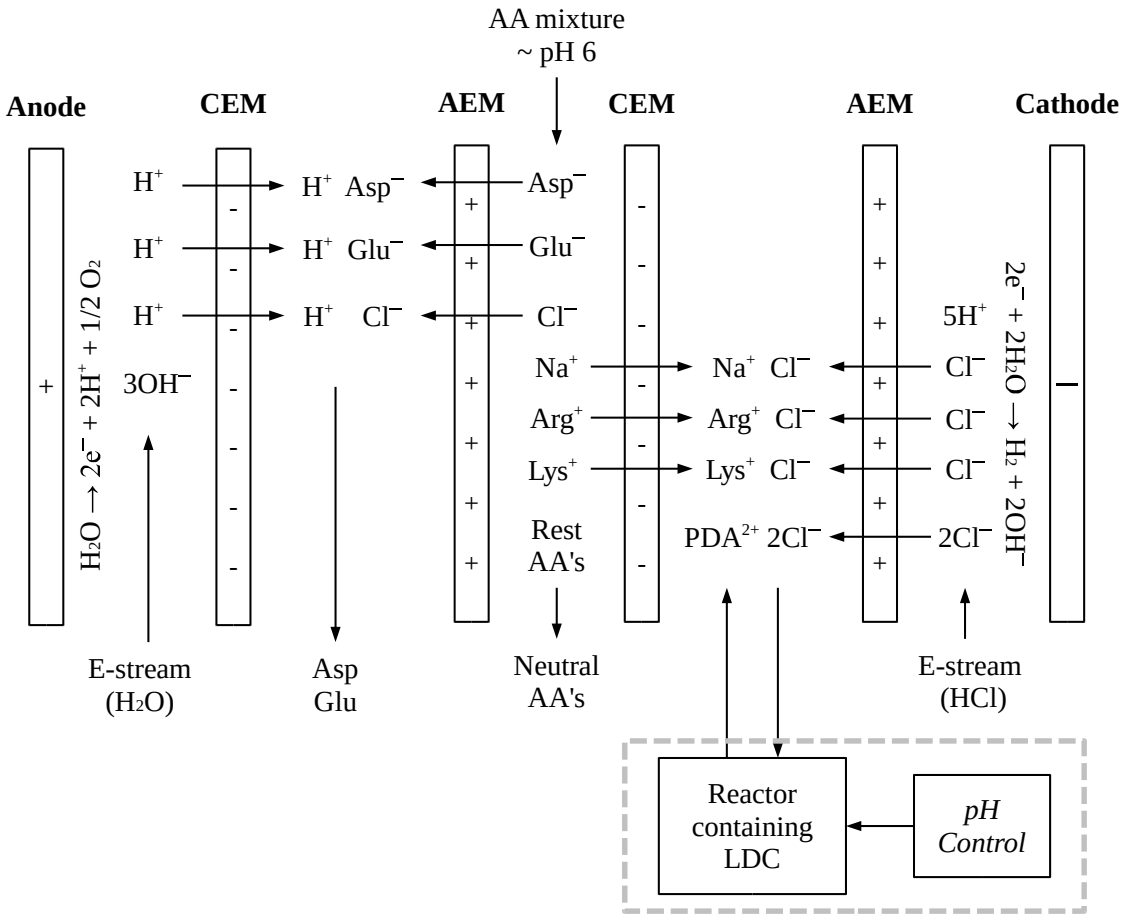

Fig. 2.2 ED separation of AAs and the combination of enzymatic modification reactor for basic AA stream. CEM stands for cation exchange membrane and AEM stands for anion exchange membrane.

2.2 Experimental

2.2.1 Materials

LDC (from *Bacterium cadaveris*, type I), sodium alginate (low viscosity), PLP·H_2O (≥ 97%), HCl standard solution (volumetric solution, 0.1 M), Arg·HCl (reagent grade, ≥ 98%), Lys·HCl (reagent grade, ≥ 98%) and Lys·2HCl (≥ 98%) were bought from Sigma-Aldrich. Quick Start Bradford dye reagent and Bovine Serum Albumin standard set were from Bio-Rad. Other

chemicals were used as received. Milli-Q (Millipore) purity water was used to make all the solutions.

2.2.2 Software

The pH and charge behavior estimation was done by software CurTiPot Version 3.3.2 (2008) for MS-Excel® 1997-2007. The software contains a database of dissociation constants.

Model fitting of operational stability data was done by WinCurveFit 1.1.8. A double exponential model was used.

2.2.3 U-HPLC

Sample preparation: 500 µL of sample (or standard 0.5-0.025 mM) was taken, put into an Eppendorf safe-lock tube and mixed with 400 µL of methanol. Subsequently 100 µL of internal standard solution (0.4 mM of taurine) was added to correct for injection errors of the U-HPLC measurement. After this pre-treatment the sample was filtered over a 0.2 µm filter (Spartan 13/0.2 RC, Whatman) and placed in a 1.5 mL vial, to be ready for analysis. The derivatization of the AAs occurs in the needle of the Dionex U-HPLC. The derivatization procedure is as follows; draw 50 µL ultra-pure water, draw 1 µL air, draw 2 µL reagent A (OPA-MET), draw 2 µL sample (or standard) solution, mix in needle 6x, wait 60 seconds, wash needle externally, draw 2 µL reagent B (FMOC), mix in needle 6x, wait 15 seconds, wash needle externally, draw 4 µL injection diluent, mix in needle 6x and inject on column.

Chromatography: The analysis was performed by using Dionex U-HPLC instrument (Dionex Corporation, Sunnyvale, CA, USA) consisted of a Ultimate 3000 RS (Rapid Separation) pump and a Ultimate 3000 Autosampler, a Ultimate 3000 column compartment with a thermo stable column area, and a Ultimate 3000 variable wavelength detector, operating with the Dionex Chromeleon™ 6.8 software.

The AAs where separated using an Acquity UPLC® BEH C18 reversed phase column (1.7 µm particle size, 2.1 x 50mm) and a Acclaim® 120 C18 guard column (5µm, 2.0x10mm), flow rate, 1 mL/min. The column temperature was maintained at 65° C. Mobile phase A consisted of 10 mM sodium phosphate, 10 mM sodium borate and 2 mM sodium azide (buffer adjusted with HCL to

pH 7.8). Mobile phase B consisted of acetonitrile, methanol and ultra-pure water 45:45:10 (v/v). Detections were performed simultaneously, by using the variable wavelength detector at 338 nm and 263 nm (data collection rate: 4.0 Hz, time constant 0.18 s).

Gradient, expressed as% solvent B: 0-6.98 min: 2-57% B, 6.98-7.80 min: 57-100% B, 7.80-9.49 min: 100% B, 9.49-9.72 min: 100-2% B, 9.72-10 min 2% B.[17]

2.2.4 Bradford Protein assay

Bradford protein assays were performed using the Bio-Rad Protein Assay Kit II with BSA standard. The absorbance was determined at 595 nm using a Multiskan Spectrum microplate spectrophotometer (Thermo Labsystem).

2.2.5 Immobilization

120 mg LDC powder was suspended in 12 mL sodium alginate solution (2 wt%) The LDC suspension was added dropwise in portions of 2 mL to 40 mL of $CaCl_2$ solution (1 wt%) to form calcium alginate beads with a diameter of approximately 1.5 mm. The beads were stored in sodium acetate buffer (10 mM, pH 6.2) containing 50 mM $CaCl_2$ and 10 μM PLP at 4 °C. The samples were wrapped in aluminum foil to protect them from light.

2.2.6 Activity assay

The initial rate of native LDC was assayed using a pH-stat method as described in literature.[18] The titrations were performed in a titration vessel with a thermostatic jacket (±0.1 °C) and monitored using a Methrohm 718 STAT Titrino. The substrate solution was prepared by dissolving 109.6 mg Lys·HCl in 17.80 mL MilliQ water, after which 200 μL 5 mM PLP·H_2O solution was added.

At 37 °C, the substrate solution was added to the titration vessel and brought to the required pH using either HCl or NaOH solution. To initiate the reaction, 1 mL LDC solution (20.0 mg mL^{-1}) was added to the substrate solution, resulting in a reaction mixture with 30 mM Lys and 50 μM PLP. A solution of 0.1 M HCl was used as titration solution. The initial rate, expressed as U/mg

(μmol H^+ min^{-1} mg^{-1}), was determined as the function of the slope of the linear part of the titration curve.

To study the pH dependent activity, assays were performed at pH 4.5, 5.0, 5.5, 5.8, 6.0, 6.5 and 7.0 at 37 °C. Similarly, to study the K_M value of Lys, activity assays were performed at 30 °C with 0.25, 0.50, 1.00, 5.00 and 30.00 mM of Lys. To study the Arg and PDA inhibition at 30 °C, assays were performed with 0.50, 1.00, 2.00 and 30.00 mM of Lys in combination with 0, 15 and 30 mM of Arg·HCl or PDA·2HCl. Additionally, to investigate the effect of Arg concentration, assays were performed with of 0.1, 0.2, 0.3 and 0.6 M Arg·HCl. Finally, to investigate the effect of ionic strength, activity assays were performed with 0.03, 0.1, 0.2, and 1 M NaCl.

2.2.7 Operational stability

The operational stability of native and immobilized enzyme was determined by performing fed batch reactions at 30, 37 and 45 °C over a period of 24 hours. The reaction setup was similar to that previously described in the Activity assay section.

The reaction vessel was filled with 20 mL 30 mM Lys·HCl and 50 μM PLP·H_2O solution and subsequently the pH was adjusted to 5.8 using a NaOH solution. At the required temperature, 20.0 mg LDC or an equivalent of immobilized enzyme was added to initiate the reaction. The titration solution was an aqueous solution of 0.1 M Lys·2HCl and 50 μM PLP·H_2O (50 μM). The activity (U/mg) in time was determined as the function of the tangent slope of the titration curve at given points in time. The residual activity is defined as the activity at the given point in time relative to the initial activity.

To study the effect of Arg on the operational stability of immobilized LDC (ILDC), the operational stability was determined in the presence of 30 mM Arg·HCl at 30, 37 and 45 °C over a period of 24 hours. The standard titration solution was supplemented with 0.1 M Arg·HCl to maintain the level of Arg during the reaction. Using U-HPLC analysis, the concentrations of Arg, Cad and Lys were determined at the beginning and the end of the reaction.

2.3 Results and discussion

2.3.1 Characterization of the native enzyme

As the decarboxylation reaction leads to an increase in pH due to loss of CO_2 and production of amines, an effective pH control is important for the enzyme assay. Here a pH-stat method was applied thereby maintaining the optimum pH of the reaction by titrating with HCl.[18] From our experimental data the optimal pH was found to be pH 5.8 at 37 °C which is in accordance with literature.[16].Between pH 5.5 and pH 6.0 similar initial rates are observed but this decreases sharply out with this pH range.

At constant pH and temperature (pH 5.8 and 30 °C) the enzyme was found to have a K_m value of 0.78 mM for Lys (lit.,[16] *ca.* 0.38 mM) and that competitive product inhibition by PDA (K_i = 9.68 ± 0.78 mM) takes place. No activity of LDC for Arg is observed, which is in accordance with literature,[19] however if the concentration of Arg (in the form of Arg·HCl) exceeds 30mM, the relative activity towards Lys starts to decrease. As NaCl, which is generated during protein hydrolysis, could be introduced into the potential separation-reaction process (Fig. 2.2), its effect on the enzyme was also studied. Again in the range of 20-30 mM, NaCl has little or no effect on activity, although activity is seen to decrease at higher concentrations (Fig. 2.3). In both these cases the reduction in activity is suggested to be related to the increasing ionic strength of the solution. Other reports also show reduction in the activity of LDC as a function of ionic strength.[20] This is possibly attributed to the formation of (partially unfolded) aggregates that are less active.[21]

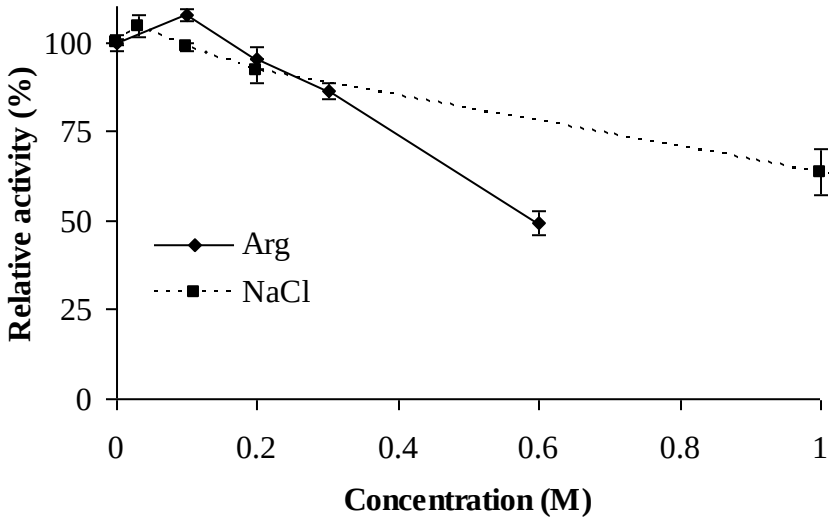

Fig. 2.3 The effect of Arg and NaCl on the LDC activity. The error bars represent standard deviation.

The effect of batch and fed batch reactor configurations on activity was determined. When the pH of a solution of 30 mM Lys·HCl was maintained using an aqueous solution of Lys·2HCl (100 mM), compared to $HCl_{(aq)}$ (100 mM), comparable initial rates but higher activities were obtained over longer periods of time. This is due to the substrate being continuously added therefore maintaining a substrate concentration above the K_m value. The volume of the Lys·2HCl solution added with time was monitored. This should indicate the progress of the reaction and this was confirmed by analysis using Ultra High Pressure Liquid Chromatography (U-HPLC) of the PDA concentration with time. Therefore the use of the pH-stat titration can be a reliable online method to assay the initial rate and operational behavior of LDC while having the pH controlled. This method was also applied in subsequent experiments where Arg was present in the system.

2.3.2 LDC immobilization

Immobilization is considered to be an effective way to simplifying the downstream processing due to the ease of separation of the enzyme from the products of the reaction and of enhancing the stability of the enzyme.[22] This will result in a cost reduction of the enzyme. Covalent

immobilization of LDC on Sepabeads EC-HFA according to literature procedure,[13] was attempted but no activity was obtained. Due to the large molecular weight of LDC (10^6 Da),[16] immobilization by physical entrapment in calcium alginate was carried out. This method has been previously reported for the successful use of GAD.[7] From several immobilization experiments, the supernatant consistently showed no residual protein, as indicated by use of Bradford test and activity assay. This indicates that the immobilization yield was very close to 100%. To characterize the behavior of the immobilized enzyme, fed batch reactions were carried out at 37 °C and pH 5.8 using Lys·2HCl as the titrating solution as aforementioned. The immobilized enzyme gave the same initial rate as the native enzyme, while activity was maintained over a prolonged reaction time (Fig. 2.4). No protein was found in the reaction solution, suggesting no enzyme leakage from the beads occurs during operation. Similar conclusions were also obtained when the reactions were carried out at 30 °C and 45 °C, though at a higher temperature both the native and immobilized enzyme have higher initial rates but decay more rapidly. The effect on stability during storage of the enzyme was also examined. Enzyme beads stored in acetate buffer at 4 °C for 5 weeks showed only a loss of *ca.* 10% of the initial rate. This loss is similar to the native enzyme after only 4 hours when suspended in water and stored at 4 °C. Thus it can be concluded that calcium alginate can completely entrap the enzyme while retaining activity and improving stability during operation and storage.

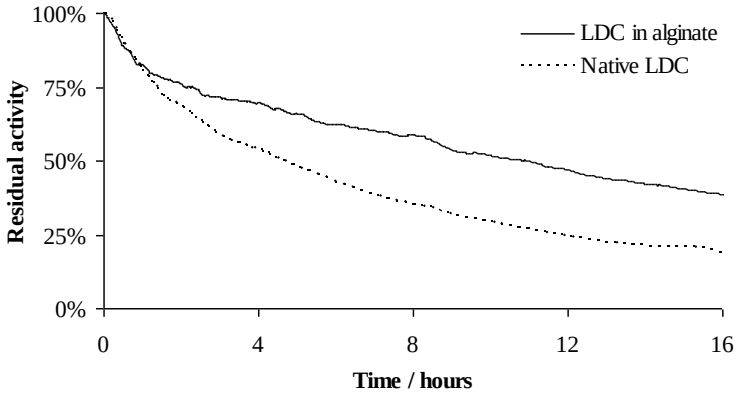

Fig. 2.4 Effect of immobilization on the operational stability of LDC over a 16hr period at pH 5.8 and 37 °C.

2.3.3 Effect of Arg

As described earlier (Fig. 2.2), it is envisaged that Lys and Arg will be fed to a reactor containing Lys and Arg during an ED process. While the behavior of Lys in both substrate and feed are known, the effects of the presence of Arg in the substrate solution and in the feed remain to be explored.

2.3.4 The presence of Arg in the substrate

A substrate solution of 30 mM of Lys and Arg was titrated with 100 mM of Lys·2HCl. The specificity of the reaction was confirmed by U-HPLC analysis before and after reaction which showed that the number of moles of PDA increased with time while the number of moles of Lys and Arg remained constant (Table 2.1).

Table 2.1 Moles of Arg, Lys and PDA at 0 hour and 24 hour at different reaction temperatures (Fig. 2.5) as determined by U-HPLC.*

Temperature	30 °C	37 °C	45 °C
Arg, 0 h (mmol)	0.59 ± 0.01	0.59 ± 0.01	0.60 ± 0.01
Arg, 24 h (mmol)	0.58 ± 0.01	0.60 ± 0.02	0.64 ± 0.01
Lys, 0 h (mmol)	0.53 ± 0.01	0.57 ± 0.01	0.57 ± 0.01
Lys, 24 h (mmol)	0.55 ± 0.01	0.59 ± 0.03	0.64 ± 0.01
PDA, 0 h (mmol)	0	0	0
PDA, 24 h (mmol)	0.37 ± 0.01	0.61 ± 0.01	0.61 ± 0.05

* Values (mean ± standard deviation, n = 2).

From Fig. 2.5 it is seen that when the substrate solution contained Lys or Lys and Arg, an increased temperature can increase the initial rate and decrease the operational stability. Such behavior is typical in the use of (immobilized) enzymes.[7] Substrate solutions containing Arg

show similar rates when operated at lower temperatures *e.g.* 30°C, while at higher temperatures the initial rates are lower compared to solutions containing only Lys. This suggests that inhibition by Arg takes place. Currently no literature reports that Arg has an inhibitory effect on LDC, but it has been known that other decarboxylases (*e.g.* ODC) can be inhibited by Arg,[23] thereby similar behaviors may also occur with LDC. This indicates that a lower temperature is preferred when applying LDC in the presence of Arg.

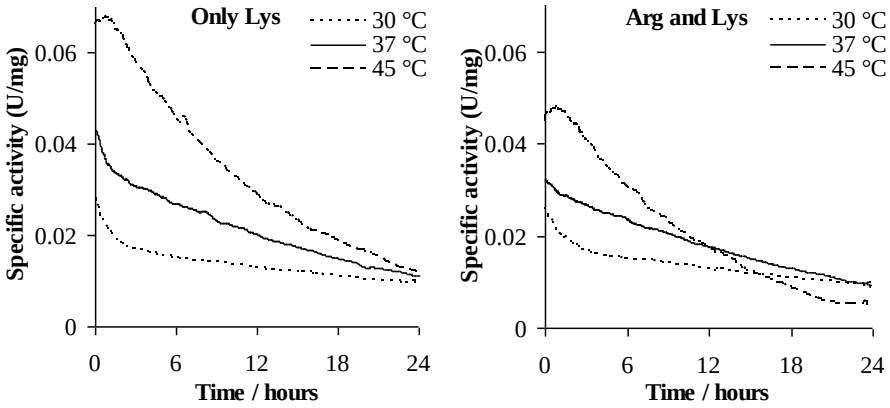

Fig. 2.5 Effect of temperature on the operational stability of ILDC in a fed batch setup with Lys or Arg and Lys in the substrate solution fed with Lys. The titration was done at pH 5.8 at 30, 37 and 45 °C for 24 hours.

Deactivation of LDC can also be observed in Fig. 2.5. It has been reported that the deactivation of LDC is believed to be linked with the loss of PLP from the enzyme.[24] However, in the current experiments additional PLP is added during titration. As seen from Fig. 2.5 the deactivation of ILDC does not appear to be first order. The deactivation curves can be fitted to a double exponential decay model (residual activity = $A \cdot e^{-at} \times B \cdot e^{-bt}$) which was applied to describe the deactivation of GAD.7 This indicates that LDC may deactivate in a similar way to GAD. According to literature, the deactivation of GAD is possibly due to the conversion of PLP to pyridoxamine 5'-phosphate which then dissociates from the enzyme to form the apoenzyme.[25]

Interestingly, experiments carried out at 45 °C appear to show an initial increase of activity. At even higher reaction temperatures *e.g.* 50 °C, this phenomenon is more pronounced (data not

shown). To examine if the increase in activity was due to a temperature gradient between the internal part of the alginate beads and solution, the immobilized enzyme was pre-incubated for 1 hour at 45 °C before addition of the substrate. However, the initial increase in the activity remained. This would suggest that changes in temperature between the bead and the solution are not responsible for the phenomenon. Another possible explanation could be diffusional limitation of the immobilization material. According to literature, diffusion within alginate can depend on electrostatic forces as the alginate matrix is negatively charged.[26] At the pH of the reaction (pH 5.8), Arg, Lys and PDA are all positively charged. Under these conditions it may be possible that they interact with the alginate matrix resulting in problems with diffusion. Since the calcium ion is responsible for the crosslinking of the immobilization material we added $CaCl_2$ (30 mM) to the substrate solution (45 °C) and observed that the initial increase became more pronounced. As this phenomenon was not observed when $CaCl_2$ was used with the native enzyme, it is suggested that the initial increase at higher temperatures was due to interaction of charged amino acids and PDA with the immobilization material.

2.3.5 Lys and Arg in substrate and feed

Ultimately, a Lys and Arg mixture would be fed to a solution containing Lys and Arg. As both the substrate and feed streams are a mixture of Arg and Lys, this has been called "self titration". Arg in the substrate solution was shown earlier not to influence the specific enzyme activity at 30 °C and indeed presence in the feed also shows very similar activity (Fig. 2.6 A). By using the aforementioned double exponential model, the residual activity and the accompanying PDA production for this process were derived (Fig. 2.6 B).[7] As indicated in the figure, most of the Lys conversion takes place over a 4 day period (corresponding to a residual enzyme activity of 5%). U-HPLC results reflected that in the self titration setup Arg and PDA were accumulated, from the feed stream and the conversion respectively, and the number of moles of Lys with respect to Arg and PDA were reduced. This increases the purity of the product and simplifies the ED separation.

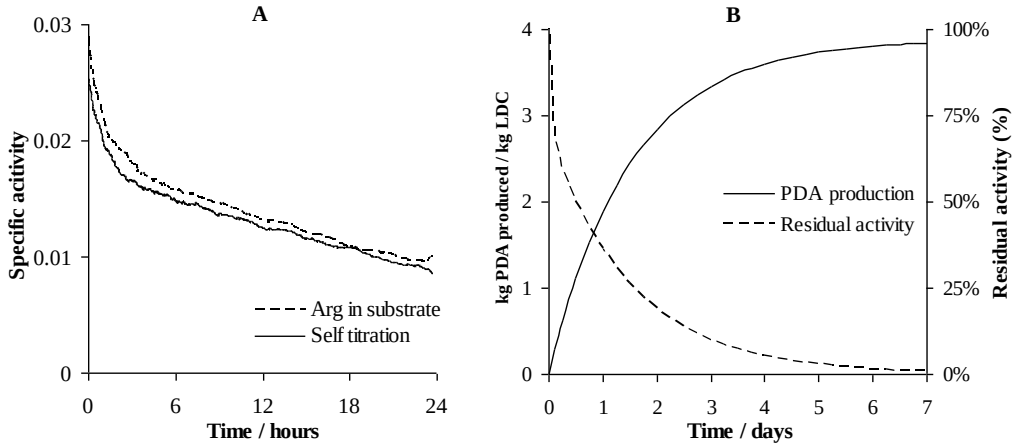

Fig. 2.6 A. Comparison of titrated by Lys·2HCl with Arg in substrate and self titration at 30 °C and pH 5.8 for 24 hours, and **B.** Estimated residual activity curve and the PDA production curve corresponding to the self titration setup.

2.3.6 Process costs

Process cost can be divided into amino acid, vessel, enzyme and other (hydrolysis and ED) costs.

In the case of amino acid cost, one could assume that the cost of protein is *ca.* 200 €/ton.[1,4] If all AAs from the protein could be isolated and have a specific added value application then the cost of each amino acid per ton could be similar to the protein source. Considering the molecular weights of Lys and PDA, the Lys cost would be *ca.* 300 €/ton of PDA.

In this study an enzyme with a very low activity was used (0.0294 U/mg). However, the activity of pure LDC has been reported to be 85.7 U/mg (30 °C, pH 5.8)[16] From Fig. 2.6 B, 1 kg of enzyme produces 2.85 kg of PDA in 48hrs, but this could increase to *ca.* 8.3 tons using 1 kg pure LDC. However, the use of a pure enzyme in a large scale application is unlikely but an enzyme extract could have an achievable activity of 50%, which would result in the use of 0.24 kg of LDC for the production of 1000 kg of PDA. A weight ratio of enzyme : alginate of 0.5 in immobilized enzymes has been reported for other enzymes and this was also used in the current work.[27] The solution used to prepare the beads contained 2 w/v% alginate and this would result in a total bead volume of 0.024 m^3. As the volume ratio of the bead to reactor is 0.1, a volumetric productivity of 87.5 kg PDA m^{-3} hr^{-1} is achievable. However such a potentially high productivity

would lead to inhibition problems which would affect the rate but this may be overcome by increasing the volume of the vessel or using a (chemostat) cascade of reactors to ensure a lower PDA concentrations are maintained.[28] By the choice or design of reactor to maintain maximum productivity, the costs for the operation of the reactor can be lowered and should not contribute to more than 10% of the cost of the product.

The prices of (an enzyme extract of) LDC for industrial production and dry alginate are *ca.* €100 /kg of equivalent pure enzyme and 6 €/kg respectively.[29] Thus the corresponding enzyme cost and alginate costs would be *ca.* 12 and 3 €/ton of PDA respectively.

Since BDA and ethanediamine, analogues of PDA, are estimated to cost *ca.* €1600 /ton,[1] then the reactor and immobilized enzyme costs are only a small fraction and the margin for protein hydrolysis and ED separation could be around €1000 /ton of product, which is considered to be reasonable.

2.4 Conclusions

The aim of this article was to design a fed batch bio-process of Lys to PDA in a mixture of basic AAs potentially obtained from an ED process. This was carried out to modify the charge allowing product formation (to PDA) to be coupled with a potential separation from the remaining basic AAs by ED. The reaction using LDC was found to be specific but was inhibited by high concentrations of Arg (>30 mM), NaCl (>30 mM) and PDA (K_i *ca.* 10 mM). It was possible to overcome the product inhibition by maintaining a sufficiently high Lys concentration. Immobilization of the enzyme did not result in a change in the activity and improved the operational stability over a 16 hour period. The presence of Arg had little effect on the activity when the operating temperature was 30 °C. At higher temperatures (37 and 45 °C) it was shown that a significant decrease in the specific activity and a reduction in operational stability took place. Based on process design and cost estimation, the associated cost in using ILDC is not a barrier in such a product formation and separation process using amino acids. In conclusion it is considered that such a route offers a more techno-economically efficient method for the production of chemicals. This is attributed to the use of biomass raw materials, which eliminates

the use of ammonia and extreme reaction conditions, to produce amines while offering the possibility to couple production with separation by ED.

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Chapter 3

Separation of L-aspartic acid and L-glutamic acid mixtures for use in the production of bio-based chemicals

This Chapter is based on

Teng Y, Scott EL and Sanders JPM

Separation of L-aspartic acid and L-glutamic acid mixtures for use in the production of bio-based chemicals

J Chem Technol Biotechnol **87**:1458–1465 (2012)

Abstract

Amino acids are promising feedstocks for the chemical industry due to their chemical functionality. They can be obtained by the hydrolysis of potentially inexpensive protein streams such as the byproduct of biofuel production. However, individual amino acids are required before they can be used for the further production of chemicals. Here we studied the separation of Asp and Glu mixture which can be isolated from protein hydrolysis solutions at low pH or from electrodialysis of complex amino acid mixtures.

In this research, Glu was converted into pGlu which can be separated from the mixture of Asp and Glu due to its higher solubility in water. The conversion was carried out under aqueous or melt conditions. Under aqueous conditions, the conversion was studied as a factor of time, temperature and the amount of Glu. The conversion was specific with high yield and not effected by Asp. After pGlu was separated from Asp and residual Glu by solubility difference, it can be transferred back to Glu through hydrolysis. The conversion of Glu to pGlu is specific and can be applied to separation Asp and Glu for their use in the production of bio-based chemicals.

3.1 Introduction

Consumption of limited reserves, geopolitical and environmental problems, as well as the requirement of reducing CO_2 emissions are driving people to search for alternatives to fossil fuels for the production of transportation fuels and chemicals.[1] Thus, biomass and biorefinery are attracting more and more attention.[2] For example, the European Union estimated that up to one quarter of its transport fuel could be replaced by biofuel by 2030.[3] However, most of the applications of biomass have focused on biofuels while the potential as a feedstock for functionalized chemicals is far less explored. Traditionally, chemicals are made from crude oil by cracking it into hydrocarbon building blocks and then reacting with additional chemicals like ammonia and chlorine in order to functionalize them. Such production routes are very energy intensive and generate a lot of rest streams and CO_2 emissions. To create new production routes to industrial chemicals, one can think of using biomass as raw materials. Some examples of biobased chemicals being studied are polylactide and 1,3-propanediol.[4,5] but these developments are mainly based on fermentation requiring the use of glucose as the feedstock. In case of making nitrogen containing chemicals, it could be interesting to use AAs which already contain functional groups like carboxyl groups and primary amine groups. This inherent functionality could help to save the energy and extra chemicals needed to produce functionalized chemicals. AAs can be obtained by the hydrolysis of potentially inexpensive proteins obtained from the byproduct of biofuel production or from other agriculture waste streams. Considering the potential growth of biofuel production, this will generate a very large quantity of protein containing by product, therefore such production route would be very interesting.[1]

Some chemical production methods based on AAs have been reported, such as acrylamide could be obtained from the conversion of Asp,[6] and *N*-methylpyrrolidone and acrylonitrile could be produced from Glu.[7,8] However, these studies were based on the use of single AAs while the hydrolysis of protein results in a mixture of up to 20 AAs. Since some of them, such as Asp and Glu, could be more useful to produce chemicals, while others, like Thr and Met,[9] are more interesting as animal feed, isolation to individual AAs becomes a major challenge before they can be used for further applications. Some traditional methods to separate AAs include the use of ion

exchange chromatography and reactive extraction,[10,11] However, these methods have limited success in large scale separations as they have been only reported for small scale separation or analytical applications.[12,13] Potentially, AAs can be separated into acidic, basic and neutral AA streams by electrodialysis (ED).[14,15] The further separation inside these streams is difficult due to the similarities in IPs and solubility. To aid separation, specific modification of AAs which changes the charge behavior could be applied to aid the separation. If such modification directly leads to a product with industrial interest, the production and separation steps could be integrated into one process that might offer a more techno-economically efficient route for the production of chemicals.[16]

Here the separation of an acidic AA stream containing Asp and Glu from a potential ED process was studied. As previously described, both AAs are interesting starting materials to produce nitrogen containing chemicals, so it would be beneficial to separate them or their products thereof. The main difficulty for the separation is that Asp and Glu have similar IPs (pH 2.77 and pH 3.22 respectively) and solubility in water.[17] To solve this, specific modification to a compound with a change in solubility behavior could be applied. For example, the conversion of Glu to pGlu could allow separation by change in the solubility in water (Fig. 3.1). Then pGlu can be isolated and converted back to Glu. While pGlu has applications for chiral synthesis and as a chemical,[18,19] by carrying out conversion and separation to produce Glu and Asp, more possibilities for the utilization of the AAs could be achieved. However, at the high temperatures required for reaction the deamination of Asp to FUM could also take place.[20,21] Thus it is important to explore the reaction conditions to achieve specificity and prevent FUM formation.

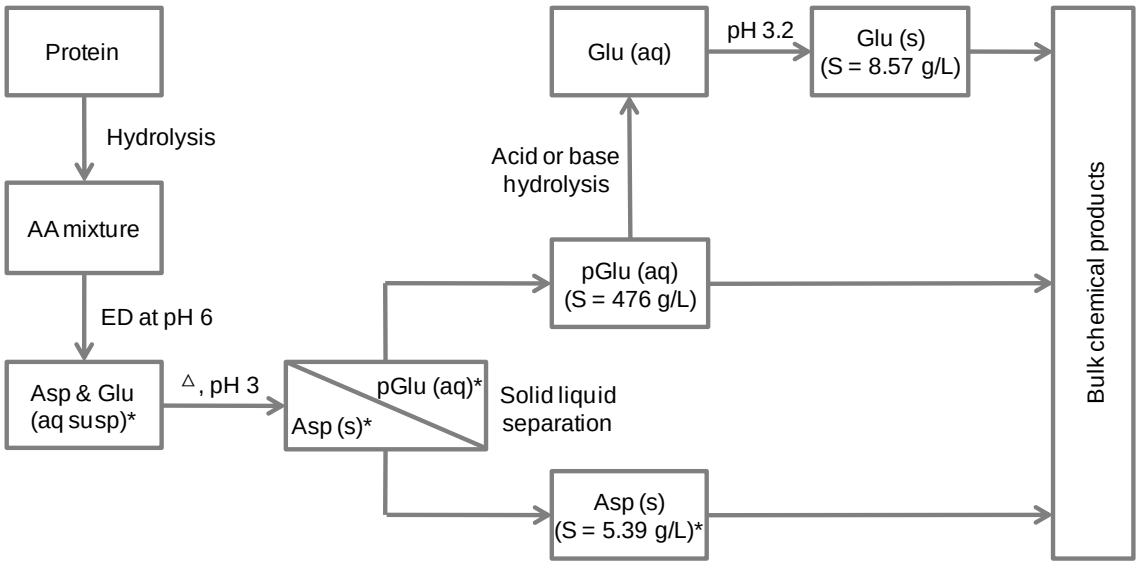

Fig. 3.1 The conversion of Glu to pGlu and the hydrolysis of pGlu to Glu as a means for the separation of Asp and Glu.

*: (aq susp): aqueous suspension; (aq): aqueous phase; (s): solid; (S): water solubility.

To achieve the optimal conditions of specific conversion of Glu to pGlu, and hence separate it from Asp, a number of reaction conditions were studied (*e.g.* time, temperature and the quantity of Glu) as well as the stability and influence of Asp in the process. To perform the separation, the optimal conditions for specific conversion of Glu to pGlu were applied to the mixture of Asp and Glu. After separation, the hydrolysis of Glu to pGlu was also studied. Deuterated water was used as the solvent and the rate of conversion and composition of the reaction mixture was analyzed by ^{1}H-NMR.

In summary, this article focuses on the separation of acidic AAs (Asp and Glu) by the specific conversion of Glu to pGlu in order to influence a change in solubility and allow separation. Various conditions were studied in order to achieve the maximum conversion and the best separation. Finally, hydrolysis of pGlu to Glu was studied in order to design a potential separation process of Asp and Glu.

3.2 Experimental

3.2.1 Materials and equipment

Asp (≥ 98%), Glu (≥ 99.5%), pGlu (≥ 99.0%) were obtained from Sigma, deuterium oxide (99% atom% D) and sulfuric acid-d_2 (96~98 wt% solution in D_2O, 99.5 atom% D) are from Aldrich. All chemicals were used as received.

Reactions carried out at 80 and at 99 °C were performed in an Eppendorf® thermal mixer equipped using 1.5 mL polypropylene Eppendorf® tubes as the reaction vessel. When the reaction (using Glu in suspension) was complete, the homogeneous, clear reaction mixture obtained was diluted to 8 mg/mL using hot D_2O to avoid the precipitation of residual Glu at room temperature. This was allowed to cool to room temperature and the sample analyzed by ^{1}H-NMR.

Reactions carried out at temperatures higher than 99 °C were performed in a Parr Series® 500 Multiple Reactor System equipped with stainless steel autoclaves (75 mL internal volume) and the reaction process was controlled by SpecView software V2. Reactions were performed using the same method as above and ^{1}H-NMR (400 MHz, D_2O) was used to analysis the reaction solution.

3.2.2 The conversion of Glu to pGlu under aqueous conditions

Glu (70.0 mg, 0.48 mmol) was added to 10.0 mL of D_2O and dissolved completely at room temperature to form a clear stock solution (7.0 mg/mL of D_2O).

To study the conversion of Glu to pGlu as a function of time, temperature and weight:volume of Glu:water, experiments were carried out as listed in Table 3.1. The experiments carried out at 80 and 99 °C were carried out by adding 1.0 mL of the stock solution (described above), or 1.0 mL of D_2O with the corresponding weight of Glu to form a suspension, to Eppendorf® tubes and reacted in an Eppendorf Thermal Mixer®. When reaction was carried out with suspensions, after a period of 4 hours the reaction mixture turned into a clear solution. It was noted that with suspensions containing lower amounts of Glu that the mixture turned into a clear solution more rapidly. Reactions carried out at 120 °C were carried out by adding 5.0 mL of the stock solution or 1.00 g of Glu and 5.0 mL of D_2O (200mg Glu / mL D_2O) to the autoclaves and reacted. ^{1}H-

NMR: δ 2.00-2.21 (m, 2H of Glu and 1H of pGlu, γ-CH_2 of Glu and 1 of β-CH_2 of pGlu), 2.26-2.59 (m, 2H of Glu and 3H of pGlu, β-CH_2 of Glu, γ-CH_2 of pGlu and 1 of β-CH_2 of pGlu), 3.74-3.84 (t, 1H, α-CH), 4.23-4.34 (q, 1H, α-CH), 7.786 (s, 1H, NH_2) (see A1 in Appendix A).

Table 3.1 A list of experiments to study the conversion of Glu to pGlu as a function of temperature, time and Glu to D_2O ratio.

Temperature (°C)	Time (hours)	Glu to D_2O ratio (mg/mL)
80	4, 8, 16 and 24	7
99	4, 8, 16 and 24	7, 50 and 100
99	24	150 and 200
120	24	7
120	0, 1, 2, 3, 4, 8 and 24	200

3.2.3 Conversion of Glu to pGlu under melt conditions

In a typical procedure, Glu (735.7 mg, 5.00 mmol) was added to a glass test tube and was reacted for 15 minutes in an oil bath maintained at 185 °C. After this time the reaction mixture was completely molten. The molten Glu is yellowish and water droplets were formed near the opening of the tube. The test tube was then transferred to an oil bath maintained at a temperature of 150 °C. Heating was continued for a further 45 minutes.

Percentage conversion as determined by ^{1}H-NMR: 100 mol%; isolated yield: 102 wt% (due to presence of water). ^{1}H-NMR: δ 1.92-2.23 (m, 1H, β-CH_2), 2.25-2.40 (m, 2H, γ-CH_2), 2.40-2.56 (m, 1H, β-CH_2), 4.25-4.43 (q, 1H, α-CH) (see A2 in Appendix A).

3.2.4 Stability of Asp

Under aqueous conditions, Asp (904.6 mg, 6.80 mmol) and D_2O (5.0 mL) were added to an autoclave and heated to 120 °C for 3 hours. After that the reaction suspension was collected and dried by freeze drying. The obtained dry white product was in white powder form and was analyzed by ^{1}H-NMR. Where reactions were performed in the melt, Asp (666.5 mg, 5.00 mmol)

was added to a glass test tube and heated to 180 °C for 45 minutes. Asp kept its original white color and powder form during and after the incubation. ^{1}H-NMR: δ 2.81-3.00 (m, 2H, β-CH_2 of Asp), 3.92-4.06 (q, 1H, α-CH of Asp), 6.272 (s, 2H, CH of maleic acid), 6.716 (s, 2H, CH of FUM) (see A3 in Appendix A).

Under melt conditions of Glu, the stability of Asp was also studied. Asp (665.5 mg, 5.00 mmol) was added to a glass tube and incubated at 185 °C for 15 minutes and then moved to an oil bath maintained at 150 °C. Heating was continued for a further 45 minutes. During the whole process, Asp kept its white powder form and no water droplets were found inside the tube. ^{1}H-NMR: δ 2.81-3.01 (m, 2H, β-CH_2), 3.94-4.06 (q, 1H, α-CH) (see A3 in Appendix A).

3.2.5 Hydrolysis of pGlu to Glu

To study the reaction under acidic conditions, pGlu (129.1 mg, 1.0 mmol) and 1.0 mL of H_2SO_4 solution in D_2O (1 M or 2 M) were added to an Eppendorf® tube and reacted at 99 °C for 1, 2, 4 and 6 hours. ^{1}H-NMR: δ 1.89-2.11 (m, 2H of Glu and 1H of pGlu, γ-CH_2 of Glu and β-CH_2 of pGlu), 2.12-2.51 (m, 2H of Glu and 3H of pGlu, β-CH_2 of Glu, β-CH_2 of pGlu and 1 of β-CH_2 of pGlu), 3.86-3.98 (t, 1H of Glu, α-CH), 4.13-4.22 (q, 1H of pGlu, α-CH) (see A4 in Appendix A).

To study the reaction under basic conditions, pGlu (129.1 mg, 1.0 mmol) and 1.0 mL of NaOH solution in D_2O (2 M) were added to an Eppendorf® tube and reacted at 99 °C for 1, 2, 4 and 6 hours. ^{1}H-NMR: δ 1.52-1.81 (m, 3H of Glu and 1H of pGlu, 1 of β-CH_2 and 2 of γ-CH_2 of Glu, and 1 of β-CH_2 of pGlu), 1.81-1.94 (q, 1H of pGlu, γ-CH_2), 1.97-2.13 (t, 1H of Glu and 1H of pGlu, β-CH_2 of Glu and γ-CH_2 of pGlu), 2.17-2.41 (q, 1H of pGlu, β-CH_2), 3.02-3.14 (t, 1H of Glu, α-CH), 3.97-4.07 (q, 1H of pGlu, α-CH) (see A4 in Appendix A).

3.2.6 Selective formation of pGlu as a means of separation of Asp and Glu (aqueous conditions)

During conversion under aqueous conditions, Asp (904.6 mg, 6.80 mmol), Glu (1000 mg, 6.80 mmol) and D_2O (5.00 mL) were added to the autoclave and reacted at 120 °C for 3 hours. After that, the reaction suspension was cooled to room temperature and the clear solution and the precipitates were separated by centrifugation. The precipitate was washed twice with 5.0 mL of

D_2O. The washing solution was combined with the original solution fraction. Both the precipitate and the solution phases were dried by freeze drying, weighed and dissolved in D_2O for ^{1}H-NMR analysis. Weight of the dry precipitate: 693 ± 27 mg; Composition (in mol%): 78 ± 1% of Asp, 6 ± 1% of Glu, 17 ± 1% of pGlu; Overall recovery of Asp: 60 ± 1%; ^{1}H-NMR: δ 2.00-2.17 (m, 2H of Glu and 1H of pGlu, γ-CH_2 of Glu and 1 of β-CH_2 of pGlu), 2.28-2.55 (m, 2H of Glu and 3H of pGlu, β-CH_2 of Glu, γ-CH_2 of pGlu and 1 of β-CH_2 of pGlu), 2.79-3.01 (m, 2H of Asp, β-CH_2), 3.74-3.83 (t, 1H of Glu, α-CH), 3.94-4.06 (m, 1H of Asp, α-CH), 4.24-4.33 (q, 1H of pGlu, α-CH) (see A5 in Appendix A). Weight of the solid obtained from the solution: 1096 ± 16 mg; Water content of the solid determined by Karl Fischer titration (150 °C): 5.7 ± 0.5 wt% (corresponding dry weight: 1034 ± 10 mg); Composition (in mol%): 13 ± 1% of Asp, 6 ± 1% of Glu, 81 ± 1% of pGlu; ^{1}H-NMR: δ 2.03-2.18 (m, 2H of Glu and 1H of pGlu, γ-CH_2 of Glu, and 1 of β-CH_2 of pGlu), 2.27-2.58 (m, 2H of Glu and 3H of pGlu, β-CH_2 of Glu, γ-CH_2 of pGlu and 1 of β-CH_2 of pGlu), 2.81-2.99 (m, 2H of Asp, β-CH_2), 3.83-3.88 (t, 1H of Glu, α-CH), 4.05-4.11 (m, 1H of Asp, α-CH), 4.26-4.34 (q, 1H of pGlu, α-CH) (see A5 in Appendix A).

The solid obtained from drying the reaction solution was used for further hydrolysis experiments following a similar procedure as described before. The solid (129.1 mg, containing Asp and some remaining Glu) was added to 1.0 mL of H_2SO_4 solution in D_2O (2 M) and reacted at 99 °C for 2 hours. The reaction solution was then analyzed by ^{1}H-NMR. The precipitation of Glu was carried out by adjusting the pH to 3.2 (IP of Glu) using NaOH solution in D_2O (5M). The precipitate was collected by centrifugation, dried under vacuum and both fractions analyzed by ^{1}H-NMR. Composition of the hydrolysis products in solution (in mol%): 12 ± 1% of Asp, 88 ± 1% of Glu; ^{1}H-NMR: δ 1.98-2.19 (m, 2H of Glu, γ-CH_2), 2.40-2.66 (m, 2H of Glu, β-CH_2), 2.90-3.07 (m, 2H of Asp, β-CH_2), 3.93-4.05 (t, 1H of Glu, α-CH), 4.22-4.29 (t, 1H of Asp, α-CH) (see A5 in Appendix A). Composition of the precipitate from hydrolysis solution (in mol%): 6 ± 1% of Asp, 94 ± 1% of Glu; ^{1}H-NMR (precipitated hydrolysis product): δ 2.07-2.26 (m, 2H of Glu, γ-CH_2), 2.48-2.67 (m, 2H of Glu, β-CH_2), 2.88-3.03 (m, 2H of Asp, β-CH_2), 3.78-3.89 (t, 1H of Glu, α-CH), 4.01-4.08 (s, 1H of Asp, α-CH).

Incomplete precipitation of the Glu was attributed to the small scale of the experiment. Precipitation of products was then carried out at larger scale using a mixture of the same

composition containing Asp (439 mg, 3.3 mmol, 12 mol%) and Glu (3619 mg, 24.6 mmol, 88 mol%) in 30 mL of H_2SO_4 (2 M), the pH was then adjusted to pH 3.2 (IP of Glu) using NaOH solution (5 M) and cooled to 4 °C to allow precipitation. The flask was then placed at room temperature overnight to dissolve any sodium sulfate which might have been precipitated. The precipitate was collected by centrifugation and dried under vacuum. Weight obtained: 3904 ± 8 mg; Composition according to ^{1}H-NMR: 93 mol% of Glu; Glu recovery by precipitation after hydrolysis: 101 ± 1%. Overall recovery of Glu: 83 ± 1%.

3.2.7 Selective formation of pGlu as a means of separation (melt conditions)

During the conversion under melt conditions, Asp (665.5 mg, 5.00 mmol) and Glu (735.7 mg, 5.00 mmol) were added to a glass test tube and reacted under melt conditions as previously described. When the reaction was complete, 3.00 mL of hot D_2O was added to the melt to dissolve the pGlu product. After cooling to room temperature, the solution was separated from the white precipitate by centrifugation and the precipitate washed twice with 1.00 mL of D_2O. The washing solutions were combined with the original pGlu fraction. The precipitate and the solution fractions were freeze dried, weighed and dissolved in D_2O for ^{1}H-NMR analysis. The solution fraction was also analyzed by gel permeation chromatography (GPC) according to reported method.[22] Weight of the dry precipitate: 532 ± 21 mg; Composition (in mol%): 100% of Asp (no other components were detected by ^{1}H-NMR); Overall recovery of Asp: 80 ± 3%. ^{1}H-NMR (precipitate): δ 2.83-3.01 (m, 2H, β-CH_2 of Asp), 3.95-4.06 (q, 1H, α-CH of Asp) (see A6 in Appendix A). Weight of the solid obtained from the solution: 741 ± 26 mg; Water content of the solid determined by Karl Fischer titration (150 °C): 3.8 ± 1.4 wt% (corresponding dry weight: 714 ± 35 mg); Composition (in mol%): 7 ± 1% of Asp, 93 ± 1% of pGlu; GPC: weight average molecular weight of the oligomers is 1255 ± 53 Da; ^{1}H-NMR: δ 1.87-2.20 (m, 1H of pGlu, β-CH_2), 2.23-2.39 (m, 2H of pGlu, γ-CH_2), 2.39-2.56 (m, 1H of pGlu, β-CH_2), 2.92-3.02 (m, 2H of Asp, β-CH_2), 4.13-4.20 (m, 1H of Asp, α-CH), 4.22-4.40 (q, 1H of pGlu, α-CH) (see A6 in Appendix A).

The solid obtained from drying the reaction solution was used for further hydrolysis experiments, precipitated and analyzed by ^{1}H-NMR following a similar procedure as described.

The precipitation and analysis of Glu was carried out in the same way as in former section. Composition of the hydrolysis product in solution (in mol%): 9 ± 1% of Asp, 91 ± 1% of Glu; ^{1}H-NMR: δ 1.98-2.20 (m, 2H of Glu, γ-CH_2), 2.22-2.65 (m, 2H of Glu, β-CH_2), 2.96-3.02 (t, 2H of Asp, β-CH_2), 3.92-4.05 (t, 1H of Glu, α-CH), 4.23-4.29 (t, 1H of Asp, α-CH) (see A6 in Appendix A). Composition of the precipitate from hydrolysis solution (in mol%): 7 ± 1% of Asp, 93 ± 1% of Glu; ^{1}H-NMR (precipitated hydrolysis product): δ 1.98-2.17 (m, 2H of Glu, γ-CH_2), 2.37-2.57 (m, 2H of Glu, β-CH_2), 2.79-2.93 (m, 2H of Asp, β-CH_2), 3.69-3.79 (t, 1H of Glu, α-CH), 3.90-4.00 (q, 1H of Asp, α-CH).

Incomplete precipitation of the Glu was attributed to the small scale of the experiment. Precipitation of products was then carried out at larger scale using a mixture of the same composition containing Asp (359 mg, 2.7 mmol, 9 mol%) and Glu (3884 mg, 26.4 mmol, 91%) in 30 mL of H_2SO_4 (2 M), the pH was then adjusted to pH 3.2 (IP of Glu) using NaOH solution (5 M) and precipitation was carried out as described. The precipitate was collected by centrifugation and dried under vacuum. Weight obtained: 4178 ± 9 mg; Composition according to ^{1}H-NMR: 90 mol% of Glu; Glu recovery from hydrolysis: 98 ± 1%. Estimated overall recovery of Glu: 101 ± 5%.

3.3 Results and discussion

3.3.1 Study on the Conversion of Glu to pGlu

The water solubility of pGlu is 476.0 g/L (at 13 °C), while the solubility of Asp and Glu are 4.95 g/L and 8.61 g/L (at 25 °C) respectively.[17] If Glu could be specifically converted to pGlu in a mixture of Glu and Asp, the pGlu formed should be able to be separated due to its enhanced solubility in water. As reported, the conversion of Glu to pGlu can be easily carried out by having Glu incubated with water at temperature higher than 150 °C.[23,24] However, under such conditions the deamination of Asp could also take place.[20,21] The main deamination product of Asp is FUM.[25] FUM has a similar solubility in water as Asp and Glu,[17] therefore it further complicates the mixture to be separated and should be eliminated (Fig. 3.2). The hydrothermal deamination of Asp starts at 110 °C and becomes pronounced at 150 °C,[21] so the conversion should not be

carried out at too high temperature. On the other hand, literature reported that pGlu can be found in vinasse, a by-product of sugar industry.[26] This indicates that even at temperatures which enable the distillation of ethanol, the conversion of Glu to pGlu takes place. Based on this, the temperatures studied were 80, 99 and 120 °C. The reaction under aqueous conditions is in equilibrium,[27] so there is an optimum set of conditions for the conversion to reach equilibrium. Mixtures containing higher amounts of Asp and Glu are desired, as after reaction the pGlu formed has a high solubility in the aqueous phase, and that the unreacted Asp (and Glu) has a low solubility in water and is effectively precipitated therefore improving the overall separation. Thus the impact of Glu to water ratio was also explored. Related to this, if water could be removed during the conversion, it might be possible that the conversion could go to completion, therefore it is also interesting to study the conversion under melt conditions taking care that Asp is stable under such conditions.

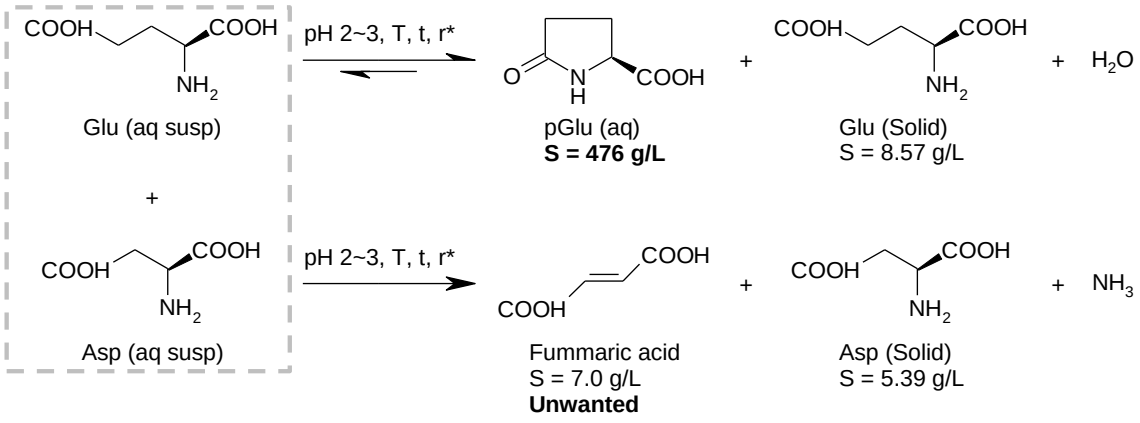

Fig. 3.2 Conversion of Glu to pGlu and Asp to FUM. Specificity for the formation of pGlu should be achieved by applying the most selective reaction conditions.

* Temperature (T), time (t) and the Glu to water ratio (r).

3.3.2 Conversion of Glu to pGlu under aqueous conditions

Fig. 3.3 A shows the effect of time, temperature and Glu to D_2O ratio on the conversion of Glu to pGlu. It is seen that conversion is influenced by time, temperature, and not by amount of Glu. From Fig. 3.3 B, it is seen that the ratio of Glu to D_2O shows little difference on the conversions

obtained at 99 °C. At 120 °C a similar observation is seen, although the overall conversion is much higher. From Fig. 3.3 B however, it can be seen that the optimal ratio of Glu to D_2O appears in the range of 50 to 150 mg/mL when reactions were carried out at 99 °C for 24 hours, while in comparison reactions carried out at 120 °C showed a consistently high conversions over the whole range. This may be explained by the greater solubility of Glu at higher temperatures and the higher rate of reaction to highly soluble pGlu. Since a higher amount of Glu is desired to aid separation, as described earlier, 200 mg/mL could be applied with no negative influence on the reaction. At such a ratio, the equilibrium and maximum conversion (86 mol%) is reached after 3 hours. Since the aim is to apply the conversion to induce the separation of Asp and Glu, it is necessary to check the effect of Asp on the conversion rate as well as the stability of Asp. At 99 °C, the presence of Asp does not appear to alter the reaction kinetics (data not shown). A further study about the stability of Asp was also carried out.

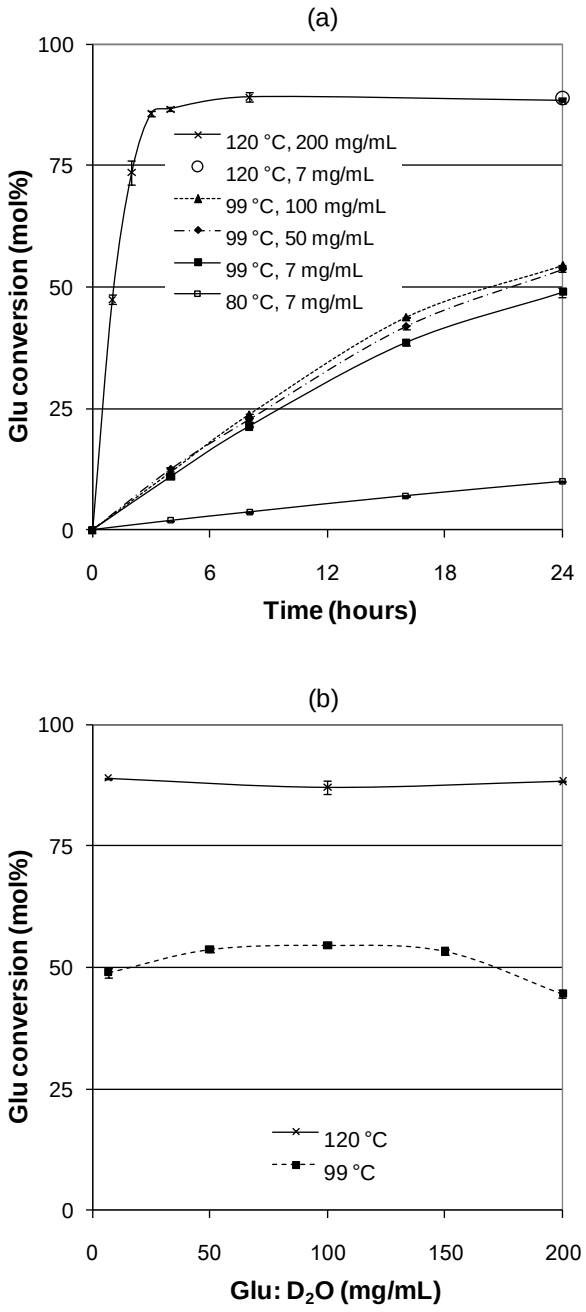

Fig. 3.3 A. The conversion of Glu to pGlu under aqueous conditions as a function of time, temperature and Glu to D_2O ratio (in w/v). The error bars represent standard deviation. **B.** The effect of the amount of Glu on the conversion of Glu to pGlu under aqueous conditions for 24 hours at 99 °C and 120 °C.

It has been reported that elevated temperatures will lead to an increase in the rate of racemization and deuterium exchange of AAs at the α-CH, this includes pGlu.[28,29] In this case, it could cause problems if pGlu would be used for chiral synthesis. It also has a negative impact when pGlu is hydrolyzed to Glu and further used for enzymatic reactions, as most of those enzymes react specifically with AAs with an L-configuration as their substrate.[6,30]

Acidic hydrolysis of proteins can also lead to racemization of AAs,[31] therefore it is also possible that under our experimental conditions (pH 2~3, native pH of Glu and pGlu) that this also takes place. In our experiments, the deuterium-proton exchange at the α-CH was also observed. As seen from Fig. 3.4, the experimental integration of β-CH_2 and γ-CH_2, are larger than the theoretical values calculated from the integration of α-CH. Since the exchange takes place at α-CH, the conversion of Glu obtained from ^{1}H-NMR results should be calculated based on the integration of the β-CH_2 and γ-CH_2.

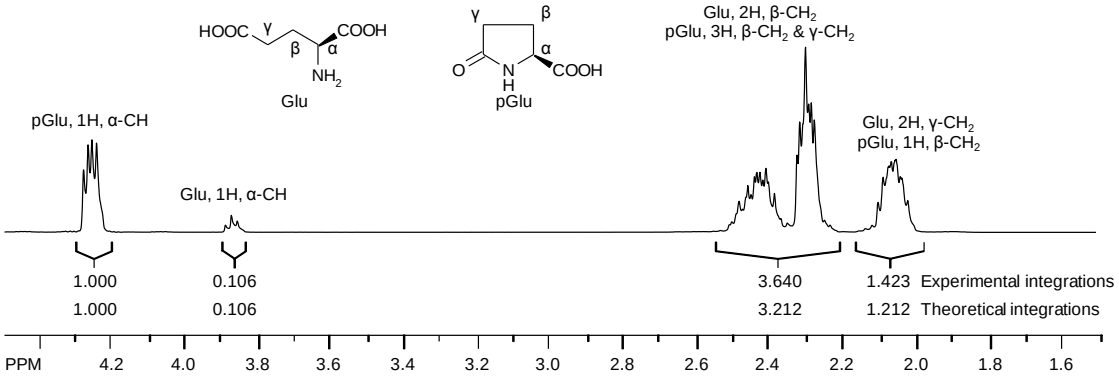

Fig. 3.4 ^{1}H-NMR spectra showing the deuterium-proton exchange on the α-CH after the conversion of Glu to pGlu. The reaction conditions are: 200 mg Glu/ mL D_2O, reacted for 24 hours at 120 °C.

As well as the issue of racemization/proton exchange, the rate of deamination of Asp will also increase with time and temperature. For example, after 2 hours reaction in water, Asp was found to be quite stable from 110 to 150 °C, but at 180 °C more than 50% is deaminated.[21] Since the aim is to use the reaction as an aid for the separation, it should be specific enough when applied

to the mixture of Asp and Glu. The stability of Asp (904.6 mg / 6.80 moles in 5 ml) was studied under the desired reaction conditions (120 °C, 3 hours). Deuterium-hydrogen exchange on the α-CH of Asp was also observed. Therefore, deamination was estimated based on the integration of the β-CH_2 and γ-CH_2. Only trace of FUM and maleic acid were detected in the ^{1}H-NMR. This is similar to what Sohn found after 2 hours reaction time.[21] It can be concluded when the mixture of Asp and Glu used, then reaction would be specific for the desired separation.

3.3.3 Hydrolysis of pGlu to Glu

Although pGlu helps the separation and has applications in chemical industry,[18,19] ideally Glu should be recovered from pGlu as the former could be used for making chemicals.[8,30] Since the conversion of Glu to pGlu is reversible in water and pH-depended,[27] the recovery of Glu from pGlu was achieved by hydrolysis using acid (H_2SO_4) or base (NaOH) (Fig. 3.5). As might be expected, using the same concentrations the acidic hydrolysis of pGlu is more rapid. It was observed that the conversion of pGlu was found to increase with acid concentration. This is probably due to protonation of the primary amine group of Glu pulling the reaction equilibrium to Glu formation. The optimal condition to recover Glu from pGlu is by reaction for 2 hours in 2 M of H_2SO_4.

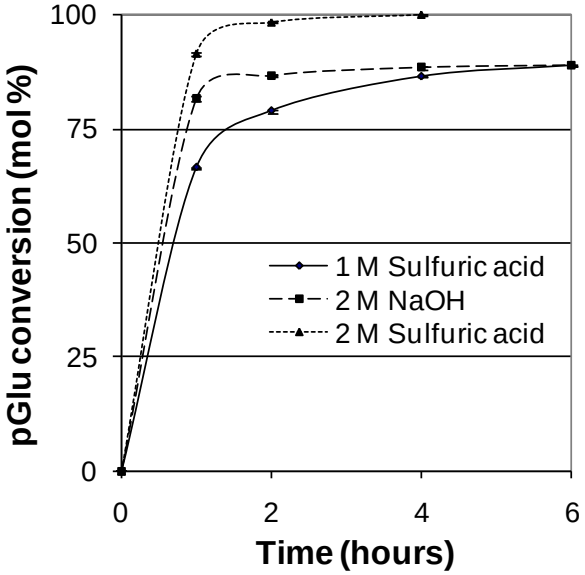

Fig. 3.5 Conversion of pGlu to Glu under acidic and basic conditions at 99 °C. 1.0 mmol of pGlu and 1 mL of H_2SO_4 (1 M or 2 M) or NaOH (2 M) solution in D_2O was used for the reaction.

3.3.4 Selective formation of pGlu as a means of separation (aqueous conditions)

The conversion of Glu to pGlu as a means of separating acidic AAs was studied by adding the same number of moles of Asp into the reaction system. As shown in Table 3.2 the major components of the precipitate and the solution fractions are Asp and pGlu respectively, indicating a preliminary separation was achieved. In both fractions, only a trace amount of FUM was found. The amount of Asp present in the solution is greater than expected arising from the solubility of Asp in water. The increased amount may be attributed to incomplete precipitation and/or enhanced solubility in pGlu. Here high conversions of Glu to pGlu are obtained (91 mol%) and this is comparable those obtained without Asp present (86 mol%), indicating that Asp has little impact on the conversion. The total number of moles of Glu and pGlu obtained is higher than that of Glu added. This is attributed to errors in determining composition based on the integrations by ^{1}H-NMR. As well as this it is also possible that the water content is under estimated by the Karl Fischer titration. This is because the temperature used was 150 °C to avoid possible reaction of

unreacted Glu, so complete removal of water may not have been achieved. By comparison, even if reactions were carried out under melt conditions (185 °C for 15 minutes and continued at 150 °C for 45 minutes) the weight of pGlu was still more than expected due to the presence of residual water. The losses in Asp are attributed to physical losses and that some Asp remains in the aqueous phase.

The results from the hydrolysis experiments indicates that all the pGlu was converted to Glu and that deamination of Asp did not occur, this may be due to the stability of Asp under acidic conditions and the low temperatures used.[20] However, under hydrolysis conditions Asp and Glu remained soluble in water as a salt. Here, $NaOH_{(aq)}$ was added to adjust the pH to the IP of Glu to allow the most favorable conditions for the precipitation of Glu. The results show that the precipitation contains much less Asp than the reaction solution and that overall the purity of Glu is increased to 94 mol% (Table 3.2). In small scale experiments the mass recovered is low due to physical losses and so larger scale precipitation of the mixture (with the same composition) was required to obtain a more accurate value. It is worth noting that the amounts of sodium sulfate present is lower than the solubility (at 20 °C it is *ca.* 200 g/L in water), and should not be a problem for the purity of the Glu precipitated.

Table 3.2 Mole balance of the AAs before the formation of pGlu, after the separation and the composition of the hydrolysis product of the solution part.

Separation procedure	**Aqueous conditions**			**Melt conditions**		
	Asp (mmol) (mol%)	**Glu (mmol) (mol%)**	**pGlu (mmol) (mol%)**	**Asp (mmol) (mol%)**	**Glu (mmol) (mol%)**	**pGlu (mmol) (mol%)**
Before conversion (mmol)	6.78	6.78	0	5.00	5.00	0
After conversion (dry precipitate)	4.04 ± 0.10 (78 ± 1%)	0.29 ± 0.03 (6 ± 1%)	0.88 ± 0.09 (17 ± 1%)	3.99 ± 0.16 (100%)	0 (0%)	0 (0%)
Overall recovery of Asp	*60 ± 1%*	–	–	*80 ± 3%*	–	–
After conversion (dry solid obtained from solution)	1.06 ± 0.09 (13 ± 1%)	0.46 ± 0.03 (6 ± 1%)	6.39 ± 0.02 (81 ± 1%)	0.36 ± 0.03 (7 ± 1%)	0 0%	5.15 ± 0.24 (93 ± 1%)
Composition of hydrolyzed solution fraction (mol%)	12 ± 1%	88 ± 1%	0%	9 ± 1%	91 ± 1%	0%
Composition of precipitate from hydrolysis (mol%)	6 ± 1%	94 ± 1%	0%	7 ± 1%	93 ± 1%	0%
Glu recovery by precipitation after hydrolysis*	–	*ca.* 100%	–	–	*ca.* 98%	–
Overall recovery of Glu	–	*83 ± 1%*	–	–	*101 ± 5%*	–

* based on the results of the larger scale precipitation

3.3.5 Selective forming of pGlu as a means of separation (melt conditions)

The main limitation of converting Glu to pGlu under aqueous conditions is that it never is complete. That is because water is used as the solvent, but it is also the product of the conversion.

However, running the conversion under melting conditions does not have such problem as it do not require any solvent for the ionization of Glu and the water formed will be removed from the reaction system immediately due to the evaporation. As well studied by Feng, the best reaction conditions for the conversion is 45 minutes at 150 °C.[29] By repeating the reported procedure with only Glu presented, it was found that the product obtain is absent of the signal of Glu according to ^{1}H-NMR, so apparently the conversion is 100%. On the other hand, Asp was found to be stable when it was incubated at 185 °C for 45 minutes. So the conversion is possible to be used as a means for the separation. This was studied by applying the reported conditions on the mixture of Asp and Glu (1 : 1 in mole ratio).

From Table 3.2 it can be seen that after conversion, the precipitate obtained is highly pure Asp. This is attributed to the fact that water was added after reaction and the lower temperatures and shorter contact times do not allow Asp to be solubilized. In the solution, the majority is highly soluble pGlu but also contains a small amount of Asp (7 mol%). From the ^{1}H-NMR spectrum of this fraction it was found that the peaks were consistent with Asp although they were quite broad. It was speculated if this was due to (partial) formation of oligomers during the high temperature reaction where water was removed. From GPC the presence of oligomers with a weight average molecular weight of *ca.* 1300 Da were confirmed. Hydrolysis of the solution fraction resulted in complete conversion of pGlu to Glu. As under melt conditions the conversion of Glu appears to be 100%, this improves the separation and hence the overall recovery of the final Glu isolated (see Table 3.2).

Using a process of separation based on reaction and change in water solubility difference, it was found that the conversions of Glu to pGlu under both aqueous and melting conditions are specific to help the separation of a mixture of Asp and Glu. It is possible to recover Glu from pGlu by hydrolysis under acidic and basic conditions with high yields. This research focuses on the separation of a mixture containing Glu and Asp which could be readily obtained by ED separation or pH-dependent precipitation of protein hydrolysates. It may be considered that the method described might be extended to more complex mixtures of AAs containing Glu in the form of protein hydrolysates. In practice, the similarities in water solubility and thermal stability of the AAs would limit the application of the method described.

3.4 Conclusions

The aim is to design a process based on the formation and hydrolysis of pGlu which could be used as a means of separating Asp and Glu derived from biomass. The specific formation of pGlu can be done under both aqueous and melt conditions. After hydrolysis, Glu obtained from both conditions are similar in purity (*ca.* 94 mol%), but under melt conditions the overall recovery of Asp and Glu are both about 20 mol% higher and Asp obtained is highly pure. Such a process offers potential for the separation of Asp and Glu and to use them for making chemicals.

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Chapter 4

The selective conversion of glutamic acid in amino acid mixtures using glutamate decarboxylase – a means of separating amino acids for synthesizing biobased chemicals

This Chapter is based on

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The selective conversion of glutamic acid in amino acid mixtures using glutamate decarboxylase – a means of separating amino acids for synthesizing biobased chemicals

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Abstract

Amino acids (AAs) derived from hydrolysis of protein rest streams are interesting feedstocks for the chemical industry due to their functionality. However, separation of AAs is required before they can be used for further applications. Electrodialysis (ED) may be applied to separate AAs, but its efficiency is limited when separating AAs with similar isoelectric points. To aid the separation, specific conversion of an AA to a useful product with different charge behavior to the remaining compounds is desired. Here the separation of L-aspartic acid (Asp) and L-glutamic acid (Glu) was studied. L-Glutamate α-decarboxylase (GAD, type I, EC 4.1.1.15) was applied to specifically convert Glu into γ-aminobutyric acid (GABA). GABA has a different charge behavior from Asp therefore allowing a potential separation by ED. Competitive inhibition and reduced operational stability caused by Asp could be eliminated by maintaining a sufficiently high concentration of Glu. Immobilization of GAD doesn't reduce the enzyme's initial activity. However, the operational stability was slightly reduced. An initial study on the reaction operating in a continuous mode was performed using a column reactor packed with immobilized GAD. As the reaction mixture was only passed once through the reactor, the conversion of Glu was lower than expected. To complete the conversion of Glu, the stream containing Asp and unreacted Glu might be recirculated back to the reactor after GABA has been removed. Overall, the reaction by GAD is specific to Glu and can be applied to aid the ED separation of Asp and Glu.

4.1 Introduction

Today's chemical industry relies on fossil fuels as both an energy and carbon source. However, increasing fuel, energy and material demand coupled with depleting and limited reserves, fluctuating and overall increasing price as well as environmental arguments with regards to CO_2 emission and geopolitical issues, are driving people to find alternatives for fossil fuels.[1] As a result, biomass as a renewable resource for the production of fuels and chemicals is attracting more attention.[2] Since biomass has a complex composition, efficient biorefinery processes are required for the efficient separation and isolation of suitable fractions to marketable products for various applications.[3] Thus fractions for food or animal feed can be obtained, as well as those for non-food applications such as energy, fuels and chemicals. Currently, the major focus of using biomass is for the production of biofuels. For example, the U.S. Energy Information Administration (EIA) has shown that in 2011 the globe fuel ethanol production was *ca.* 545 million barrels (*ca.* 87 million m^3) and that of biodiesel production was *ca.* 147 million barrels (*ca.* 23 million m^3).[4] However, the potential of using biomass to make chemicals has been less well studied.

There are several reasons why the chemical industry can consider the replacement of fossil fuels with biomass. Biomass reduces dependency on fossil fuels,[2] and it is considered to be carbon neutral, therefore it could contribute to reducing greenhouse gas emissions.[6] Most importantly it is the only reliable carbon source besides fossil fuels to produce chemicals and materials.[5] As well as this it also contains functional groups which are required for producing organic chemical products.[1] For example, lactic acid and 1,3-propropanediol can be produced by fermentation and used to make polymers and glycerol can be used as a feedstock for the production of epichlorohydrin.[8-10] It has also been described that the inherent functionality could lead to a reduction in energy, heat transfer, processing steps and extra chemicals needed to produce functionalized chemicals.[11] In the case of nitrogen containing chemicals, amino acids (AAs) could also be considered as their functional groups, amine and carboxyl groups, as well as phenyl (*e.g.* phenylalanine) and hydroxyl groups (*e.g.* serine) give rise to products that are similar to those currently being produced.[1]

AAs are the building blocks of proteins, which could be obtained from hydrolysis of potentially inexpensive proteins obtained from the by-product of biofuel production. These include dried distillers grains with solubles (DDGS) from bioethanol production and press cake from rapeseed.[4] Considering the possible growth of biofuel production in the future, this will give a large volume of protein containing by-products, therefore making AA feedstocks more available.[1] Other possible AA sources can be agricultural waste streams (*e.g.* feather meal and vinasse) and non-food part of crops (*e.g.* grass juice).[12,13] For example Brazil produces about 275 billion liters of cane vinasse per year as a by-product of bioethanol production, which is equivalent to 2.7 million tons of residual crude protein.[12]

The production of chemicals from AAs is relatively new and most of these studies focus on the use of single AAs. For example, the production of acrylamide from L-aspartic acid (Asp),[6] and *N*-methylpyrrolidone and acrylonitrile from L-glutamic acid (Glu) via the formation of γ-aminobutyric acid (GABA).[8,7] Currently single AAs are often produced by fermentation which would make the cost of production of bulk chemicals prohibitive.[1,10] Another approach is to obtain them from inexpensive hydrolyzed protein as described earlier. Thus the issue of separation of individual AAs becomes a key issue. Known methods of AA separation include chromatography, reactive extraction and ED.[18-21] The latter is very promising as it is a continuous process which may be carried out on large scale (*e.g.* desalination of sea water and whey).[22]

In principle, ED could separate AAs into acidic, basic and neutral AA streams according to charge difference.[20] However, a further separation within each stream remains difficult due to the similarities in chemical and physical behavior (*e.g.* pH dependent charge and solubility). For example, acidic AAs including Asp and Glu have comparable isoelectric points (2.77 and 3.22, Fig. 4.1), and their solubility in water are both small and similar (5.36 and 8.57 g/L at 25 °C).[23,24] To help further separation, one can think of introducing specific modification which changes this behavior. If the modification product itself is a useful chemical product or intermediate, then an integrated *in-situ* product formation and recovery (ISPR) process can be created which saves process steps and energy input.[11,25]

In this article, we describe the specific modification of Glu in the presence of Asp by applying L-glutamate α-decarboxylase (GAD, type I, EC 4.1.1.15) which specifically converts Glu to

GABA, which can be further converted to other chemicals as previously derscribed.[8,7] Additionally GABA has a significantly higher isoelectric point (pH 7.19) and solubility in water (1300 g/L).[23,24] For example, at pH 3.5 GABA (positively charged) can be separated from remaining Asp and Glu (almost neutral) by charge difference using ED (Fig. 4.1), or by solid-liquid separation.[26] It has been found that high recoveries (90%) of Asp and Glu can be obtained using ED however further separation was not possible. When Asp and GABA were used as a medium then separation was indeed possible.[21]

Although the kinetics and immobilization of GAD was known,[7] the use of it as a means to carry out a specific reaction within a mixture to deliberately modify charge and aid potential product separation is novel. Here the enzyme specificity and inhibitory effect of Asp present were studied. To determine optimal reaction conditions, the effect of Asp on the operational stability of GAD and immobilized GAD (IGAD) was also explored using a mixture of Asp and Glu as both the substrate and titrating feed solution. For potential use of the reaction in a separation process initial studies on a continuous reaction employing a column reactor containing immobilized IGAD was investigated.

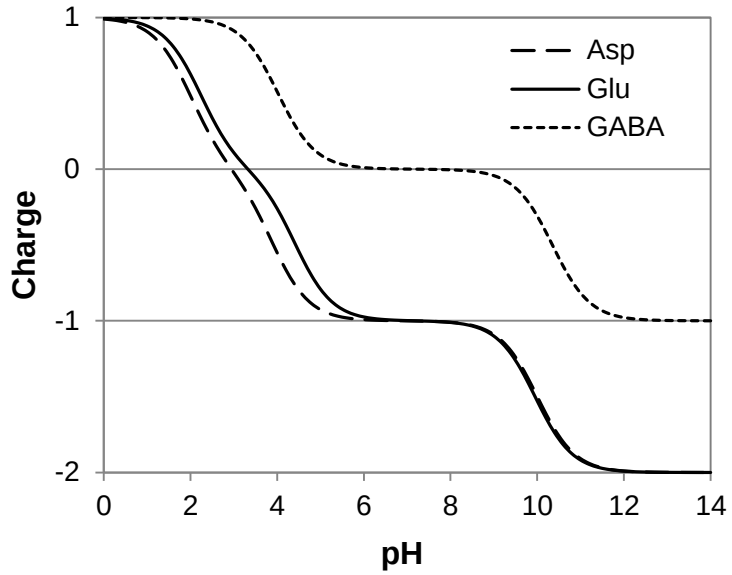

Fig. 4.1 Charge behaviors of Asp, Glu and GABA as a function of pH.[23]

4.2 Experimental

4.2.1 Materials

L-Glutamate α-decarboxylase (type I, from *E. coli*, EC 4.1.1.15), sodium alginate (low viscosity), L-aspartic acid (≥ 98%), L-glutamic acid (≥ 99.5%), pyridoxal 5'-phosphate monohydrate (PLP·H_2O, ≥ 97%), HCl standard solution (volumetric solution, 0.1 M) were ordered from Sigma-Aldrich. Calcium carbonate (≥ 99.0%) was ordered from Fluka®. Bovine Serum Albumin standard set and Quick Start Bradford dye reagent were obtained from Bio-Rad. All the solutions were made with Milli-Q (Millipore) purity water at room temperature.

4.2.2 Software for pH simulation

CurTiPot 3.5.4 (Feb/2010) for MS-Excel® was used to simulate pH values of amino acid(s) solutions.

4.2.3 Analysis

In the Bradford protein analysis, the absorbance of samples was determined at the wavelength of 595 nm by a Multiskan Spectrum® microplate spectrophotometer (Thermo Labsystem®).

Ultra-high pressure liquid chromatography (U-HPLC) was used to analyze the amino acids. Dionex U-HPLC instrument (Dionex Corporation, Sunnyvale, CA, U.S.) was used and the method employed was previously described.[27] For sample preparation, the sample or standard solution (500 μL, 0.5-0.025 mM), methanol (400 μL) and internal standard solution (0.4 mM of taurine, 100 μL) were added to an Eppendorf safe-lock tube and mixed. The derivatization reagents used were *ortho*-phthalaldehyde, ethanethiol and 9-fluorenylmethyl chloroformate. The separation of AAs were performed using an Acquity U-HPLC® BEH C18 reversed phase column and a Acclaim® 120 C18 guard column at the flow rate of 1 mL/min at 65 °C. The detection was performed using a variable wavelength detector at 338 nm.

4.2.4 GAD immobilization

Immobilization was carried out by entrapment of GAD in calcium alginate based on the reported procedure.[7] Solid GAD (10 mg) was dissolved in 5 mL of sodium acetate buffer (0.2 M, pH 4.6). 5 mL of sodium alginate solution in water (4 wt%) was added and mixed well to form a GAD solution (1 mg/mL). The GAD solution was added dropwise in portions of 1 mL to 10 mL of $CaCl_2$ solution (1 wt%) to form calcium alginate beads with a diameter of approximately 1.5 mm. The supernatant from the immobilization liquor was analyzed by a Bradford protein assay. The beads were stored in sodium acetate buffer (10 mM, pH 4.6) containing $CaCl_2$ (50 mM) and PLP (0.5 mM) at 4 °C.

4.2.5 Assay to determine initial activity

The initial activities of native and IGAD was assayed using a pH-stat method according to the reported procedure.[7] The titrations were performed in a titration vessel with a thermostatic jacket (± 0.1 °C) and monitored using a Metrohm 718 STAT Titrino®. In a typical procedure, the substrate solution was Glu (20 mM) solution containing PLP·H_2O (0.5mM); the titrating solution was $HCl_{(aq)}$ (0.1 M) or $Glu_{(aq)}$ (50 mM) (fed batch titration); the enzyme solution was 10 mg/mL of GAD solution in sodium acetate buffer (0.2 M, pH 4.6) containing PLP·H_2O (0.5 mM). At 40 °C, the substrate solution was added into the titration vessel and brought to pH 4.6 by adding enough NaOH solution (5 M) when native GAD was used, or by adding powder form $CaCO_3$ when IGAD was used. Afterwards, 0.1 mL of the enzyme solution or IGAD containing 1.0 mg of GAD was added to the substrate solution to start the reaction. The initial activity was expressed as U/mg (μmol H^+ min^{-1} mg^{-1}) and was determined as the function of the slope of the linear part of the titration curve (time: ~20 min).

To determine the K_m value to Glu, the concentration of Glu in substrate solution was adjusted to 1.0, 2.0, 5.0, 10.0, 20.0 and 40.0 mM and the initial activities of GAD at these concentrations were determined by above pH-stat method using $HCl_{(aq)}$ (0.1 M).

To determine the K_i value to Asp, assays were performed with 2.0, 5.0, 10.0, and 20.0 mM of Glu in combination with 0, 20.0, and 50.0 mM of Asp by pH-stat method titrated using $HCl_{(aq)}$ (0.1 M).

4.2.6 Operational stability assay

The operational stability was expressed as the residual activity as a function of time. The residual activity is defined as the ratio of the enzyme activity (U/mg) to the initial activity. To determine the operational stability of native GAD and IGAD and the effect of Asp in a mixture, titration experiments were carried out as listed in Table 4.1. The setup and procedure were the same as the initial activity assay except that the reaction time was extended to 24 hour to obtain the activity data continuously. In case of assay IGAD, $CaCO_{3(s)}$ was used instead of $NaOH_{(aq)}$ to increase the pH of the substrate solution. In all experiments, $PLP{\cdot}H_2O$ was added to the substrate to 0.5 mM. Model fitting of operational stability data was performed by WinCurveFit® 1.1.8 using double exponential model as reported.[28]

Table 4.1 Titration employing pH-stat method to determine the operational stabilities of native GAD and IGAD and the effect of Asp at pH 4.6 and 40 °C.

Expt.	**1**	**2**	**3**	**4**	**5**
Aim	Operational stability of native GAD	Effect of Asp on the operation stability of native GAD	Operational stability of native GAD under self-titration	Operational stability of IGAD	Operational stability of IGAD under self-titration
Enzyme	Native GAD	Native GAD	Native GAD	IGAD	IGAD
Substrate solution	20 mM of Glu	20 mM of Asp and 20 mM of Glu	20 mM of Asp and 20 mM of Glu	20 mM of Glu	20 mM of Asp and 20 mM of Glu
Titrating solution	50 mM of Glu	50 mM of Glu	20 mM of Asp and 20 mM of Glu	50 mM of Glu	20 mM of Asp and 20 mM of Glu
Titration method	Fed batch	Fed batch	Self-titration	Fed batch	Self-titration

4.2.7 The reaction using IGAD in column reactor

Immobilization of GAD (70 mg) was performed using the method as described earlier. IGAD beads were packed into a glass liquid chromatography column (Sigma-Aldrich, 74 mL, jacketed). The substrate solution was prepared by dissolving Asp (37.27 g), Glu (41.20 g), PLP·H_2O (1.730 g) and $CaCl_2$ (0.777 g) in 14 L of water to produce a solution with concentration of Asp (20 mM), Glu (20 mM), PLP·H_2O (0.5 mM) and $CaCl_2$ (0.5 mM). A powder form of $CaCO_3$ was added into the substrate solution to adjust the pH to pH 4.0. To start the reaction, the reactor was heated to 40 °C by circulating water through the water jacket for 2 minutes. This was followed by the continuous pumping of the substrate solution into the reactor at a flow rate of 25 mL/min. A ~2 mL sample of the solution was taken every 10 minutes in the first hour and every 30 minutes

in the rest 7 hours. During the reaction, air bubbles were formed on the IGAD beads, indicating the formation of CO_2. The samples were analyzed by U-HPLC according to reported procedure.[27]

4.3 Results and discussion

Decarboxylation of AAs increases the pH of the reaction solution, so effective pH control is required to maintain the pH so that a high enzyme activity can be retained. An effective pH can be achieved by titrating with, for example, 0.1 M HCl (pH-stat titration). As reported by Lammens *et al.*, GAD from *E. coli* shows no product inhibition. Therefore a fed batch titration can be applied to study the activity of GAD over time (operational stability).[7] This was performed by titrating the reaction with a concentrated solution of Glu (50 mM) to maintain both the pH and the substrate concentration. Moreover, in a mixture of AAs, if the enzyme is specific and inhibition is not significant, a pH-stat method using the mixture as both the substrate and titrating solution (self-titration) can be applied to study the effect of the mixture on the operational stability.[11] Therefore, a solution containing a mixture of Asp and Glu (both 20 mM) was used in the self-titration experiments. While the enzyme assay was carried out in fed batch mode, the enzymatic reaction was also carried out in continuous mode to study the performance of GAD (activity, conversion rate) for potential application in a separation process *e.g.* ED.

4.3.1 Enzyme kinetics

The native GAD was studied at pH 4.6 and 40 ºC as described in literature.[7] It was found that the initial activity of the enzyme derived from the titration rate differs with the type of acid added (Table 4.2). This is because the activity results were obtained from the pH-stat titration based on the rate of addition of protons to the reaction mixture. However, different acids vary in proton dissociation. Also, the proton dissociation of the acid titrated is affected by the buffering effect of the substrate solution. Thus, the activity obtained by titration is apparent and correction is needed to convert the apparent activity into the enzyme activity which is the rate of production of GABA. When a certain amount of Glu was converted, the amount of acid required to compensate the pH change can be calculated by CurTiPot software (see supplementary material of this

article). The calculation was made by replacing a certain amount of Glu with the same amount of GABA (to simulate the decarboxylation of Glu) and then gradually increasing the amount of acid added until the pH returns to the original value (to simulate the titration). The ratio of the amount of acid to be added to that of the Glu converted is defined as the correction factor. If these factors are applied, the obtained initial activities become comparable (Table 4.2), indicating that such a correction method is suitable in the assay of GAD. From here on, all the activities shown were after correction.

Table 4.2 The correction factors for different titration methods and the corresponding initial activities of GAD.

Substrate solution	Titrating solution	Correction factor	Initial activity of native GAD (U/mg)	
			From titration	Corrected
20 mM of Glu	0.1 M of HCl	0.79	0.64 ± 0.02	0.81 ± 0.02
20 mM of Glu	50 mM of Glu	1.45	1.23 ± 0.03	0.85 ± 0.02
20 mM of Asp and 20 mM of Glu	50 mM of Glu	1.44	1.16 ± 0.09	0.80 ± 0.03
20 mM of Asp and 20 mM of Glu	20 mM of Asp and 20 mM of Glu	1.13	0.98 ± 0.07	0.86 ± 0.06

The apparent K_m of GAD is 1.6 mM (lit.,[7] 2.0 mM) and V_{max} is 0.87 U/mg (Fig. 4.2). The initial activity of GAD is similar in the range of pH 3.5 to pH 4.6. The enzyme shows no activity for Asp and is specific for Glu, and this is in accordance with other results reported by literature.[29] GAD is found to be inhibited by Asp. However, since the inhibition is competitive and K_i (72.2 ± 7.1 mM) >> K_m (1.6 mM) (Fig. 4.2), the inhibition caused by Asp can be eliminated by maintaining a sufficiently high concentration of Glu. For example, according to kinetic data, when Asp and Glu (20 mM of each) is used, the recovery of the initial activity is *ca.* 90%. However, if lower concentrations of Asp and Glu (2 mM of each) are applied, the recovery of the initial activity is only *ca.* 55%.

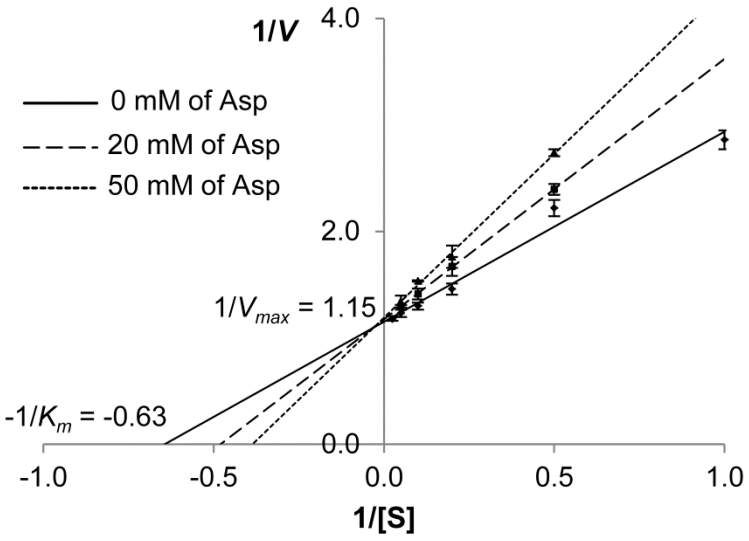

Fig. 4.2 K_m of GAD and determination of K_i of Asp on GAD by Lineweaver-Burk plot. $1/K_{m(apparent)}$ at 20 and 50 mM of Asp were 0.48 and 0.38 mM, therefore K_i = 72.2±7.1 mM. *V*: enzyme activity (U/mg); [S]: concentration of Glu (mM). Errors are the standard deviations based on duplicate measurements.

4.3.2 Effect of the presence of Asp

To study the effect of Asp on the operational stability of native GAD and IGAD, titrations were carried out at 40 ºC and pH 4.6 (Table 4.1) and the corresponding results shown in Table 4.3.[28]

Table 4.3. The initial activity and operational stability of GAD and IGAD. The operational stability data (A_0, B_0, a, and b) were obtained by fitting the residual activity as a function of time (t) according to a double exponential decay function (Lit.,[28] $\boldsymbol{Residual\ activity = A_0 e^{-at} + B_0 e^{-bt}}$).* Errors are the standard deviations of duplicate measurements.*

Expt.	1	2	3	4	5
Enzyme	Native GAD	Native GAD	Native GAD	IGAD	IGAD
Substrate solution	20 mM of Glu	20 mM of Asp and 20 mM of Glu	20 mM of Asp and 20 mM of Glu	20 mM of Glu	20 mM of Asp and 20 mM of Glu
Titrating solution	50 mM of Glu	50 mM of Glu	20 mM of Asp and 20 mM of Glu	50 mM of Glu	20 mM of Asp and 20 mM of Glu
Initial activity (U/mg)	0.85 ± 0.02	0.80 ± 0.03	0.86 ± 0.06	0.91 ± 0.01	0.93 ± 0.02
A_0 %	76.9 ± 2.0	76.9 ± 2.8	78.0 ± 2.0	79.3 ± 2.3	81.3 ± 1.3
B_0 %	23.1 ± 2.0	23.7 ± 2.8	22.0 ± 2.0	20.7 ± 2.3	18.8 ± 1.2
a	0.010 ± 0.002	0.007 ± 0.001	0.052 ± 0.002	0.022 ± 0.005	0.066 ± 0.001
b	1.81 ± 0.40	3.08 ± 0.61	5.97 ± 2.11	4.55 ± 0.14	6.71 ± 0.69

* A_0 and B_0 are coefficients of initial activities; a and b are activity constants (h^{-1}).

The deactivation of GAD (Fig. 4.3) includes a steep deactivation in the first hour followed by a gradual decline thereafter. As suggested by Meeley and Martin, the deactivation of GAD is not first order but can be described as the sum of two exponential deactivation processes.[28] According to Lammens *et al.*, this is due to the multiple PLP binding sites of GAD that

deactivate at different rates.[16] Thus, in Table 4.3 we used a double exponential decay function to modeling the operational stability of GAD.

The effect of the presence of Asp was studied by the addition of Asp to the substrate solution to produce a solution containing Asp and Glu (both 20 mM). As expected, the titration curve is similar to the curve obtained when no Asp is present (Fig. 4.3 A). This is due to the concentration of Glu being maintained sufficiently high by the fed batch titration to eliminate competitive inhibition by Asp.

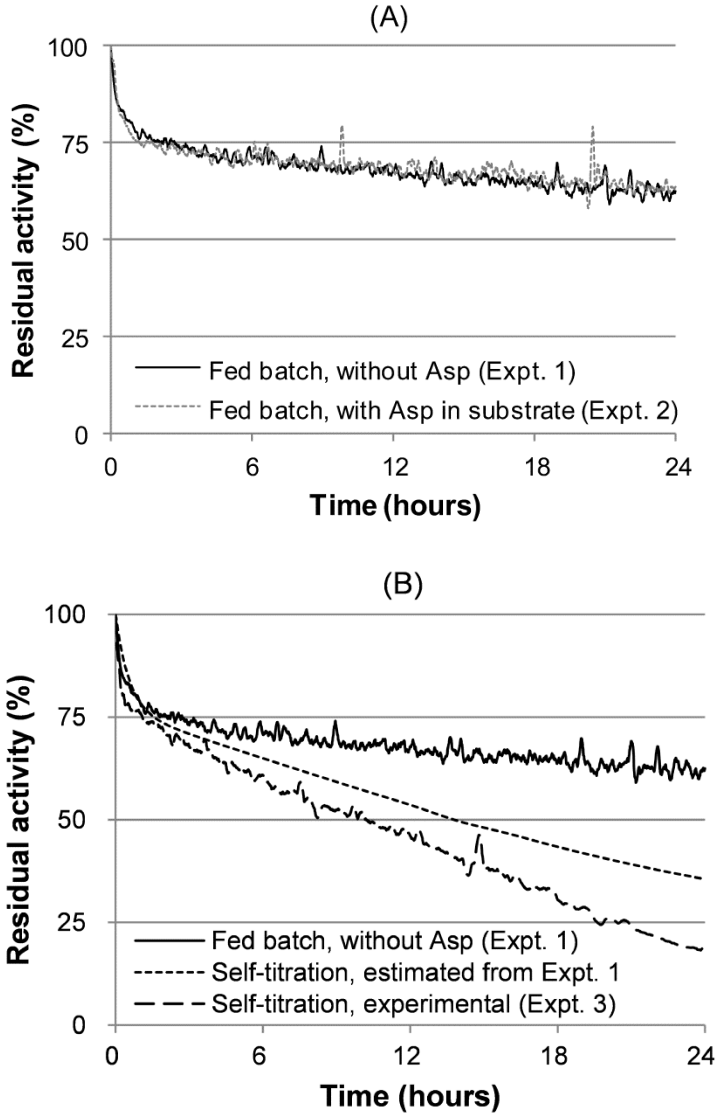

Fig. 4.3 A. Residual activity of native GAD in the presence or absence of Asp. **B**. The residual activity of native GAD assayed by fed batch titration, by self-titration, and by estimation based on the results of Expt. 1 in Table 4.3.

For potential use of GAD as a means to separate Asp and Glu by selective conversion to GABA and utilize ED,[21] a mixture of Asp and Glu as the feed solution is required. Thus the self-titration method, which uses a mixture of Asp and Glu as both the substrate and feed solution,

was applied. Self-titration is shown to be a reliable assay method as the initial activity obtained is comparable with those from other titration methods (Expt. 3 *vs.* Expt. 1 and 2, Table 4.3). The operational stability of native GAD in self-titration was significantly lower than in fed batch titrations (Fig. 4.3 B). When IGAD was used instead of native GAD, its operational stability by self-titration was also lower than that of fed batch titration (Expt. 5 *vs.* Expt. 4, Table 4.3). It is proposed that the faster reduction of activity in self-titration is related to Asp inhibition. As 50 mol% of the acid titrated was Glu (the mole ratio of Asp to Glu was 1:1), and the number of moles of the acid titrated was 1.13 times that of Glu (Table 4.2), therefore only 0.57 times of Glu consumed was compensated. Thus the concentration of Glu continues to decrease during reaction. The constant concentration of Asp, allied with the decreasing concentration of Glu, makes the competitive inhibition by Asp more pronounced. If this assumption is correct, then the residual activity of self-titration can be estimated from the fed batch titration. However, the estimated residual activity of the self-titration is higher than that obtained experimentally (Fig. 4.3 B), therefore other reasons should also account for the loss of residual activity in self-titration. This may be explained by the presence of Asp as the inhibitor also reduces the stability of the enzyme. Similar phenomenon was reported on L-lysine decarboxylase when L-arginine as the inhibitor was added to the reaction mixture.[11]

4.3.3 Effect of immobilization

Immobilization was shown to be an effective way of enhancing stability and usability of an enzyme.[30] It has been reported that GAD can be immobilized by entrapment in calcium alginate and that the operational stability is enhanced. Other methods, such as immobilization on Eupergit

showed similar behavior.[7] However, due to the more favorable costs for potential large scale production, the use of calcium alginate was applied here. The immobilization was found to be complete, as no protein was detected by a Bradford protein assay in the supernatant ($CaCl_2$ solution). After immobilization the initial activities of IGAD are comparable to that of the native enzyme (Table 4.3).

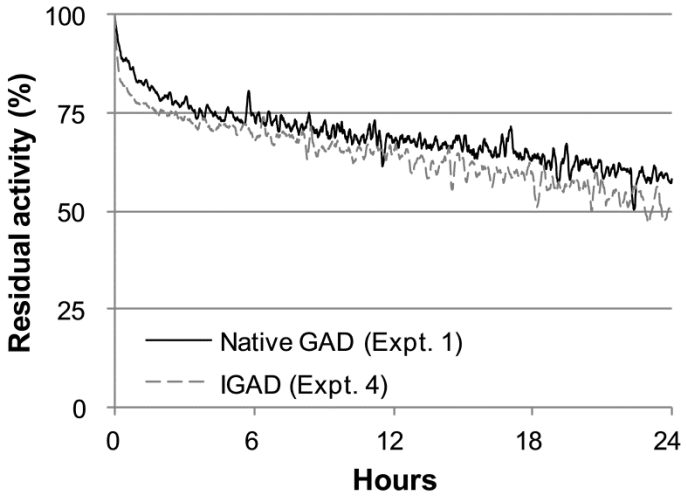

Fig. 4.4 The operational stability of native GAD and IGAD in a fed batch setup at 40 °C and pH 4.6.

Contrary to previous reports,[7] here the operational stability of IGAD is not enhanced compared with that of native GAD (Fig. 4.4). This may be related to small differences in the experimental method. In the reported procedure, the substrate solution was prepared by adding $NaOH_{(aq)}$ to reach pH 4.6. As Ca^{2+} in the alginate beads could be exchanged by Na^+,[31] $CaCl_2$ was also added to maintain bead strength. This would result in the introduction of Cl^- which is known

to enhance GAD activity.[32] For any potential application of GAD in combination with ED separation, extra ions would also be transported and reduce efficiency. Ideally they should be avoided. Therefore $CaCO_{3(s)}$ instead of $NaOH_{(aq)}$ and $CaCl_2$ was used to adjust the pH, resulting in only the introduction of Ca^{2+} to maintain the rigidity of alginate beads. This may however lead to diffusional limitation.[33] Thus a lower operational activity after immobilization was obtained.

From above, the addition of Cl^- ($CaCl_2$) might be necessary to maintain the operational stability of GAD and bead rigidity but only at a concentration which would be acceptable for any subsequent ED. Alternatively immobilization on epoxy resins could maintain stability and reduce diffusional limitation although the impact on the cost of production at large scale may be prohibitive. Another possible option could be the use of a membrane reactor which retains the native enzyme inside the reactor.[34]

4.3.4 Initial studies on the continuous mode reaction

For potential integration with ED, a continuous reactor is preferred. Here the reactor was built by packing IGAD beads in an HPLC column. To simulate the out flow of acidic AAs from an ED stack, a substrate solution containing Asp (20 mM) and Glu (20 mM) was pumped continuously through the column reactor (Fig. 4.5). A flow rate of 25 mL/min was applied according to the reported condition which was used for ED.[21] The eluent was collected and analyzed by U-HPLC, showing that the concentration of Asp, Glu and GABA reached steady state after 20 minutes (Fig. 4.6). The concentration of GABA formed at the steady state is *ca.* 0.54 mM (0.0835 g/h, 1.19 $kg \cdot m^{-3} \cdot h^{-1}$) which is significantly lower than that estimated from the enzyme activity (2.3 mM), indicating that the diffusional limitation might have taken place. As studied by Martinsen

et al., the diffusion coefficient of calcium alginate increases with the flow rate, and plateaus after reaching a certain flow rate.[33] To eliminate the possible diffusional limitation, it is better to consider other immobilization methods with less diffusional problems, for example covalent binding on epoxy resins.[7]

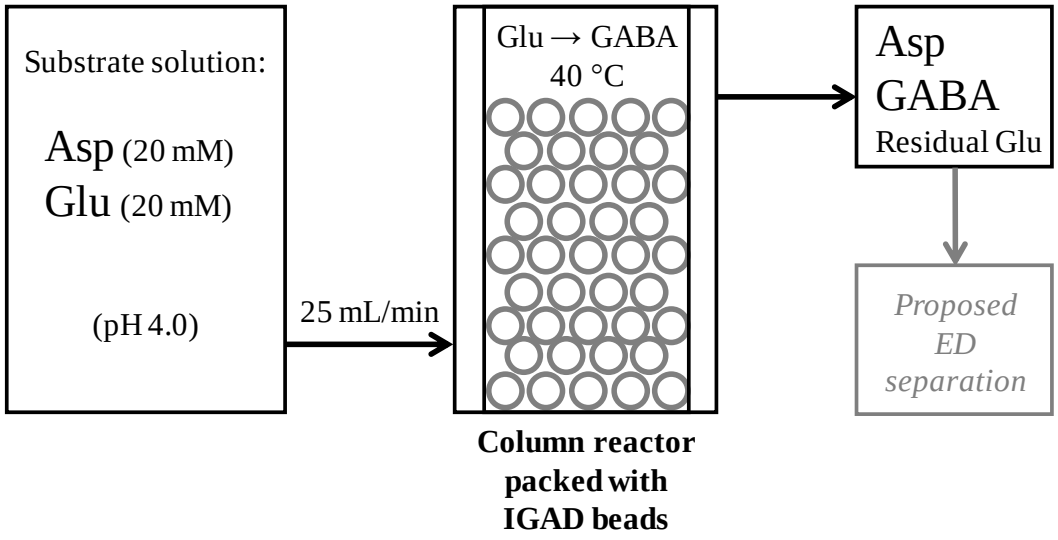

Fig. 4.5 The specific formation of GABA from a mixture of Asp and Glu in a column enzymatic reactor and for possible integration with ED separation.

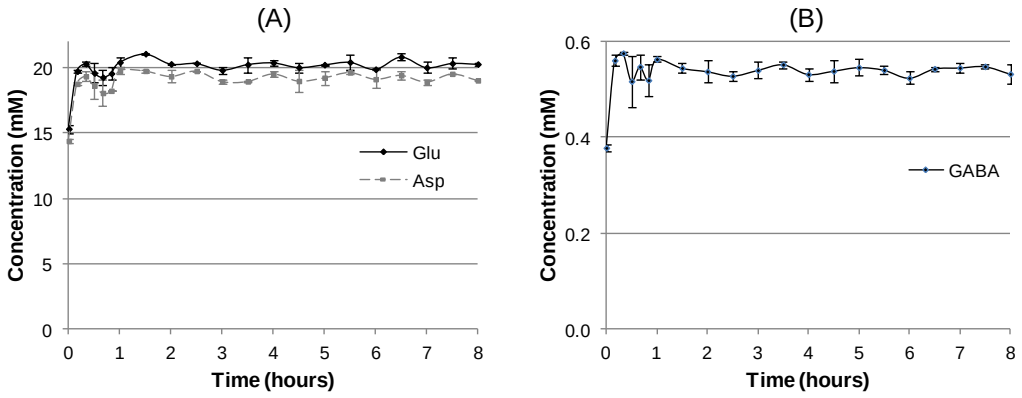

Fig. 4.6 The concentration of Asp and Glu (A), and GABA (B) eluted from the column reactor containing IGAD (volume: 0.07 L).

Such a column reactor may be integrated with a continuous ED process. The stream containing Asp and remaining Glu after removal of GABA could be recirculated back to the reactor until Glu conversion is complete. It can be calculated from kinetic results that GAD will still maintain approximately half of its original activity when the concentration of Asp and Glu are 20 mM and 2 mM respectively (corresponding to 90% of Glu conversion). Thus the overall conversion of Glu can be enhanced by circulating the mixture of Asp and Glu around the column reactor and the ED stacks, and the inhibition of Asp is not a problem for the column reactor to convert small concentration of Glu under high concentration of Asp. After an ED separation of GABA, recirculation of the stream containing Asp and residual Glu can be envisaged thereby increasing conversion of Glu and increasing the overall separation. The use of potentially cost efficient immobilized enzymes (€5 per ton GABA products),[16] operationally stable IGAD reagents, and the implementation of a column reactor with low (running and investment) conversion costs (~€0.2/m^3.hr)[35] also aids separation. In an ED separation of GABA and Asp it was shown to occur with high recoveries (~90%) and low energy consumption (1.1 kWh/kg).[21] This would allow possible recirculation of dilute streams for conversion, concentration and product separation. Due to the potentially low process costs it might be considered to operate

small scale decentralized facilities to avoid the transport logistics related to dilute aqueous biomass sources.

4.4 Conclusions

The aim was to design a bio-process to convert Glu to GABA in a mixture of acidic AAs. Here the use of GAD was employed. The kinetics and specificity of GAD to a reaction mixture containing Asp and Glu was characterized by pH-stat titration. It was shown that desired reaction was specific to Glu although Asp leads to competitive inhibition of GAD and reduces its operational stability. However, as K_i (72.2 mM) >> K_m (1.6 mM), the inhibition can be eliminated by maintaining a sufficiently high concentration of substrate, such as feeding the reactor with a solution of Asp and Glu (20 mM of each). Immobilization by calcium alginate is complete without loss of initial activity, though the operational stability not improved after prolonged reaction times. However, this immobilization method also leads to diffusional limitation, which reduces the operational activity of the immobilized enzyme compared to the native enzyme. When a continuous mode reaction was carried out for potential integration with an ED separation, conversion took place but was lower than expected. This is attributed to diffusional limitation effects. Further research can focus on enhancing the conversion yield of the enzyme reactor and its integration with ED.

Acknowledgements

We would like to thank Dr. Tijs Lammens (Wageningen University, The Netherlands) for his suggestions concerning the enzyme assay and Dr. Kitty Nijmeijer and Dr. Olga M. Kattan Readi (Twente University, The Netherlands) for their fruitful discussions on ED separation. This project is financed by Technology Foundation STW, The Netherlands.

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Chapter 5

Simultaneous decarboxylation of L-serine and deamination of L-phenylalanine as a means to separate neutral amino acids

This Chapter is based on

Teng Y, Scott EL, Susan C.M. Witte-van Dijk and Sanders JPM

Simultaneous decarboxylation of L-serine and deamination of L-phenylalanine as a means to separate neutral amino acids

Submitted

Abstract

Amino acids (AAs) obtained from the hydrolysis of biomass derived proteins are interesting feedstocks for the chemical industry. They can be prepared from the byproduct of biofuel production and agricultural wastes. They are rich in functionalities needed in petrochemicals, providing the opportunity to save energy, reagents, and process steps. However, their separation is required before they can be applied for further applications. Electrodialysis (ED) is a promising separation method, but its efficiency needs to be improved when separating AAs with similar isoelectric points. Thus, specific conversions are required to form product with different charges. Here we studied the enzymatic conversions which can be used as a means to aid the ED separation of neutral AAs. A model mixture containing L-serine, L-phenylalanine and L-methionine was used. The reactions of L-serine decarboxylase and L-phenylalanine ammonia-lyase were employed to specifically convert serine and phenylalanine into ethanolamine and *trans*-cinnamic acid. At the isoelectric point of methionine (pH 5.74), the charge of ethanolamine and *trans*-cinnamic acid are +1 and –1, therefore facilitating potential separation into three different streams by ED. Here the enzyme kinetics, specificity, inhibition and the operational stabilities were studied, showing that both enzymes can be applied simultaneously to aid the ED separation of neutral AAs.

5.1 Introduction

Traditionally, petrochemicals are produced using large amounts of oil and gas as carbon and energy sources. However, challenged by the depletion of fossil fuels, global warming, energy insecurity and issues surrounding environmental pollution, the current chemical industry needs to shift to more sustainable and carbon neutral production routes. [1-3] To avoid these drawbacks, one can think of replacing fossil fuels with carbon neutral biomass, which is the only reliable carbon source to produce chemicals and materials. However, sustainable and cost competitive production routes to chemicals should also be used.[4]

Due to the complex composition of biomass, biorefinery is required to separate and convert it into fractions which can be used or converted into marketable products.[3] One example is the production of biofuels from grains and seeds. Here bio-ethanol and fatty acid esters are produced as well as protein rich rest streams such a dried distillers grains and solubles (DDGS) and presscakes, which are used as animal feed.[5] In the case of bio-diesel production, glycerol is also produced. While biomass has been used to produce fuels its application to produce bulk chemicals has received less attention. Some examples are known such as the use of glycerol to produce epichlorohydrin and the fermentation of sugars and glycerol to produce 1,3-propanediol.[6,7] However other biomass, such a proteins and amino acids already contains functional groups that are needed to form bulk chemicals.[1-3] If it can be used as a feedstock to produce chemicals, the process steps, energy demand and CO_2 emission in the chemical production can be reduced.[4] For instance, 1,4-butanediamine can be prepared from microbial fermentation.[8] However, this production process requires the input of glucose as the feedstock and ammonia (salt) as the nitrogen source, which could increase the product cost and might make it less competitive compared with the corresponding petrochemical routes.[1] To save the input of extra reagents, biorefinery process using amino acids (AAs) as the starting material can be considered.[9] Other functionalities of AAs can be utilized include the carboxyl, hydroxyl (*e.g.* in Ser) and phenyl (*e.g.* in Phe and Tyr) groups.[1]

AAs can be obtained from hydrolysis of biomass derived proteins. Potentially important protein sources include the byproducts from biofuel production, such as DDGS and oilseed press

cake.[4] Other agricultural rest streams such as leaf proteins and grass juice may also be used.[10] Assuming that 10% of the global transportation fuel will be substituted by biofuels, in 2020 the protein byproducts available would be 100 million tons.[1] Considering the production of bulk chemicals such as acrylic acid (1.5-2 million tons per year[1]) and the yearly global consumption of proteins (*ca.* 25 million tons),[4] this quantity of available protein is sufficient to allow large production of bulk chemicals without posing problems to the need of food.

At present, most reported chemicals synthesized from AAs start with a single AA, such as the preparation of 1,4-butanediamine from ornithine and the production of *N*-vinylpyrrolidone from glutamic acid.[11,12] However, a mixture of AAs is obtained from protein hydrolysis. Therefore separation becomes a critical issue before they can be used for chemical production. Traditional separation methods, such as chromatography and reactive extraction, are costly and limited to small scale.[18,19] In contrast, electrodialysis (ED) is a promising separation technology which can be performed at large scale and in a continuous mode, such as the commercial desalination of sea- and brackish water (*e.g.* ENR® Process, at the capacity of *ca.* 3500 m^3/day).[15] In the case of AAs, ED can separate them into neutral, acidic and basic streams based on the difference of their isoelectric points (IP).[21] Still, further ED separation inside those streams is difficult due to the IP similarities.[21-18] To assist the separation, specific conversions of AAs can be employed to modify their charge, and preferably the conversion can lead to product with industrial interest).[9,20]

In this article, we studied the specific conversion methods to aid the separation of neutral AAs. These neutral AAs are supposed to be derived from a proposed ED separation of hydrolyzed proteins. It is proposed that one of these AAs can be decarboxylated and the other deaminated, therefore at around pH 7, the charge of the former will be positive and that the latter negative. A model mixture containing L-methionine (Met), L-serine (Ser) and L-phenylalanine (Phe) has been used (Fig. 5.1). Here, L-Serine decarboxylase (SDC) was employed to specifically decarboxylate Ser into ethanolamine (ETA),[20] and L-phenylalanine ammonia-lyase (PAL, in *Rhodotorula glutinis* whole cells, E.C. 4.3.1.5) was applied to specifically deaminate Phe into *trans*-cinnamic acid (CA).[21] Currently, ETA is used as an intermediate in the textile, detergent and plastics industries,[1] and CA can be further decarboxylated into styrene which is exclusively used to prepare poly(styrene).[22] At the IP of Met (pH 4.75), the net charge of ETA and CA are +1 and –1,

so they could be potentially separated from Met into different streams by ED. Met should not be converted during these reactions as it is a valuable compound can be directly used in animal feed.[23] If the rate of deamination and decarboxylation can be the same, the pH of the reaction will be maintained without the need of an extra pH control.

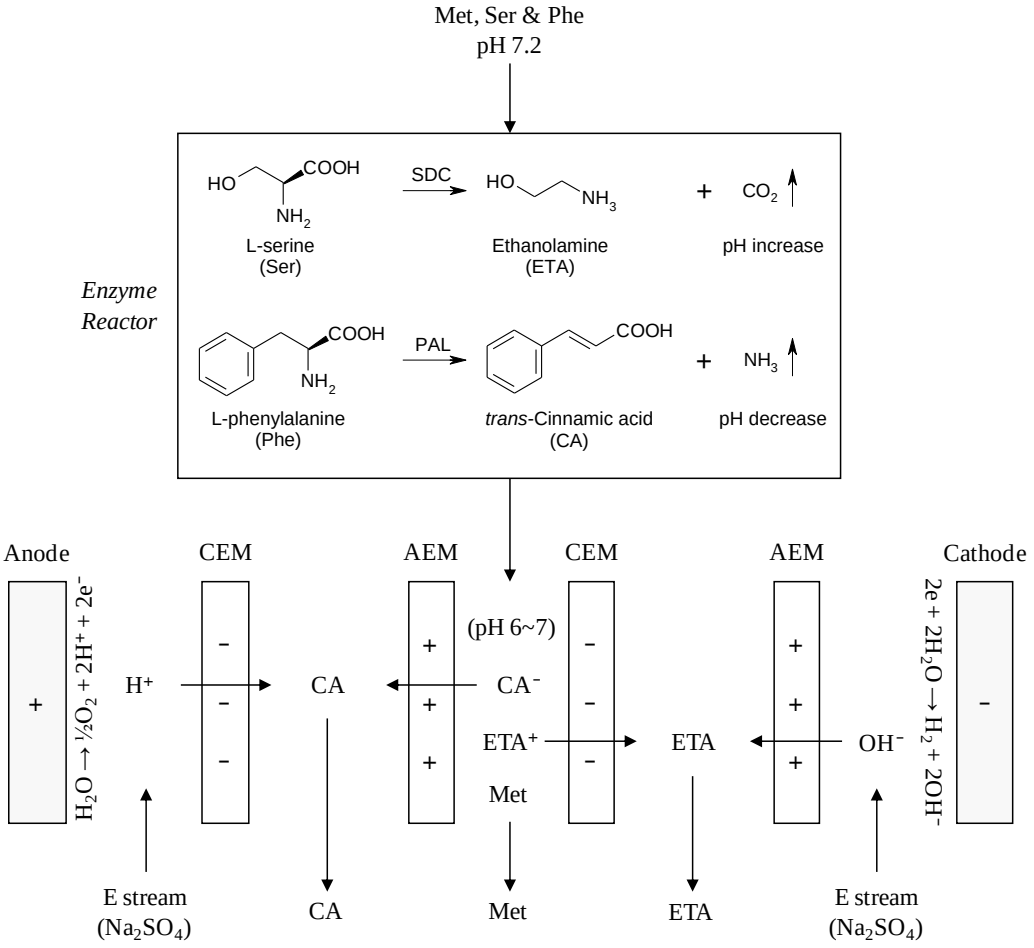

Fig. 5.1 Potential application of specific conversion applying SDC and PAL as a means of separating Met, Ser and Phe by ED.

In this study, the activity of PAL was enhanced by permeabilization of the yeast cell wall. The enzyme kinetics, specificity, inhibition, and operational stabilities (the relative activity as a

function of reaction time) of SDC and PAL were studied individually. Finally, the two enzymes were combined together and reacted in the AA mixture.

5.2 Experimental

5.2.1 Materials and equipment

The pET28/AtSDC His-tagged constructed cDNA containing the *Arabidopsis thaliana* SDC gene was kindly provided by Dr. Michael J. Ziemak from IFAS, University of Florida, USA. Yeast cells (*Rhodotorula glutinis*) were kindly provided by Marinella van Leeuwen from Food and Biobased research, Wageningen UR, the Netherlands. Pyridoxal 5'-phosphate monohydrate ($PLP \cdot H_2O$, ≥97%), L-serine (≥99%), L-phenylalanine (≥99%), L-methionine (≥99.5%), ethanolamine (≥99%), and hexadecyltrimethylammonium bromide (CTAB, ≥99%) were ordered from Sigma-Aldrich®. Other chemicals were used as received. Milli-Q (Millipore®) purity water was used to make all solutions. All the reactions were performed in Eppendorf® polypropylene tubes (1.5 mL). Eppendorf Thermomixer® was used for mixing and maintaining the reaction temperature.

5.2.2 Analysis

The concentration of AAs, ETA and CA was analyzed by a Dionex® U-HPLC instrument (Dionex Corporation, Sunnyvale, CA, USA) equipped with an Acquity UPLC BEH C18 reversed phase column (particle size: 1.7 μm, 2.1×150 mm) and VanGuard Acquity UPLC BEH C18 guard column (particle size: 1.7 μm, 2.1×5 mm) based on a previously reported method.[9]

5.2.3 Preparation of SDC

The potassium phosphate buffer (0.1 M, pH 8.0) used to dissolve SDC contains Na_2EDTA (1.0 mM), dithiothreitol (5.0 mM), PLP (0.1 mM) and glycerol (10% by weight). According to the reported procedure,[20] the plasmid (pET28b-SDC) containing the *A. thaliana* SDC gene was transformed into *E. coli* BL21 (DE3), resulted in an expression of a protein with an N-terminal

histidine tag. Then Ni-NTA chromatography was used for the purification of the enzyme.[20] The concentration of the obtained enzyme stock solution contained 0.1 mg SDC per mL.

5.2.4 SDC assay

In a typical procedure, 150 μL of the enzyme stock solution was added into an Eppendorf® tube. At 37 °C, the reaction was initiated by adding 600 μL of Ser solution (62.5 mM) in potassium phosphate buffer (0.1 M, pH 7.2, containing 0.1 mM of PLP). Therefore at the start of the reaction, the concentration of Ser in the reaction mixture was 50.0 mM. The reaction was maintained at 37 °C with a mixing rate of 900 rpm. At given time intervals, 20 μL of the reaction mixture was taken. The reaction was quenched by adding 20 μL of trichloroacetic acid (TCA) solution in water (10% by weight) and analyzed by U-HPLC.

To determine the initial activity, samples were taken at 0, 5, 10, 15 and 20 min. The enzyme unit is defined as the amount of ETA formed (in μmol) per minute. Thus the enzyme activity is defined as U/mg. To study the operational stability, the reaction continued for 2.5 h and samples were taken every 5 min. To study the specificity and inhibition of the enzyme, assays were performed with Ser (20.0 mM) in combination with Phe (20.0 mM), Met (20.0 mM), and CA (8.0 mM).

5.2.5 Preparation of PAL containing yeast cells

The yeast *R. glutinis* stock culture was used as the microbial source of PAL. The resting cells which have PAL activity were prepared according to reported procedure.[21] The pale pink cell pellet obtained was made into a 0.1 g/mL wet cell suspension by adding enough potassium phosphate buffer (0.1 M, pH 7.2) and mixing well on a Cole-Palmer Roto-Torque® Heavy Duty rotator. This suspension was stored at 4 °C.

The permeabilization of yeast cells was performed based on the reported method.[24] In a typical procedure, 1.00 mL of the wet cell suspension was added to an Eppendorf® tube (1.5 mL). The buffer solution was removed from the cells by centrifugation at 6000 g for 10 min at room temperature. Then 0.9 mL of CTAB solution (0.1% by weight) was added to the cells. The mixture was stirred in an Eppendorf Thermomixer® for 2 hours at 900 rpm at room temperature.

After that, CTAB solution was removed using aforementioned centrifugation method and 0.9 mL of potassium phosphate buffer (0.1 M, pH 7.2) was added to form a cell suspension (~0.1 g/mL). The cell suspension was stored at 4 °C.

5.2.6 PAL assay

In a typical procedure, 600 μL of the cell suspension was added into an Eppendorf® tube. At 37 °C, the reaction was initiated by adding 400 μL of Phe solution (50.0 mM) in potassium phosphate buffer (0.1 M, pH 7.2). Thus at the start of the reaction, the concentration of Phe in the reaction mixture was 20.0 mM. The reaction was performed at 37 °C with a mixing rate of 900 rpm. At given time intervals, 20 μL of the reaction mixture was taken. The reaction was quenched by adding 20 μL of trichloroacetic acid (TCA) solution in water (10% by weight) and the concentration of Phe was analyzed by U-HPLC according to the reported method.[25]

The concentration of CA was determined by U-HPLC using the same columns and pump flow (0.4 mL/min) as that for AAs and ETA.[25] The sample solution injected was 1 μL. Eluents used were: eluent A (10 mM of disodium phosphate buffer at pH 7.8), and eluent B (acetonitrile, methanol and Milli-Q water in the volume ratio of 60:40:20). The gradient applied was (expressed as eluent B): initial composition: 20% B, 0-2.5 min: 35% B, 2.5-2.6 min:100% B, 2.6-5 min: 100% B, 5-5.1 min: 20% B, 5.1-7min: 20% B. The wavelength for the detection was 270 nm.

To measure the initial activity, samples were taken at 0, 10, 20, 30 and 40 min. The enzyme unit is defined as the amount of CA formed (in μmol) per minute. Thus the enzyme activity is defined as U/mg. To study the operational stability, the reaction was running for 2.5 h and samples were taken every 10 min since the beginning. To study the specificity and inhibition of the enzyme, assays were performed with of Phe (20.0 mM) in combination with Ser (20.0 mM), Met (20.0 mM) and ETA (20.0 mM).

5.2.7 Simultaneous reaction of SDC and PAL in one reaction system

In an Eppendorf® tube 490 μL of yeast cell suspension containing PAL and 115 μL of SDC solution were added, and thus the volume ratio of them was 4.26. The substrate used was the

water solution of Ser (50.0 mM), Phe (50.0 mM) and PLP (0.1 mM). The pH of the substrate solution was brought to pH 7.2 by adding concentrated KOH solution (5 M). At 37 °C, the reaction was initiated by adding 395 μL of the substrate solution. Thus the final concentration of Ser and Phe were 20 mM. The reaction was maintained for 2.5 h at 37 °C at the mixing rate of 900 rpm. The reaction was quenched by adding 20 μL of trichloroacetic acid (TCA) solution in water (10% by weight) and analyzed by U-HPLC.

To study the impact of Met on both the enzymes activity and operational stability, it was added when both enzymes were present in the same reaction system. The procedures were the same as above except that the substrate solution used contained Ser (50.0 mM), Phe (50.0 mM), PLP (0.1 mM) and Met (50 mM). Thus, in the reaction mixture the final concentration of Ser, Phe and Met were 20 mM.

5.3 Results and discussion

5.3.1 Decarboxylation of SDC

SDC is a PLP dependent enzyme which catalyzes the decarboxylation of Ser to ETA.[20] This reaction is considered to be the main source of ETA in plants.[26] It has been reported that recombinant cells with SDC expressed (such as *E. coli* and *Pseudomonas putida*) can be used for the fermentative production of ETA.[27,28] However, As reported by Foti *et al.*, the formed ETA was toxic to the cells, leading to decreased yield and cell growth rate.[28] These problems can be tackled if soluble SDC is employed. Also, by using soluble enzymes, extra chemicals (*e.g.* glucose) required for the fermentation process can be saved and the diffusional limitation caused by the cell well can be avoided. In addition, the use of SDC as a means of separating AAs is novel.

SDC was assayed at pH 7.2 in buffer containing PLP (0.1 mM) as the cofactor.[20] Its K_m is 10.8 mM (lit.,[26] 10 mM) and V_{max} is 1.2 U/mg. When ETA was present, the apparent K_m obtained (8 mM) was not higher than the K_m value, indicating that no product inhibition occurs. This is in accordance with what reported in literature.[26] As displayed in Table 5.1, after Phe, Met, and CA were added to the reaction of SDC individually, their concentration were not changed with the

elapse of time. This proved that the enzyme is specific to Ser. Also, the activity of SDC was not influenced by Phe and Met. In contrast, when CA (8.0 mM) was added to the substrate solution containing 20 mM of Ser), the activity of SDC reduced by nearly 30%, indicating that it might be an inhibitor. Inhibition of an organic acid on an AA decarboxylase had been observed for L-glutamate α-decarboxylase (GAD), which was explained by the interaction between the carboxyl group and the active site of the enzyme.[29] Whether this could explain the inhibition on SDC would be an interesting topic for future study. However, the phenomenon itself indicates that it might be beneficial to limit the concentration of CA when the reaction of SDC is integrated with that of PAL. Possibly, this could be achieved by performing the removal of CA by ED simultaneously with the decarboxylation of Ser.

Table 5.1 The influence of Phe, Met and CA on the initial activity (V_{init}) of SDC and the concentration of these compounds at different reaction time.*

Extra compound added	**Control****	**Phe (20 mM)**	**Met (20 mM)**	**CA (8 mM)**
V_{init} (U/mg)	0.81 ± 0.01	0.81 ± 0.02	0.81 ± 0.03	0.58 ± 0.11
$c_{0\ min}$	–	19.7 ± 0.1	19.6 ± 0.1	7.9 ± 0.1
$c_{10\ min}$	–	19.7 ± 0.1	19.6 ± 0.2	7.9 ± 0.1
$c_{20\ min}$	–	19.7 ± 0.1	19.5 ± 0.2	7.9 ± 0.1
$c_{30\ min}$	–	19.7 ± 0.1	19.7 ± 0.3	8.0 ± 0.1
$c_{40\ min}$	–	20.0 ± 0.4	20.2 ± 0.1	8.1 ± 0.2

* Values (mean ± standard deviation, n = 2), c_x: the concentration of the extra compound at the reaction time x.

** Control: react with only Ser (20 mM) at pH 7.2 and 37 °C.

In this research, it is desired that the decarboxylation of Ser and the deamination of Phe can be integrated in one reaction system at the same reaction rate, so the same amount of ETA and CA can be generated, bringing benefits in maintaining pH without the need for additional acid or base

and avoids the formation of salts which can reduce the efficiency of a subsequent ED separation. Therefore it is necessary to study the operational stability of both enzymes. The operational stability of SDC was expressed as the relative activity as a function of time. As shown in Fig. 5.2, the deactivation of SDC appears to be exponentially, and the corresponding half-life is around 80 min. This is significantly shorter than that of GAD (*ca.* 16 h, assayed at pH 4.6 and 40 °C) and L-lysine decarboxylase (*ca.* 14 h, assayed at pH 5.8 and 37 °C) which follows the double exponential deactivation,[9,12] but its deactivation behavior is comparable to that of L-aspartate α-decarboxylase (half-life is about 70 min as assayed at pH 7.5 and 30 °C) which also deactivates exponentially.[30] It is noticeable that most AA decarboxylases use PLP as a cofactor which is less stable at alkaline pH.[31] However, as extra PLP was present in the reaction mixtures, it can be safely concluded that the deactivation of these enzymes may due to other mechanism rather than the decomposition of PLP. Enzyme immobilization onto Sepabeads® was tried to enhance the enzyme stability,[30] but this attempt was not successful. Based on the operational stability of the native enzyme, the amount of ETA produced can be estimated by Equation (1), where n_{ETA} is the amount of ETA produced (in μmol), V_{SDC} is the volume of the enzyme solution added (in mL), a_{SDC}^{init} is the initial activity of SDC (in U/mg), and *t* is the reaction time (in min).

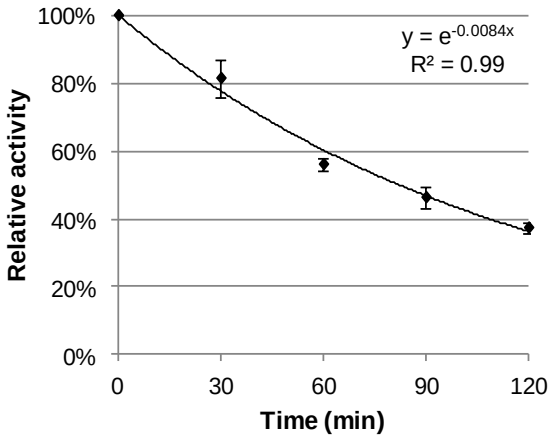

Fig. 5.2 The relative activity of SDC as a function of time.

$$n_{ETA} = V_{SDC} \cdot 0.1 \cdot \alpha_{SDC}^{init} \cdot \int_0^t e^{-0.0084t}\, dt = V_{SDC} \cdot 0.1 \cdot \alpha_{SDC}^{init} \cdot \left[\frac{e^{-0.0084t}}{-0.0084}\right]_0^t \quad (1)$$

5.3.2 Deamination of Phe by PAL

The deamination of Phe was carried out by whole cell bioconversion.[21,32] The assay of PAL containing yeast cells was performed by U-HPLC analysis of the CA concentration in the reaction mixture.

As reported by Srinivasan *et al.*, the cellular activity of PAL in yeast cells can be enhanced by permeabilization of the cell well with detergents such as CTAB.[24] This method was adopted here. As can be seen in Fig. 5.3, inoculation of the original yeast cells with Phe did not result in formation of CA. After treatment, the CA formation increased, corresponding to an activity of 2.5×10^{-4} U/mg. From here on, all the experimental data of PAL were obtained by using the permeabilized yeast cells.

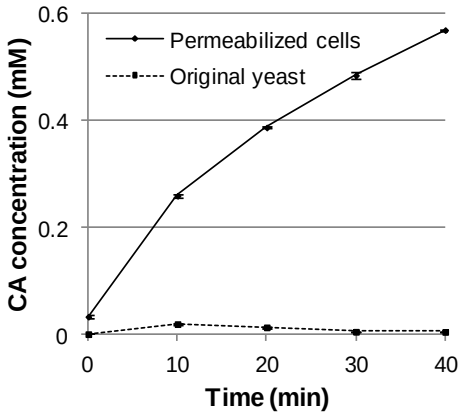

Fig. 5.3 Comparison of CA forming by yeast cells after permeabilization and the original yeast cells.

The K_m of PAL is 23.2 mM and the V_{max} is 5.4×10^{-4} U/mg. If the reaction of PAL is carried out simultaneously with that of SDC, other compounds involved would be Ser, Met and ETA. As reflected in Table 5.2, these compounds did not have an effect on Phe conversion or PAL activity, indicating that PAL is specific to Phe and is not inhibited by these compounds. Specificity is

usually a concern of whole cell bioconversion as other enzymes might also exist in the cells. The high specificity of PAL in the yeast cells may relate to the catabolic role of the enzyme under specific conditions which enables the yeast to use solely Phe as carbon source, so the cells do not need to take up other compounds from the reaction mixture.[34]

Table 5.2 The influence of Ser, Met and ETA on the initial activity (V_{init}) of PAL and the concentration of these compounds at different reaction time.*

Extra compound added	**Control****	**Ser (20 mM)**	**Met (20 mM)**	**ETA (20 mM)**
V_{init} (10^{-4} U/mg)	2.8 ± 0.1	2.9 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
$c_{0\ min}$	–	19.8 ± 0.2	20.0 ± 0.1	20.9 ± 0.1
$c_{10\ min}$	–	18.9 ± 0.1	20.0 ± 0.2	19.8 ± 0.1
$c_{20\ min}$	–	19.1 ± 0.3	19.9 ± 0.1	20.1 ± 0.1
$c_{30\ min}$	–	18.9 ± 0.1	20.0 ± 0.1	20.0 ± 0.1
$c_{40\ min}$	–	19.1 ± 0.1	20.2 ± 0.1	20.1 ± 0.1

* Values (mean ± standard deviation, n = 2), c_x: the concentration of the extra compound at the reaction time x.

** Control: react with only Phe (20 mM) at pH 7.2 and 37 °C.

The operational stability of PAL was also studied. As seen in Fig. 5.4a, after reacted for 30 min, the concentration of CA increased almost linearly, indicating that a stable enzyme activity was reached. In other words, the relative activity of the enzyme declines in the first 30 min and remains at around 33% thereafter (Fig. 5.4b). It has been reported that CA is a strong inhibitor to PAL.[33] So if the reaction time is significantly longer (*e.g.* 20 hours[21]), it can be expected that the accumulation of CA will lead to an observable decline of enzyme activity. Under our experimental conditions (reacted in 20.0 mM of Phe at pH 7.2 and 37 °C), the growth of CA concentration as a function of time ($t \leq 2.5$ h) can be fitted by two linear functions (Fig. 5.4a). Correspondingly, the formation of CA can be estimated by Equation 2, where n_{CA} is the amount

of CA produced (in μmol), V_{PAL} is the volume of SDC solution added (in mL), and t is the reaction time (in min, $t > 30$).

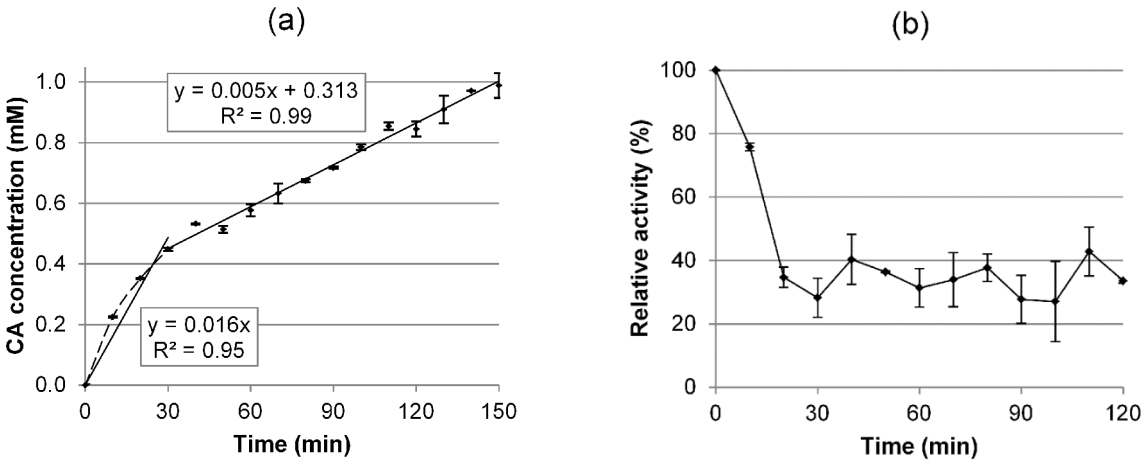

Fig. 5.4 (a) The increasing concentration of CA as a function of time due to the deamination of Phe by PAL, where the equations in the figure display the two linear fitting results; and (b) The corresponding relative activities of PAL as a function of time.

$$n_{CA} = V_{PAL}[0.025 \cdot 30 + 0.0073 \cdot (t - 30)] \quad (2)$$

5.3.3 Simultaneous reaction of SDC and PAL

Since both SDC and PAL are specific to their desired substrates and are not inhibited by Met, it is possible to carry out both reactions simultaneously. As described earlier, ideally the rates of the two reactions should be similar so as to maintain a constant pH of the reaction. However, as the two reactions have different operational stabilities, the final concentration of ETA and CA will be different even if the initial rate of the ETA and CA formed is the same. So it would be more practical to have the same amount of products formed after reacted for a given time. In this case, the volume ratio of SDC solution to PAL solution can be calculated by solving simultaneous Equations (1) and (2). When the reaction time was 2 hours, the volume ratio of PAL solution to SDC solution was found to be 4.26. In the simultaneous reactions, an SDC solution and PAL

containing yeast cell suspension were added to the reaction mixture, and the operational stabilities of both enzymes are studied.

As illustrated in Fig. 5.5a, when SDC was reacted with PAL in the absence of Met, its operational stability was improved. This might due to the presence of CA as the possible inhibitor. The stabilization of an enzyme by an inhibitor has been reported with other enzymes. For example, Burton observed that D-amino-acid oxidase from sheep kidney can be stabilized by its inhibitor L-leucine and sodium benzoate.[35] Whether SDC is stabilized by CA can be an interesting topic for future study. However, when Met was also present in the simultaneous reaction, the relative activity of SDC became similar to when it reacted individually. Probably this was because Met could reduce the operational stability of SDC, so when its destabilization effect on SDC could cancel the stabilization effect led by CA. The reduction of an AA decarboxylase's operational stability due to a non-substrate AA has been reported before, such as L-arginine to L-lysine decarboxylase and L-aspartic acid to L-glutamate α-decarboxylase,[132] but the impact of Met on the operational stability of SDC still needs to be further exploited.

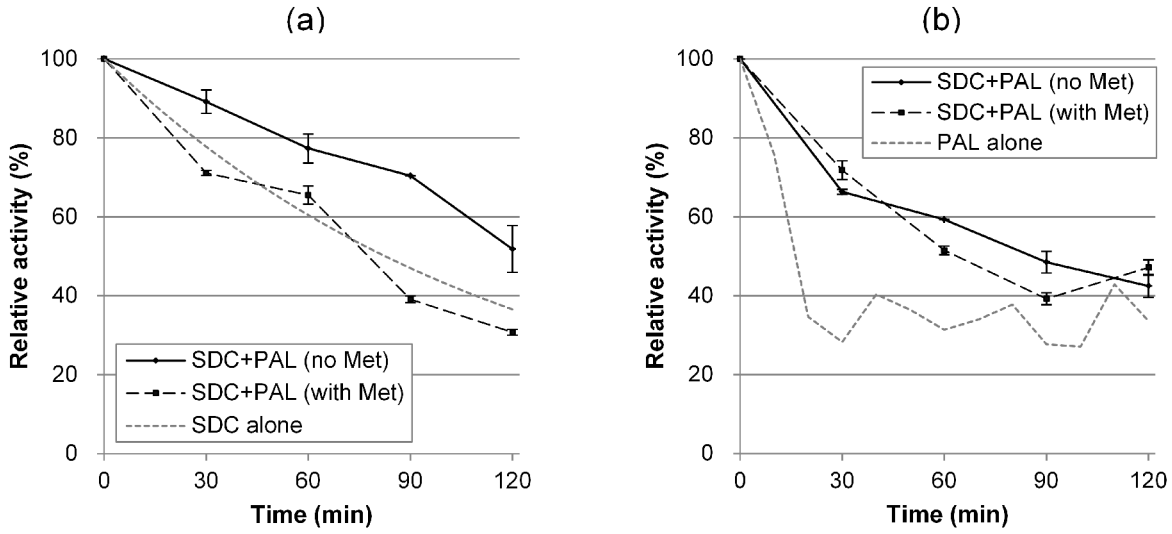

Fig. 5.5 (a) The relative activity of SDC as a function of time when it was reacted with PAL simultaneously (SDC+PAL) *vs.* when it was reacted individually (alone) (b) The relative activity of PAL as a function of time in the simultaneously experiment *vs.* when it was reacted individually.

On the other hand, in the simultaneous reaction the deactivation of PAL slows down in the first 0.5 h regardless of the adding of Met (Fig. 5.5b). Possibly PAL was stabilized by the small amount of ETA. When Met was added, its concentration kept stable during the 2 hours reaction (20.4 ± 0.3 mM), which proves that both enzymes did not react with Met.

To sum up, the operational stability of SDC and PAL can be influenced by Met and each other's conversion. So it is necessary to study these influences before the two reactions can be well performed in one reaction system.

5.4 Conclusion

This research aims at designing specific conversion methods to aid the ED separation of neutral AAs derived from biomass. The enzymes used were SDC and PAL which converted Ser and Phe into ETA and CA, allowing the maintenance of reaction pH and their subsequent separation by

the following ED process. The two enzymes were prepared and the activity of PAL in yeast cells was enhanced by permeabilization of the cell well. By U-HPLC analysis, it was found that both enzymes are specific to their native substrate and are not inhibited by Met. So they can be applied as a means to aid the ED separation of Ser, Phe and Met. Although CA could be an inhibitor for SDC, it is not considered to be a problem if simultaneous ED separation of it could be performed. Attempt of using the two enzymes in one reaction system was successful with both ETA and CA formed. The operational stability of SDC was improved when reacted with PAL in the absence of Met, possibly because CA can stabilize the enzyme. But this was not observed if Met was added, probably because Met can make the enzyme less stable. These findings indicate that it is necessary to further study the operational stability of SDC in presence of CA and Met.

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Chapter 6

General Discussion

6.1 Introduction

Amino acids (AAs) can be used as building blocks for the synthesis of bulk chemicals.[1] However, they are usually available as a mixture of up to 20 amino acids (*e.g.* hydrolyzed protein). AAs can be separated by electrodialysis (ED) into 3 fractions of similar isoelectric points (IPs) (acidic, basic and neutral AAs) but not 20 pure compounds. If AAs are utilized as chemical feedstocks, a major challenge to be overcome is how to purify them under economically viable conditions. The aim of this research is to explore specific conversion methods of AAs with similar IPs to aid their further separation by ED and at the same time perform a reaction towards the desired end product. This will be possible when conversions change the charge of a specific AA while leaving the charge of the other AAs unaltered. In this chapter, the results presented in previous parts are summarized and integrated into a general discussion of the research. In the end, some conclusions are drawn and suggestions for future research are given.

6.2 General discussion and suggestions

6.2.1 The use of L-lysine decarboxylase (LDC) as a means to separate two basic amino acids

Chapter 2 describes the study on the use of a specific decarboxylase LDC as a means to aid the ED separation of basic AAs (L-arginine (Arg) and L-lysine (Lys)) by the yielding 1,5-pentanediamine (PDA). This was achieved by using LDC to selectively convert the Lys into PDA in the presence of arginine.

LDC from *Bacterium cadaveris* is a pyridoxal 5'-phosphate (PLP) dependent enzyme that has been known to react specifically with Lys.[20,16] It was found that LDC has the highest initial activity at pH 5.8, which is in accordance with literature.[20,16] Outside the range of pH 5.5 to 6.0, the enzyme activity reduces sharply. The deactivation of LDC follows a double exponential decay model (as a function of temperature) that can be used to estimate the productivity of the enzyme. Immobilization was performed by entrapment in calcium alginate beads. At different reaction temperatures, immobilization enhances the enzyme's operational stability without

reducing its initial activity. The reaction using LDC was inhibited by high concentrations of Arg (>30 mM), NaCl (>30 mM) and PDA (competitive inhibition, K_i = 10 mM). However, the inhibition caused by PDA can be overcome either by maintaining a sufficiently high concentration of Lys or indeed by the removal of PDA (*e.g.* by ED). Although we observed inhibition of LDC by Arg at 37 and 45 °C, we did not observe this at 30 °C. This is possibly due to stronger affinity between the inhibitor and the enzyme at higher temperature. This phenomenon has been reported on other enzymes before (*e.g.* cellobiohydrolase).[4] After the formation of PDA, it is possible to separate PDA from unreacted Lys and Arg by ED at pH ≈ 10. At this pH the average charge of PDA is around +1 while that of Arg and Lys are near neutrality. The cost of raw materials, enzyme and alginate are estimated to be 200, 12 and 3 € per ton of PDA respectively, leaving enough economic room for subsequent ED separation.

6.2.1.1 Proposed specific conversion of Arg by two enzymes at the same time

Lysine is an essential amino acid and used in large volumes (around 1 million tons per year) for poultry and pig feed.[1] Furthermore it has been suggested that Lys can be used to prepare caprolactam for the synthesis of nylon-6.[113] So the preservation of Lys for these applications might also be interesting. Thus, specific conversion of Arg needs to be carried out in order to change the charge to enable separation from Lys by ED. Arg can also be a useful feedstock for the synthesis of chemicals.

Könst *et al.* reported the enzymatic conversion of Arg to L-ornithine (Orn) by L-arginase and the conversion of Orn to 1,4-butanediamine (BDA) by L-ornithine decarboxylase (ODC).[6,7] As Orn is a basic AA which has a similar IP to Lys (Fig. 6.1), the conversion of Arg to Orn alone cannot aid the separation and thus decarboxylation by ODC would be necessary. As seen in Fig. 6.1, at pH 10.8 Arg has no net charge, while Lys and Orn are slightly negatively charged and BDA is positive.[8] Therefore at pH 10.8 BDA can be separated from any basic AAs by ED. BDA is a bulk chemical used as the co-monomer in the production of nylon-4,6.[1] It is currently prepared from fossil fuels with an annual production of *ca.* 10,000 tons.[1] The market value of this

chemical is high (*ca.* €3500 /ton).[9] Thus enzymatic conversions of Arg to BDA could have potential in industrial applications.

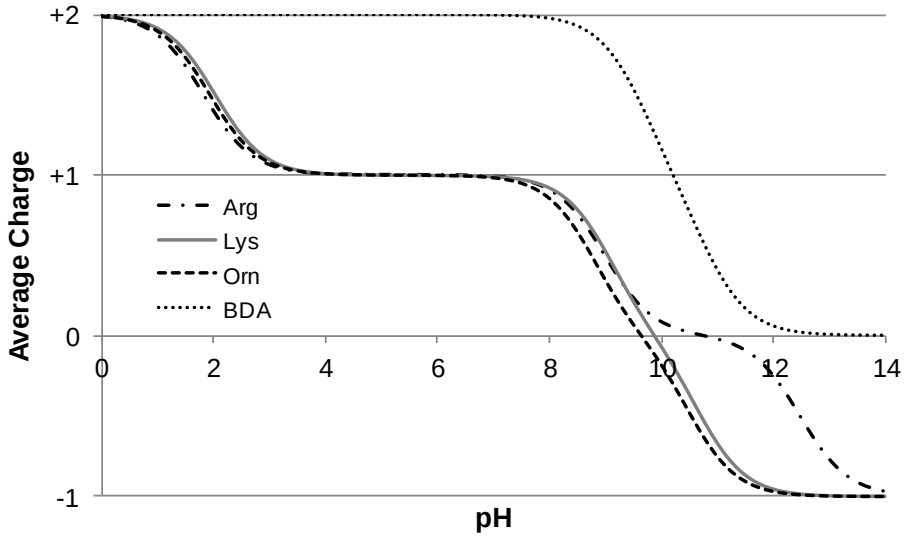

Fig. 6.1 The pH dependence of charge behavior of Arg, Lys, Orn, and BDA as calculated by CurTiPot software.[10] The *pKa* values of Orn and BDA are from literature.[8,11]

To apply the conversion of Arg to BDA, L-arginase and ODC should be specific to their substrates, should not be significantly inhibited by Lys and preferably be applied simultaneously in one pot. It is known that ODC from some sources (*e.g. Lactobacillus sp.*) also has activity with Lys.[12] Therefore development of more specific ODC is required. The rate of Orn formation should be higher than that rate of BDA to maximize activity of ODC by maintaining a suitably high substrate concentration. Since both enzymes can be covalently immobilized on Sepabeads® and their optimal pH ranges are similar (arginase: pH 8.5~11.5, ODC: pH 8~9),[6,7] the integration of them in one reaction system could be possible. This could offer the potential to run the conversions in one system and reduce process costs.

6.2.1.2 Using pH-stat titration to assay decarboxylases

Traditionally, the assay of LDC was carried out by capturing the CO_2 formed or using HPLC analysis of the PDA produced.[20,13] These assay methods are considered to be time consuming and often leads to error.[14] Also, these methods use a buffer to maintain the reaction pH. Furthermore, the presence of additional ions in the ED separation is not desired as they will also be transported along with the product to be separated, leading to a reduction of separation efficiency as well as higher costs for the electricity used. Therefore we investigated a buffer free approach. A buffer free assay could also be helpful, as an increase of ionic strength can reduce the LDC activity as have been reported by Sabo *et al.*[15] However, as described in Chapter 2, the impact of ionic strength was limited since it did not significantly reduce the enzyme activity (*ca.* 25% decrease of activity at 1M of NaCl). To avoid the use of buffer, a pH-stat method was employed. Since the principle of the method is based on maintaining a constant pH by the addition of acid (HCl), is it correct to state that the amount of HCl required is proportional to the CO_2 produced? Therefore all the factors related to pH should be considered:

1. The proton dissociation of the carboxylate group to be removed. The α-COOH of Lys has a *pKa* value of 2.04.[10] Above pH 4, the proton is fully dissociated (> 99%). Therefore the amount of carboxylate groups removed is proportional to loss of protons that can originally be dissociated from those groups.
2. The release of CO_2 from the solution. The apparent first *pKa* of carbonic acid is 6.4.[14] So below pH 6.4 the equilibrium is more favorable at releasing CO_2. The releasing of CO_2 can be more efficient if stirring is applied.
3. The proton dissociation of the acid used for titration. As Arg·2HCl and Lys·2HCl already contain HCl in their molecules, they can work the same as $HCl_{(aq)}$ by means of pH control.[10]
4. The buffer effect of all the compounds in the reaction solution. The pH of LDC reaction needed is well beyond any *pKa* values of Arg, Lys and PDA therefore the buffer effect can be ignored.

From above, it can be concluded that when applying a pH-stat method to assay LDC, the number of moles of acid added is the same as of the number of moles of AA decarboxylated. As

an assay for LDC the pH stat is ideal, but one should be careful to conclude that this method is suitable for all decarboxylases.

In Chapter 4 this pH stat method was applied to assay L-glutamate α-decarboxylase (GAD). However, the operational pH of GAD (pH 4.6) is close to the pKa_3 of L-glutamic acid (Glu) (lit.,[8] 4.2) and pKa_1 of γ-aminobutyric acid (GABA) (lit.,[8] 4.0). As the capacity of a buffer solution would increase when the pH is closer to its *pKa* values. It can be expected that this effect of Glu and GABA would be significant under the operational conditions of GAD assay. Also, when a solution of Glu (or mixture of L-aspartic acid (Asp) and Glu) was used as the titrating solution, the proton dissociations of Asp and Glu are not complete at the reaction pH. Due to these reasons, the amount of acid added is not the same as that of Glu decarboxylated. Therefore correction of the rate of titration is required to determine the real rate of decarboxylation. In Chapter 5, the reaction of L-serine decarboxylase (SDC) was performed at pH 7.2. This pH is higher than the apparent pKa_1 of carbonic acid as stated in item 2, so the release of CO_2 would not be favorable and therefore pH-stat method would not be applicable. From these examples, it can be seen that all the aforementioned four criteria should be carefully considered before pH-stat method can be applied.

6.2.1.3 Proposed integration of conversion with ED separation

Ideally, the conversion of Lys should be integrated with an ED separation process. As described in Chapter 2, at pH 6 the ED separation of a mixture of 20 AAs (produced by protein hydrolysis) is proposed to generate an aqueous stream containing Arg and Lys. This leads to a reactor that converts Lys to PDA, and after pH adjustment, another ED separation can be carried out to obtain two pure components, PDA and Arg if no residual Lys would remain). In a continuous reactor coupled to the ED, the substrate concentration and reaction pH can be maintained by the continuous addition of the substrate solution.

Fig. 6.2 shows how a continuous LDC reactor, containing ILDC, can be integrated with the subsequent separation of PDA. As a result of decarboxylation, CO_2 gas could be formed inside the column. On small (lab) scale this does not pose problems. On large (industrial) scale, gas

formation and its release should be taken into consideration.[16] When the solution of Arg and Lys is flowing through the reactor, the pH will gradually increase as a result of decarboxylation. To obtain a higher conversion of Lys, it is necessary to maintain the pH in the reactor at the optimal pH. Therefore the pH of the Arg and Lys solution is adjusted to the lower limit of LDC's optimal pH range (pH 5.5) before it was added into the reactor.

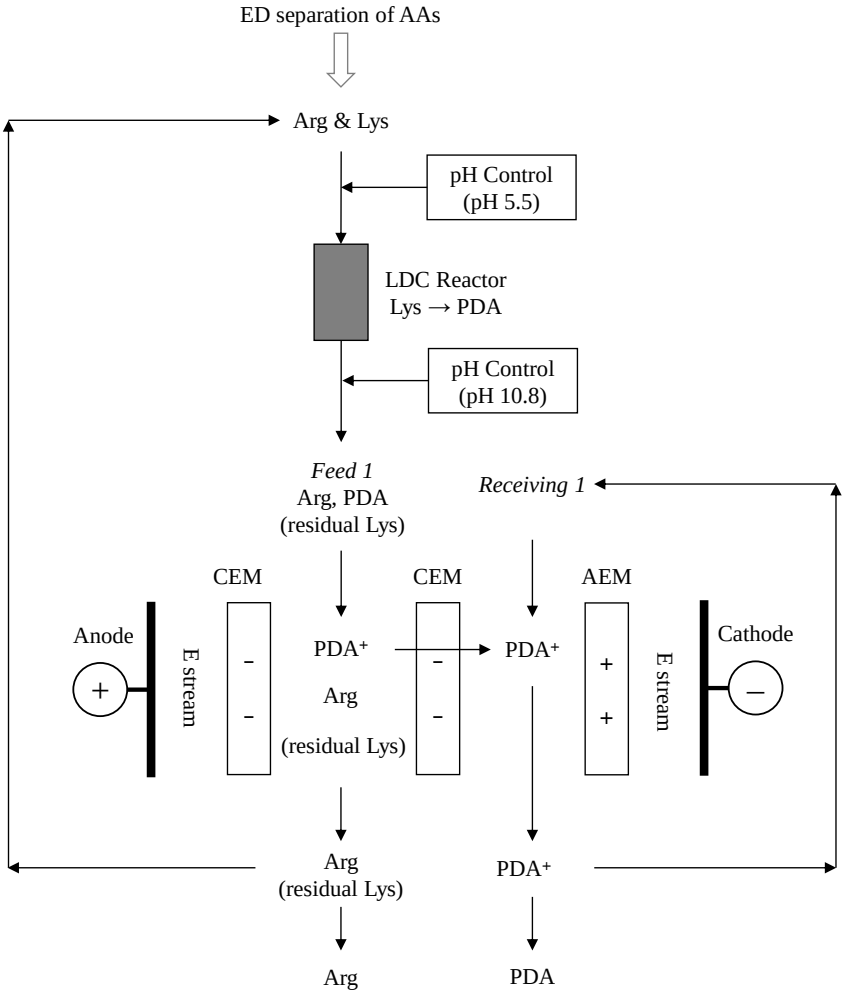

Fig. 6.2 Proposed integration of the LDC reaction and the ED separation of PDA from Arg (and residual Lys). E stream: electrode stream.

After the LDC reactor, an ED stack configuration for the separation of PDA is carried out. As stated in Chapter 1, the ED separation of PDA should be performed at 10.8 (the IP of Arg), so the

pH of Feed 1 needs to be adjusted to that value. After separation, the stream containing Arg (and residual Lys) is fed back to the enzyme reactor so the latter can still be converted. By running the process as shown in Fig. 6.2, Lys is continuously converted to PDA which is then separated from Arg and accumulated in Receiving 1. The Arg product stream (containing only a modest lysine contamination) can be obtained from the bleed stream of this feedback loop. As the unreacted Lys is sent back to the reactor, the overall conversion of Lys can be enhanced. Because of the recycling the concentration of the PDA will increase. The product stream can be obtained as the bleed stream of this recycle.

6.2.2 Specific formation of L-pyroglutamic acid from glutamic acid as a means to separate the two acidic AAs

In chapter 3, the cyclization of Glu to pGlu under (hydro)thermal conditions was used to separate acidic AAs (Asp and Glu). This separation was achieved by carefully choosing the reaction conditions to prevent the deamination of Asp, allowing only the formation of pGlu to take place. As pGlu has a much higher solubility in water (lit.,[18] 476.0 g/L) than that of Asp (lit.,[18] 5.39 g/L) and Glu (lit.,[18] 8.57 g/L), solid-liquid separation can be applied to separate pGlu from the latter two. Also, as the conversion of Glu to pGlu is reversible, the pGlu separated can be hydrolyzed back to Glu under acidic or basic conditions. Thus, the separation of Glu from its mixture with Asp can be achieved.

6.2.2.1 Application of pGlu

It has been reported that pGlu can be added to some skin care products as a humectant.[19] To overcome the drawback of being easily washed away because of its good water solubility, pGlu is reacted with medium chain fatty alcohols to form esters which can penetrate into skin and release pGlu under the effect of pyroglutamate peptidase.[20] On lab scale, pGlu can be used in the asymmetric synthesis of bioactive products for medical applications (*e.g.* Pidotimod, an immunostimulator).[21]

The pGlu can be converted to Glu which can be decarboxylated to *γ*-aminobutyric acid (GABA). This can be further converted to *N*-methylpyrrolidone by reaction in methanol in the presence of a catalyst in a one pot synthesis.[22-25] It has also been proposed that *N*-vinylpyrrolidone can also be prepared from Potentially, *N*-vinylpyrrolidone could be prepared from 2-pyrrolidone, the decarboxylation product of pGlu (reacted at pH 3 to 7 at 280 °C).[22-26] 2-pyrrolidone is traditionally reacted with ethylene to produce this product. In this way, the hydrolysis of pGlu is not required.

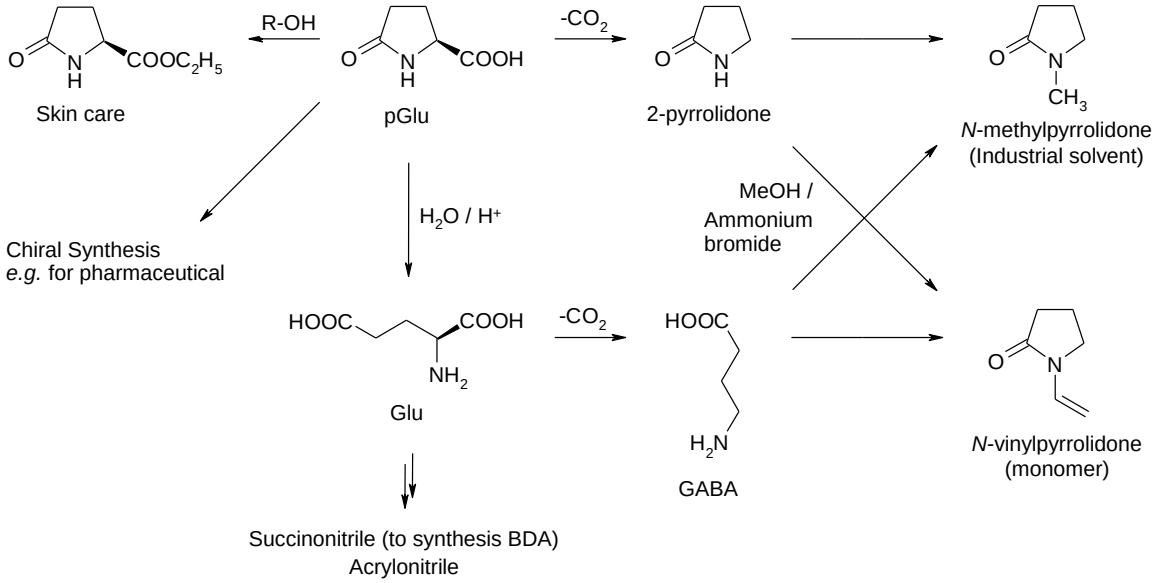

Fig. 6.3 The (potential) uses of pGlu.

As seen in Fig. 6.3, pGlu is interesting to be used for the synthesis of bulk chemicals, though. In some cases Glu is still preferred, such as the synthesis of succinonitrile and acrylonitrile). This is because in these synthesis routes the oxidative decarboxylation of Glu is employed.[24,25] However, the significance of using pGlu is that its formation aids the separation of Glu from Asp. Since the mixture of Asp and Glu can be obtained from the ED separation of hydrolyzed proteins, the formation of pGlu provides not only an important source of Glu, but also a purer stream of Asp which can also be used for chemical synthesis (*e.g.* acrylonitrile and acrylamide).[27]

6.2.2.2 Racemization

In the conversion of Glu to pGlu, racemization can take place. Under acidic conditions, the racemization of pGlu formed can be observed from ^{1}H-NMR spectrum.[28] In this study, the optimal reaction conditions of converting Glu to pGlu are in the melt at 150 °C.[29] As reported by Feng, under such conditions up to 9% of the pGlu is the D- form.[30] Chirality would be an issue when pGlu is used for the asymmetric synthesis.[21] This also will be the case if pGlu is converted back to Glu for producing chemicals by enzymatic conversion. For example, the conversion of Glu to GABA is catalyzed by GAD that is specific to the L-isomer.[22] The D-Glu formed in the separation step is not converted by GAD therefore it cannot be separated from Asp by ED.

To overcome these potential chirality issues, enzymatic conversion of D-Glu to the L-isomer may be considered. As reported by Asano *et al.*, a broad substrate specificity AA racemase (EC 5.1.1.10) from *Pseudomonas putida* uses D-Glu as a substrate.[17] However, this racemization can be inhibited by L-Glu itself.[17] So to be able to apply this method, it is necessary to use a racemase which is active at high concentrations of Glu to reduce complexity and costs. By applying chemical conversions, instead of using enzymes which are mostly stereospecific, this problem can be circumvented. For example, Lammens *et al.* reported a synthesis route of biobased succinonitrile from Glu.[25] In this process, no enzymes are used and none of the reactions depend on chirality. As aforementioned, pGlu can be decarboxylated into 2-pyrrolidone which is achiral.[26] So similarly, the production of chemicals that starts from 2-pyrrolidone, such as *N*-vinylpyrrolidone *(*or *N*-methylpyrrolidone),[22,23] would not be affected by the issue of racemization.

6.2.3 The use of GAD as a means to separate the two acidic AAs

Chapter 4 describes the study on the use of GAD as a means to aid the ED separation of Asp and Glu. This was achieved by using GAD to selectively convert the latter into γ-aminobutyric acid (GABA) in the presence of Asp.

GAD had been well studied when its natural substrate, Glu, is the only AA present in the reaction system.[22] In a similar approach to chapter 2, the main focus here is to find the optimal conditions for the specific conversion of Glu in the presence of Asp. The enzyme assay method

was pH-stat titration, but due to the reasons as stated in section 6.2.1, correction of the titration rates was necessary to obtain the actual reaction rates. GAD was immobilized by entrapment in calcium alginate based on reported method.[22] The operational stability of the immobilized GAD was studied when Asp was added to the reaction mixture.

6.2.3.1 Diffusional limitation

In Chapter 4, a column reactor was designed to be integrated with the subsequent ED separation of GABA. The substrate solution containing both Asp and Glu was pumped through the reactor to convert Glu into GABA (Expt. 1 in Chapter 4). However, the concentration of GABA obtained at steady state (0.54 mM) is significantly lower than expected (2.3 mM). This was attributed to the possible diffusional limitation caused by calcium alginate which was used as the immobilization material.[31] The low concentration of GABA may be problematic for its separation. This is because other cations with smaller size (*e.g.* Na^+ from the hydrolysis of proteins, and Ca^{2+} from $CaCl_2$ added to stabilize the alginate) present in the solution (if any) can be transported faster than $GABA^+$.[32]

To eliminate potential diffusional limitation, the method of immobilization can be improved. As reported by Lammens *et al.*,[22] GAD can be covalently bound on a Eupergit® support which does not lead to a diffusional limitation, but the drawback is that it can lead to irreversible deactivation by changing the enzyme's conformation.[33] Since the actual function of the GAD immobilization here is to retain the enzyme within the reactor rather than enhance the enzyme stability, the diffusional limitation issue could be avoided if native enzyme could also be retained. Könst *et al.* has reported a membrane reactor which can retain native enzymes inside to carrying out reactions continousely.[6] The molecular weight of GAD is about 310,000 Da.[34] Ultra-filtration membranes used for that reactor are available with a cut-off molecular weight of 10,000 Da.[35] This membrane pore size is sufficiently small to retain the GAD so that it can be used for the reaction.

6.2.3.2 Integration of two ED separations and one conversion reaction

As proposed in Chapter 4, two ED separation processes will be integrated with the column reactor which holds GAD entrapped in calcium alginate beads: upstream the reactor, ED1 configuration separates the mixture of Asp and Glu from the solution of hydrolyzed protein at pH 6; Downstream the reactor, another ED configuration (ED2) separates the GABA formed from Asp and residual Glu. The principles of both ED separations have been reported before and also discussed in Chapter 4.[36,37] Thus, an experimental setup was built as shown in Fig. 6.4.

To perform this process, *Feed 1* which contained Asp, Glu and L-alanine (Ala) (each in 25 mM) was fed into ED 1. Here Ala was added to represent of all other (neutral) amino acids that would have been present if a real protein hydrolysate had been used. At pH 6, Ala was almost neutral so it did not migrate over the membrane; Asp and Glu were negatively charged and can therefore be removed from the AA mixture (now represented by Ala). After ED1, a concentrated solution of $CaCl_2$ (4 M) was added at the rate of 0.25 mL/15mins into the stream containing Asp and Glu to maintain the structure of alginate beads. Then this stream was pumped into a column reactor that packed with GAD entrapped in calcium alginate beads. In the reactor, Glu was specifically converted into GABA. The pH of the stream flowed out of the reactor was adjusted to pH 3.2. At that pH, GABA is positively charged therefore can be separated by the subsequent ED (ED 2). Asp and unreacted Glu are almost neutral, so were not separated and were combined with the stream of *Receiving 1* to recycle the Glu. The reaction were kept running for 8 hours. Samples were taken at the all the streams flowing out of the ED stack and the reactor and were analyzed by UPLC.

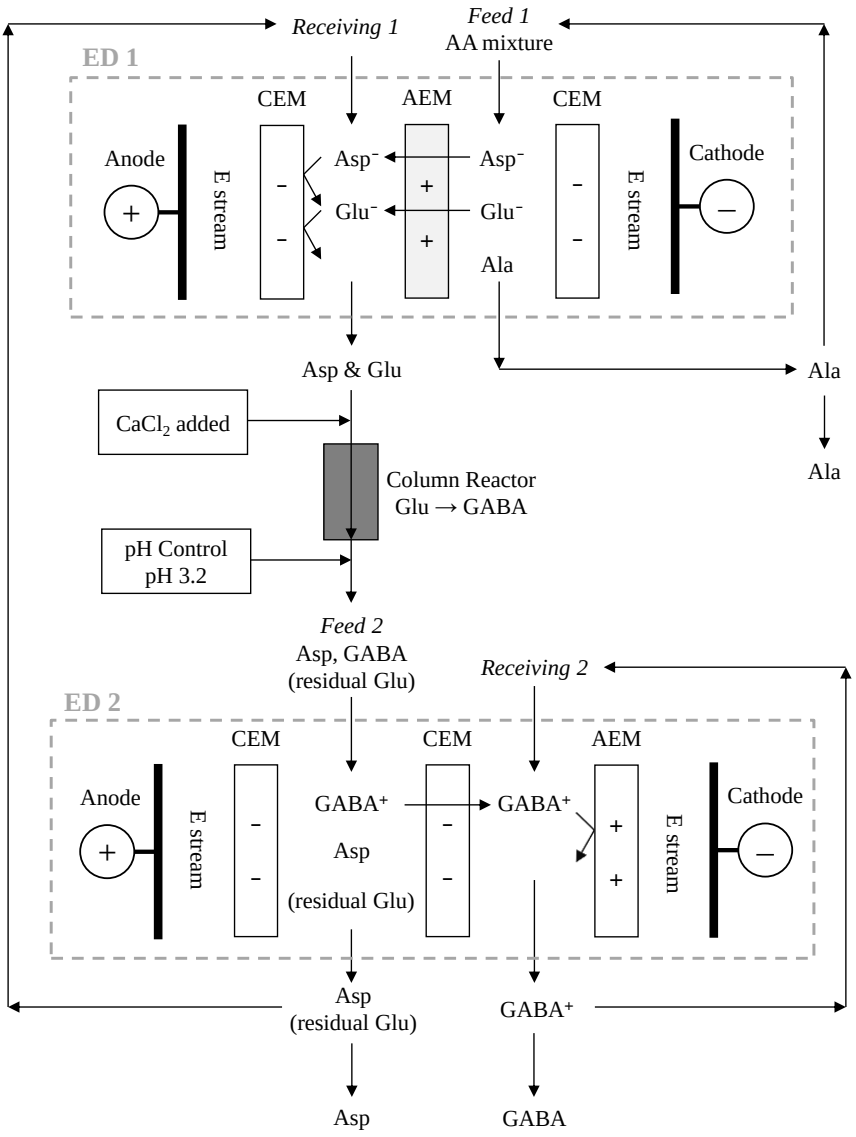

Fig. 6.4 Experimental setup for the integration of ED separations and the column reactor containing immobilized GAD. AA: amino acids; AEM: anion exchange membrane; CEM: cation exchange membrane; E stream: electrode stream (0.1 M of Na_2SO_4 solution); ED: the electrodialysis stack.

After 8 hours' reaction, *ca.* 60% of Asp and 80% of Glu had been separated from Feed 1; *ca.* 20% of the total Glu had been converted to GABA, showing that the formation of GABA took place in the column reactor. However, GABA was not separated by ED 2 possibly due to its low

concentration compared with the Na^+ and Ca^{2+} concentrations in Feed 2. Na^+ was transported from the electrode stream (0.1 M of Na_2SO_4). Here the electrode stream was used to enhance the conductivity which enhances the ion transportation and hence improves the separation efficiency. Ca^{2+} (in the form of $CaCl_2$) was added before the column reactor to maintain the structure of calcium alginate.

To improve the separation, the concentration of Na^+ and Ca^{2+} ions could be eliminated by using Eupergit® to immobilize the enzyme and not Ca^{2+}-Alginate or by the use of a membrane reactor.[6,22]

6.2.3.3 Chemical and enzymatic methods in the specific conversion of Glu

In Chapter 3 and 4, the conversions of Glu by chemical and enzymatic reactions have been discussed. These two methods were compared in Table 6.1.

Table 6.1 Preliminary comparison and potential improvements of the specific conversion methods of Glu under laboratory conditions. aq.: aqueous reaction conditions.

Property	**Chemical Conversion**	**Enzymatic Conversion**	**Potential Improvement**
Catalyst	No	GAD	–
Extra chemicals	No	PLP (enzyme cofactor) alginate $CaCl_2$ (immobilization) $CaCO_3$ (pH adjustment) Na_2SO_4 (ED)	Enzyme immobilization by covalent binding to resins to eliminate the use of salt
Reaction temperature	80-120 °C (aq.) ≥150 °C (melt)	40 °C	Develop and use more thermally stable enzyme to get increase reaction rate
Reaction pH	pH 3 (aq.)	pH 4.6	–
Production mode	Batch	Batch or continuous	–
Product	pGlu	GABA	Develop more chemical routes using pGlu as raw material
Product concentration	Up to 3.7 M (lit.,[38] solubility of pGlu)	< 0.2 M (limited by reactants' solubility)	Use enzyme with higher pH optimum to enhance the solubility of substrate.
Reaction rate	~ 430 mmol/(L·h) (aq.)	Dependant on the enzyme (5.22 mmol/(L·h) at V_{max} = 0.87 U/mg)	Addition of more enzyme or using a more active enzyme
Impact of Asp	No	Competitive inhibition (K_i = 72.2 ± 7.1 mM)	Simultaneous removal of Asp (by ED)

Separation method	Solid-liquid	ED	–
Racemization	~9% (lit.[30])	No	See Section 6.2.2
Side products	Fumaric acid (aq.) Not detected (melt)	$CaCl_2$ & Na_2SO_4 (pH adjustment in titration & ED)	Enzyme immobilization by covalent binding to resins to eliminate the use of salt
Conversion yield	~ 83% (aq.) 100% (melt)	~ 30% (one round reaction)	Use more active enzyme; Circulate the reacted stream; Enzyme immobilization by covalent binding to resins to reduce diffusional limitation
Waste water generation	Solid-liquid separation	Immobilization; ED (electrode stream)	–
Maintenance	Easy	Difficult (preservation of enzymes and sterilization)	Use more (thermal) stable enzyme
Ease of operation	Easy	Difficult (repacking of deactivated enzymes, ED membrane fouling)	Use more stable enzyme to prolong the reaction; Use ED reversal to prolong the separation

As seen in Table 6.1, the chemical conversion has the potential disadvantage that it uses a higher reaction temperature. This could lead to high energy consumption and racemization. But as discussed before, racemization would not be an issue if chemical reactions are used to convert pGlu into chemical products. Both approaches can have their advantages and disadvantages. One should study them carefully to make a good judgment on additional costs for capital, chemicals, pH adjustments, waste products *etc*.

6.2.4 The use of SDC and L-phenylalanine ammonia-lyase (PAL) as a means to separate one or more pure chemical (intermediate) products from a mixture of 16 neutral amino acids.

Chapter 5 describes the study on the use of SDC and PAL as a means to aid the ED separation of neutral AAs. SDC catalyzes the decarboxylation of L-serine (Ser) to ethanolamine (ETA) and PAL catalyze the deamination of L-phenylalanine (Phe) to *trans*-cinnamic acid (CA).[39,40] A solution containing Ser, Phe and L-methionine (Met) was used as the model mixture of neutral AAs. It was desired that the decarboxylation and deamination reaction can be performed in one reaction system at the same reaction rate to eliminate pH changes. By ED the two products formed at pH 6 can be separated from Met and into different streams.

Both enzymes were assayed by a UPLC amino acid analysis.[41] Yeast cells containing PAL activity were incubated in a hexadecyltrimethylammonium bromide (CTAB) solution (0.1 wt%) in order to permeabilize the cells and thereby enhance the PAL activity. In this model system it was shown that SDC (K_m = 10.8 mM) and PAL (K_m = 23.2 mM) are specific to Ser and Phe, respectively, therefore can aid the separation of neutral AAs. CA reduced the activity of SDC therefore it appeared to be an inhibitor of this enzyme. But this was not considered to be a problem as CA could be removed simultaneously by ED. As desired, the two enzymes can react in one reaction system, and the operational stability of SDC was better than expected. To understand this and hence to better integrate the two enzymatic conversions, the impact of CA and Met on the operational stability of SDC needs to be studied in the future.

6.2.4.1 Preliminary separation of neutral AAs

In Chapter 5, the model mixture contains 3 neutral AAs. However, the neutral AA stream separated by ED from hydrolyzed protein can contain up to 16 AAs including L-histidine (His). Here His is in fact a basic AA, but its IP (lit.,[8] pH 7.59) is close to those of neutral AAs (lit.,[8] ~pH 6) therefore it will remain with the neutral AAs. The large amount of AAs can cause problems for their conversions. For example, inhibitory effects of all neutral AAs have to be studied before an enzyme can be used for the conversion. Specificity may also be an issue. For

example, it has been reported that PAL from yeast (*Rhodotorula glutinis*) also had activity towards L-tyrosine (Tyr).[42] To avoid these issues, one can think of a preliminary separation the neutral AAs into several simpler streams.

One possible solution is separation by solubility differences. In principle this method could be applied when the solubility of AAs differs significantly. For instance, the most soluble AAs L-proline (Pro, 1625 g/L), mixed with a smaller amount of L-cysteine (Cys, 277 g/L), Ser (250 g/L), L-glycine (Gly, 239 g/L) and Ala (166.9 g/L), can be separated from the other AAs.[8,18] Also, Tyr might be easily separated due to its significantly lower solubility (0.51 g/L).[8] To further separate the remaining AAs, one can think of using the polarity difference of AAs. In this case, the use of aqueous organic solvent might be applied. For example, the solubility of L-asparagine (Asn) and Phe in water is similar (lit.,[8] 25.1 and 27.9 g/L), while in 60 V% of aqueous ethanol solution, the solubility of Asn is only *ca.* 10% as that of Phe (lit.,[43] 1.51 and 12.3 g/L), allowing them to be isolated.

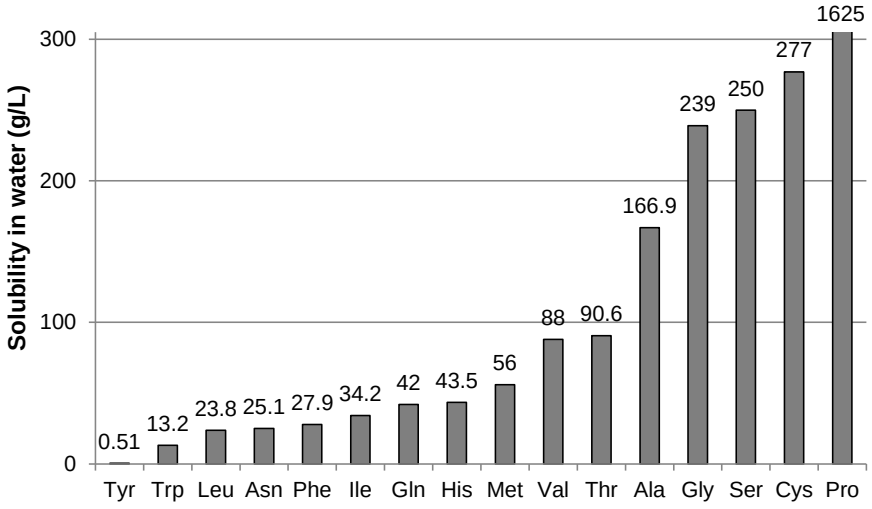

Fig. 6.5 The solubility of neutral AAs and His in water at 25 °C.[8,18] They are obtained by dissolving only the AA in water and no further addition of acid or base is performed.

Another possible solution is to apply a chromatographic method using neutral resin. Thang and Novalin reported the use of polymeric resin (Amberlite™ XAD™ 1600) to refine grass

silage juice.[44] The authors claimed that neutral resin is better than ion exchange resin as the latter could be saturated quickly if the feed contains high amounts of ions as impurities.[44] This resin separated a mixture of AAs from grass juice into three groups (Fig. 6.6). Each group contains no less than two neutral AAs therefore this resin can be used for the preliminary separation of neutral AAs. However, this method is based on size exclusion, so the consumption of resin and solvent would be significantly larger than that of ion exchange.[44] To improve this, simulated moving bed chromatography (SMB) can be considered.[45] For example, Wu *et al.* designed an SMB process to separate tryptophan and phenylalanine, with both AAs obtained at both purity and yield around 96%.[46] Park *et al.* developed a SMB process to separate L-valine (Val) from isoleucine. Here the purity and yield of Val were both 98%.[47] In a SMB operated continuously for the enantioseparation of D,L-methionine, the purities of the two individual isomers obtained over a 3 day period remained above 98%.[48] All these examples illustrate that SMB is a reliable method to separate AAs with high purity. An industrial production line employing SMB process can be at the scale of 400 tons/year.[49] In principle it would not be unrealistic to perform SMB at that scale for the stepwise separation of neutral AAs.

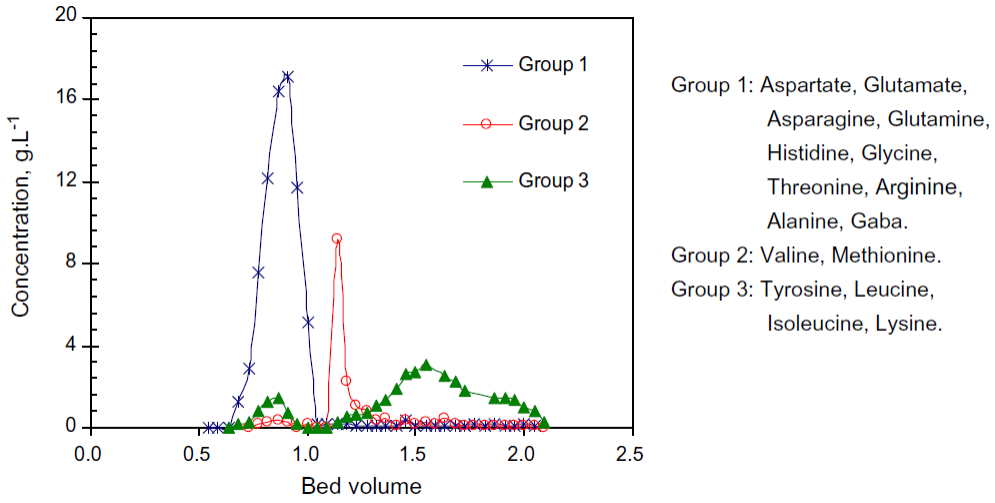

Fig. 6.6 Effluent profiles of AAs separated by XAD™ 1600 resin. This figure is originally published by Thang and Novalin.[44]

6.2.4.2 The simultaneous decarboxylation and deamination of AA

L-Phenylalanine decarboxylase (PDC, EC 4.1.1.53) and L-tyrosine decarboxylase (TDC, EC 4.1.1.25) are PLP dependent enzymes which convert Phe and Tyr into phenylethylamine and tyramine, respectively.[50] Both conversion products are used as intermediates to prepare medicines (*e.g.* Sumatriptan).[51-53] As reported, some types of PDC (*e.g.* those isolated from *Cavia aperea*, *Achromobacter sp.*, and *Micrococcus percitreus*[54]) and TDC (lit.,[55] this from *Arabidopsis thaliana*) have a pH optimum of 9, while the reaction of PAL can be carried out from pH 7 to pH 10.[54-56] So similarly to the combination of SDC and PAL as discussed in Chapter 5, the reactions by PDC and/or TDC could also be combined with that of PAL in one reaction system. As both PAL and PDC use Phe as the substrate, the combination of these enzymes would convert Phe into two different products, and both products could be separated into different streams at the same time.

L-Valine decarboxylase (VDC, EC 4.1.1.14, isolated from *Proteus vulgaris*) catalyzes the decarboxylation of L-leucine (Leu), Val and L-isoleucine (Ile) (in decreasing order of activity) to form 3-methylbutylamine, isobutylamine and 2-methylbutylamine, respectively.[57] Other types of VDC (*e.g.* this from *Cystoclonium purpureum*) may also have activity with Met and L-cysteine (Cys).[58,59] As reported, VDC from *Lysinibacillus sphaericus* has a pH optimal at 7.7,[59] so the reaction by VDC may also be combined with that of PAL.[56] To avoid the problem of the low substrate specificity, preliminary separation of Val from Leu and Ile is desired. This may possibly be done by using the neutral resin chromatography as mentioned above (Fig. 6.6).[44]

How can we control that two different enzyme systems work at the same rate during the process even when one or both of the enzymes will lose activity during the reaction? A balance of the two reaction rates can be obtained by adding one or both enzymes to obtain a system in which the pH is constant.

Besides enzymatic conversion, chemical conversions might also be applied. A known example is the hydrolysis of Asn and L-glutamine (Gln) to form Asp and Glu under acidic hydrothermal conditions.[89] Since the charge behavior and pH-depending solubility of Asp and Glu are significantly different than those of neutral AAs,[8] the hydrolysis of Asn and Gln can be used as a

means to separate them from the remaining neutral AAs. Practically, their hydrolysis already takes place when acidic hydrolysis of proteins is performed.[89]

Hamdy *et al.* reported the photocatalytic conversion of L-tryptophan (Trp) to Kynurenine by black light.[61] Kynurenine is potentially a useful chemical intermediate as it can be converted to alanine and anthranilic acid by kynureninase. The latter product can further be decarboxylated into aniline which is used as a monomer in polyurethane foams.[61] Since kynurenine is a basic AA,[62] in principle it could be separated from the remaining neutral AAs by ED at pH 6. However, the specificity of this photocatalytic conversion needs to be studied before it can be used to aid the separation of neutral AAs. This can be performed by testing the stability of the remaining neutral AAs under the same photocatalytic conditions of converting Trp.

6.2.5 Process overview

Fig. 6.7 summarized the process of separating biomass derived AAs as described in this thesis. As seen in this figure, process steps still need to be studied includes the protein hydrolysis, preliminary separation of neutral AAs, and the specific conversion of the remaining neutral AAs.

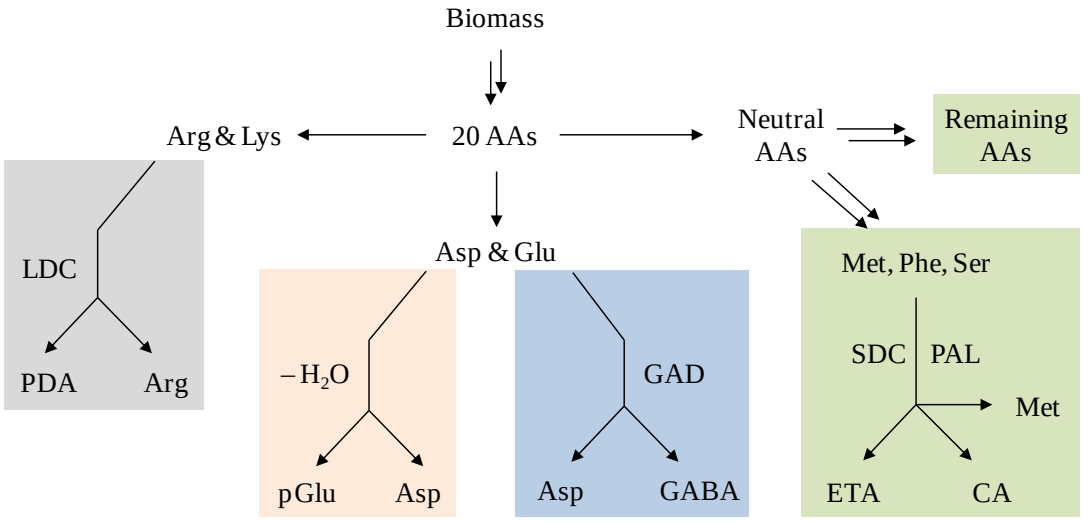

Fig. 6.7 Overview of the specific conversion of biomass derived AAs as described in this thesis.

As stated in Chapter 1, the mixture of 20 AAs is proposed to be obtained from the hydrolysis of biomass derived proteins. However, the present methods of protein hydrolysis often have drawbacks such as destroying certain AAs and leading to racemization.[89] Therefore better methods of protein isolation and hydrolysis still needs to be developed. In this case, a promising method is the enzymatic hydrolysis by proteases. This method can preserve the sensitive AAs (*e.g.* Cys and Trp) that are otherwise destroyed during chemical reaction and without inducing racemization.[90] Another advantage is that it can be used to help the extraction of protein from biomass therefore the protein extraction and (partial) hydrolysis steps may be combined.[64] However, a potential challenge might be the cost of the enzymes required. At present, proteases are mainly applied in detergents, the food industry and for laboratory purpose,[65,66] but industrial application of enzymes requires lower cost in their production.[67] Several strategies can be applied to achieve this, including the over expression of genes to enhance the enzyme yield, protein engineering to improve the enzyme activity and stability.[68] Also, enzyme immobilization can be utilized to enhance the stability and re-usability and hence reduce the enzyme cost.[33] As estimated by van Hoek *et al.*, bulk enzymes with industrial interests can be produced with the cost around €100 /kg.[68] In this thesis, it has been illustrated that AAs derived from protein hydrolysis can be useful to manufacture bulk chemicals. Therefore proteases are also of industrial interests in for the chemical production. Thus, further studies on the enzymatic hydrolysis of proteins would be beneficial to help reduce the cost of protease.

Preliminary separation of neutral AAs has already been discussed in section 6.2.4. Assuming that neutral AAs are successfully separated into a few streams, then a further separation inside these streams may still need to be assisted by specific conversions. This may possibly be achieved using the enzymes as suggested in the end of section 6.2.4.

Since Fig. 6.7 is limited in details of the reaction conditions and the unit operations, some of the potential issues cannot be seen directly from the figure. One issue is the desalination of the AA mixture. Salts can be formed due to pH adjustment of reactions or operating conditions or the ion transportation in ED (from E stream). As mentioned in section 6.2.3, the formation of salts should be avoided as it reduces the efficiency of ED separation. Another issue is the composition of the AA mixture to be separated. Depending on the sources, the amounts and types of AAs

obtained from biomass can differ significantly. This may impact the optimization of the refinery route. When the protein contains a higher percentage of a certain AA, then it may be separated by other separation methods, such as the precipitation. For example, if one focuses on the use of Glu from wheat gluten (lit.,[69] *ca.* 35% of all AAs), then acidic hydrolysis (by HCl) would be better than basic hydrolysis. This is because during acidic hydrolysis, Gln is converted to Glu.[89] Also, under acidic conditions racemization is eliminated, which favors the subsequent enzymatic conversion of Glu.[90] After hydrolysis the crystallization of Glu·HCl can be directly performed in the protein hydrolysate without pH adjustment, thus the downstream processing is simplyfied.[70]

6.3 General recommendations for future research

This thesis described some specific conversion methods of biomass protein derived AAs as a means of aiding their separation preferably by ED. Based on these conversions, a biorefinery route of synthesizing industrial chemicals from protein rest streams was proposed (Fig. 6.7). The main recommendations for future research are as follows:

6.3.1 Use of novel or alternative enzymatic conversions of AAs

In this thesis, the enzymes used are decarboxylases and ammonia lyases. To enrich the specific conversion methods used and expand potential products, it would be beneficial to identify more enzymes capable of performing suitable reactions that can change chemical or physical behavior.

As mentioned in section 6.2.4, the simultaneous decarboxylation and deamination of AAs can save the extra acid and base needed for maintaining the reaction pH, and the products formed can be separated into different streams by ED. Currently, it is known that 5 AAs can be both enzymatically decarboxylated and deaminated (Asp, His, Phe, Ser, and Tyr).[59] For other AAs, it is interesting to exploit their decarboxylases and/or ammonia lyase that can be reacted simultaneously. Subsequently, the application of the conversion product should also be studied to guarantee that the conversions would be of industrial interest.

In this thesis, all the charge modifications of acidic and basic AAs were performed on the charge groups on the α-carbon. However, some AAs also have a charged group elsewhere in the

molecule. From the view of improving product separations using ED, the removal of these groups could also be considered. Currently, it has been reported that aspartate 4-decarboxylase can remove the *β*-carboxyl group of Asp to form Ala.[59] However, Ala is already a proteinogenic AA. To make the conversion also interesting for chemical preparation, further transformation of Ala needs to be developed. For example, one can think of its decarboxylation to form ethylamine which is used in dye industry.[1] Similar approach might be used when other enzymes were found for the modification of the side charge groups.

6.3.2 The development of more robust enzyme mutants

Here "more robust" means to have higher thermal and operational stability, less susceptible to inhibition and a larger operational pH optimum. If their thermal and operational stability could be enhanced, their deactivation would be slower and the productivity can be enhanced. These improvements would facilitate the saving of energy and production costs. Also, under the operational conditions, enzymes are often exposed to many coexistent compounds (salts, conversion products, and AAs that are not involved in the reaction). These compounds could lead to inhibition which reduce the activity and hence the productivity. Therefore enzymes less susceptible to inhibition would be desired. Ideally the optimal pH range of the enzymes used would include the pH required for the subsequent ED separation. This would also eliminate salt formation. If two or more enzymatic reactions needs to be performed simultaneously in one reaction system (*e.g.* SDC and PAL as in Chapter 5), their optimal pH ranges should (partly) overlap and so here also enzymes with wider optimal pH range would be useful.

To obtain more robust enzymes, one can think of screening them from extremophiles. Extremophiles are "organisms that thrive in extreme environments", such as thermophile which grow between 60–80 °C.[71] Zambare *et al.* reported the use of thermostable cellulases from *Geobacillus sp.* for the production of bioethanol from switchgrass lignocellulose.[72] The enzyme can operate at higher temperature (up to 70 °C) promoting a good reaction rate and hence a higher conversion efficiency of switchgrass to ethanol.[72] If the enzymes mentioned in this thesis can also be used at higher temperatures, the conversion rates can be boosted and the production cost might be reduced.

Another strategy is to use genetic modification and protein engineering to enhance the enzyme stability. One example is the GAD from *E coli.* As described in Chapter 4, native GAD from *E coli.* has its optimal operational pH at 4.6.[22] By genetic modification, Pennacchietti *et al.* made a mutant of GAD which had an optimal activity at pH well above 5.7.[73] By this improvement, the substrate concentration can be significantly higher (lit.,[70] 27 wt% at pH 5.7 *vs.* 4 wt% at pH 4.6). If this mutant enzyme can be used to aid the separation of Asp and Glu, the concentration of AAs can be higher, and the subsequent ED separation of Asp and the conversion product (GABA) can be performed around pH 6.[37] This means that the reaction solution can directly be fed to the ED without a pH adjustment.

6.3.3 The issue of unwanted salts (or ions)

In this research, mixtures of pure AAs were used for experiments therefore desalination was not an issue. However, in reality the solution of AAs derived from biomass may contain salts derived from hydrolysis or the biomass itself e.g. alkali or alkaline earth metal salts. As mentioned in section 6.2.1, salts can reduce the efficiency of separation. Therefore in the future study desalination would be an important for the continuous research.

Traditionally, desalination of AAs was performed by ion-exchange chromatography. This method often leads to the absorption of part of the AAs.[74] Also, the ion-exchange resin consumed by the chromatography needs to be regenerated using concentrated acid or base. This brings costs of more chemicals, energy, and capitals.[75] Under high salt concentration the exhaustion of ion-exchange resin can be faster and the resin regeneration would be difficult.[76] Thus, chromatographic method is not preferred.

Interestingly desalination can also be performed using ED. Chen *et al.* reported the production method of Lys: firstly Lys·HCl from was reacted with NaOH in solution, and the formed NaCl was removed by ED to give a purified solution of Lys.[77] Thus, their "production" method is actually a desalination process of AA solution. Fidaleo *et al.* reported a batch mode ED to desalt soy sauce (*ca.* 15% w/v NaCl) which contains AAs as the taste and aroma-bearing components.[78] To minimize the health problems caused by high intake of salt, the authors built a lab scale ED setup to desalt the soy sauce without losing much AAs. They carefully chose the process

variables (pH, temperature, concentration, solution composition, stack assembling, and electric current density) for the desalination. As claimed by the authors, about 70% of the nitrogen (from the amino group) and 14% of chloride ions were found in desalted soy sauce, indicating that the desalination was successful.

Certainly, it would be much better to avoid the formation of salt than to desalt it afterwards. This may possibly be achieved by improvement of reaction and process design. For example, as discussed in section 6.3.2, by using the mutant of GAD which can work well above pH 5.7, the pH adjustment of reacted solution can be saved as the subsequent ED separation can be performed at pH 6. This can certainly avoid the associated formation of salt.

6.3.4 The use of CO2 for reversible pH adjustment

The production of salts is often linked to pH control by the addition of acids and bases. If the pH can be reversibly tuned by dissolving or releasing gas, the use of extra acid and base as well as the formation of salt can be avoided. Hepburn *et al.* have reported such a novel method in the bioproduction of succinic acid.[79] In their experiment, CO_2 was used to decrease the pH of the fermentation broth to below 4.2 (the pK_{a1} of succinic acid), and the increase of pH was achieved by bubbling nitrogen to drive out the CO_2. According to the authors, an elevated pressure of CO_2 can reduce the pH even further.

The use of CO_2 to alter pH might also be interesting for other biorefinery process. However, as carbonic acid is a weak acid, there will be a practical limit to further decreasing pH. Thus, the method would suit situations using weak acidic conditions. For example, it might be used for enzymatic hydrolysis of proteins which can be performed at around pH 3.[64] As the dissolved CO_2 is promoted by increasing the gas pressure, the reaction device has to be pressure resistant. This also raises the point if pH adjustment by CO_2 can be (more) economical compared to conventional methods (using adding acid). Besides CO_2, one may also think of finding other gases for pH adjustment. For example, a NH_3 gas would produce an alkaline aqueous solution.

6.3.5 Developing of new applications for the AA-based chemicals

Some of the bulk chemicals that can be synthesized from AAs have been summarized in Table 1.1 in Chapter 1. Most of them (*e.g.* BDA, NMP, and ETA) are now produced in the petrochemical industry.[1] Still, many of the AA conversion products described in this thesis, such as pGlu as described in section 6.2.2, are not yet used as a bulk chemical (or intermediate) in the current chemical industry.

Besides pGlu, another example of potentially useful product is PDA (Chapter 2). PDA is a diamine whose chemical structure is very similar to that of BDA. Currently the main application of BDA is to react with adipic acid to produce nylon-4,6.[1] As estimated, the annual production of BDA is about 10,000 tons,[1] In contrast, the use of PDA to synthesis nylon is less well studied. Kind and Witmann reported that PDA can be used to react with sebacic acid to produce nylon-5,10.[80] As nylon-5,10 displays excellent properties such as high melting point and glass transition temperature as well as low water absorption, they claimed that nylon-5,10 might be applied in the automotive industry or in high-value consumer products.[80] If the applications of nylon-5,10 can be developed, the need of PDA would be enhanced, and the separation and specific conversion of Lys (Chapter 2 of this thesis) would be commercially of interest.

As more conversion methods of AAs are developed the number of products that could be produced would increase. Exploring new applications for these AA-based chemicals would be beneficial to improve the economics of the AA separation route.

6.4 The project "electrodialysis separation of amino acids"

The research described in this thesis is part of the STW project "A novel process route for the production of building blocks for chemicals from protein sources using electrodialysis". The aim is to develop a biorefinery approach of using biomass derived AAs to produce bulk chemicals. As specific conversions of AAs and the ED separation are used, this project is also abbreviated as "EDAA" (ED separation of AAs). The research is conducted in the Chair of Biobased Commodity Chemistry at Wageningen University (this thesis) and Membrane Technology Group at University of Twente (thesis of Dr. Readi[81]). As described in this thesis, selective conversion

of AAs using enzymatic and chemical approaches can be achieved. These conversions lead to products with different charge and solubility behavior, providing opportunities for their further separation.

Regarding the acidic AAs (Asp and Glu), their separation from other AAs is known. For example, Sandeaux *et al.* recovered 98% of Asp and 88% of Glu from a protein hydrosate.[36] Kumar *et al.* isolated Glu from a mixture of Glu and Lys through ED, achieving a recovery and current efficiency of 85% and 66%.[82] However, the separation of Asp from Glu is difficult due to their similar IPs (2.85 & 3.15).[37] As studied in University of Twente, at pH 6 the Asp^{-1} (the superscript indicates the net charge) can be isolated from $GABA^{0}$ using ED with high recoveries (~90%) and current efficiency (70%).[37] This separation does not need strict pH control due to the similar charges behavior of Asp and GABA between pH 4 to 8. These results indicated that to assist separation by ED, GAD can be used to specifically decarboxylate Glu to GABA. The integration of enzymatic reactions and ED separation provides an attractive method to separate AAs, and potential products from the mixture of AAs which have similar IPs.[37]

The IP of basic AAs (Arg and Lys) are also similar (lit.,[8] 10.76 and 9.74) therefore their direct separation by ED is limited in efficiency.[83] On one hand, their ED separation can be aided with the specific decarboxylation of Lys to PDA using LDC. The pure stream of Arg can be obtained by ED at pH 12.5 and purified PDA can be obtained at either 10.9 or 10.0, but at pH 10.0 the separation of $PDA^{+1.5}$ from Arg^{0} gave the highest recovery (63%) and highest current efficiency (83%) with a low energy consumption (3 kWh/kg).[83] On the other hand, the direct ED separation of Arg and Lys can be achieved if the poisonous effect of Arg on commercial cation exchange membranes (the decline of swelling degree) can be tackled.[84] In Twente University, this was resolved by applying a tailor made cation exchange membrane with high swelling degree as well as by using ultrafiltration membranes. These approaches were also successfully applied to complex mixture of AAs, such as the isolation of basic AAs from neutral Ala.[84]

Aiming at the separating of neutral AAs, the enzymatic decarboxylation of Ser to form ETA can be utilized. However, determined by the zwitterionic nature of AAs, under the operational conditions, a small pH shift can alter the charges of AAs significantly. This declines either the recovery or the product purity. To maintain the pH without the introducing of extra ions, a tailor-

made segmented bipolar membrane (sBPM) containing both the monopolar and bipolar areas was developed.[85] In this membrane, the transport of ETA^{+1} at neutral pH (~pH 7) is allowed. Meanwhile, water was split into H^+ and OH^- which were moved to different direction under the electrical field to compensate the pH changes. Compared with using commercial cation exchange membranes, this method gave a pure stream of Ala while decreased the energy consumption by 13% (3.9 kW/kg).[85]

For the purpose of process intensification, it is desired that the AA conversion and the subsequent ED process can be integrated. Besides connecting their individual setups together, the integration can also be performed by immobilizing the enzymes in ED membranes to allow the simultaneous reaction and the removal of conversion product. Readi *et al.* used milled Relizyme^{TM} EP403/S as enzyme carriers to manufacture mixed matrix membranes (MMM). Then, GAD was immobilized in the carriers.[86] The activity of GAD on the membranes is about 50% that immobilized on unmill carrier particles. After the membrane was applied, 33% of the Glu conversion was achieved with 40% of the current efficiency. These results proved that using MMM is a promising method to combine the enzymatic conversion and ED separation simultaneously for the purpose of biorefinery.[86]

To compare the integration method using connected setups and MMM, the preliminary process design and economic evaluation have been performed.[87] The process evaluation shows that the using MMM can save about 25% of the required membrane area, which consequently saves the same percentage of capital and operating costs.[87] If MMM is used, the flux of AAs can be achieved in the project (lit.,[81] in the order of 10^{-5} mol/m^{2}s) needs to be improved by a factor of 100 to allow the whole process being profitable. However, if the membrane cost can be decreased by 40%, a flux growth by a factor of 10 is enough to guarantee the investment of the process to be paid back within 3 years. As a suggestion, future research should focuses on increasing the AA flux through ED membranes and the manufacturing of more economical membranes to reduce their cost.[87] Similar approach can be expanded to evaluation the conversion and separation of Lys and Ser.[87]

6.5 General conclusions

The aim of the research described in this thesis was to study specific conversion methods as a means to induce the separation of AAs with similar IPs (Fig. 6.7).

In case of basic AAs (Arg and Lys), LDC can be used to aid their separate by ED. Based on the operational pH, the pH-stat method using $HCl_{(aq)}$, Lys·2HCl or the mixture of Arg·HCl and Lys·2HCl (in the mole ratio of 1:1) is effective to assay the enzyme. Using a column reactor packed with immobilized LDC, the enzymatic conversion can be connected with the subsequent ED separation to form a continuous product formation and removal process, providing benefits in enhancing the overall conversion. Another method of aiding the separation is to convert Arg to BDA using L-arginase and ODC. This requires the two enzymes to be both specific to their native substrate and not be significantly inhibited by Lys.

In case of modifying acidic AAs (Asp and Glu), (hydro)thermal chemical conversion of Glu to pGlu can be used. If pGlu can be decarboxylated efficiently, it can be a useful chemical or intermediate thereof for chemical production. However, reacting under thermal conditions also leads to racemization of AAs. To address this issue, one can think of using racemase to recover the L-isomers. Also, racemization would not be a problem if chemical methods are used for the subsequent conversions of pGlu.

The separation of acidic AAs can also be assisted with GAD. The enzyme was immobilized in calcium alginate and packed into a column reactor for the integration with ED. However, diffusional limitation took place in the reactor. This prevented the building up of GABA concentration and hence eliminated its separation. To tackle this, immobilization by covalent binding on a resin or using a membrane reactor can be considered. Both the enzymatic and chemical conversion methods have pros and cons. Therefore further research is needed to carefully make good consideration on additional costs.

In case of neutral AAs, the reaction of SDC and PAL can be performed simultaneously to facilitate the separation of Ser, Phe and Met. Before applying specific conversions, it would be beneficial to perform preliminary separation of all neutral AAs. This can be done by utilizing their solubility difference or using moving bed chromatography with neutral resin. Other

enzymatic and chemical reactions may also be useful to aid the separation of neutral AAs, but specificity would be a critical aspect to study.

Based on the process overview, the future research can be focused on the investigation of new enzymatic conversion methods, the developing of more robust enzymes, desalt and the elimination of salt formation, the use of reversible pH adjust technical by gases, and the finding of more use of chemicals can be prepared from AAs.

As part of the EDAA project, the results presented here illustrate that specific conversion of AAs by enzymes and chemical methods can promote to their separation. This project will contribute to the biorefinery of biomass derived AAs to replace fossil resources in the production of bulk chemicals.

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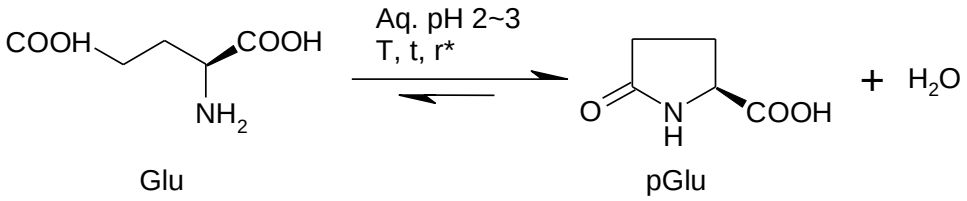
Appendix A

Supplementary material to Chapter 3

A1 The conversion of Glu to pGlu under aqueous conditions



Scheme A1 Conversion of Glu to pGlu under aqueous conditions

*: temperature (T), time (t) and the Glu to water ratio (r).

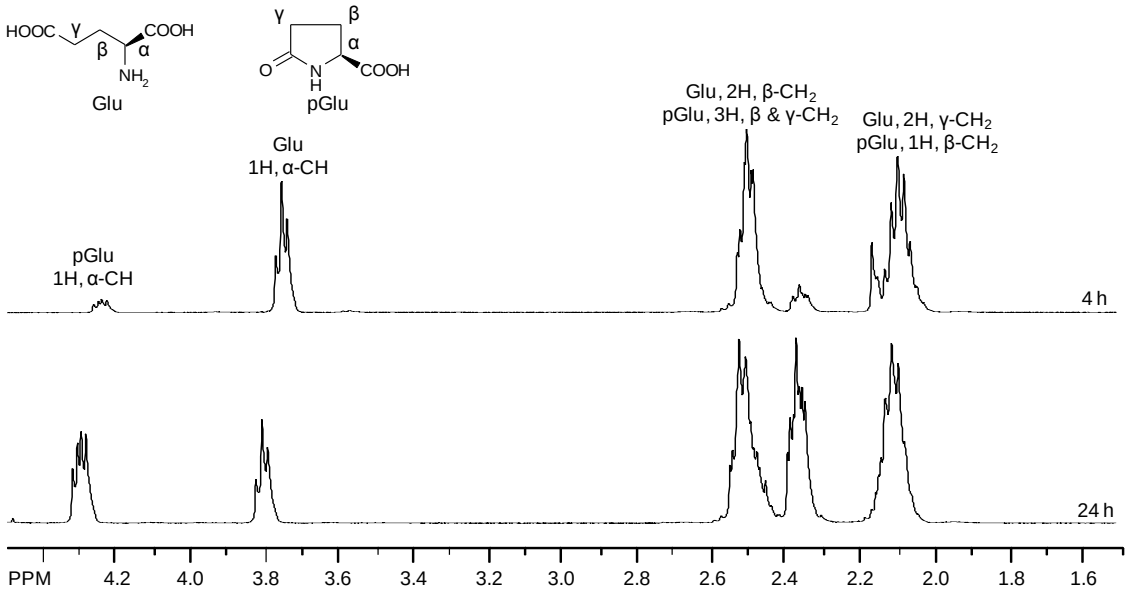
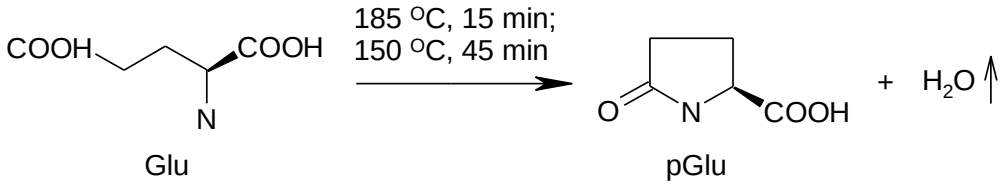


Fig. A1 ^{1}H-NMR spectra of the conversion of Glu to pGlu under aqueous conditions. In both case, 100 mg of Glu was added to 1.0 mL of D_2O and reacted at 99 °C. At other conditions (amount of Glu, time and temperature) the spectra were similar.

A2 The *conversion* of Glu to pGlu under aqueous conditions



Scheme A2 Conversion of Glu to pGlu under aqueous conditions

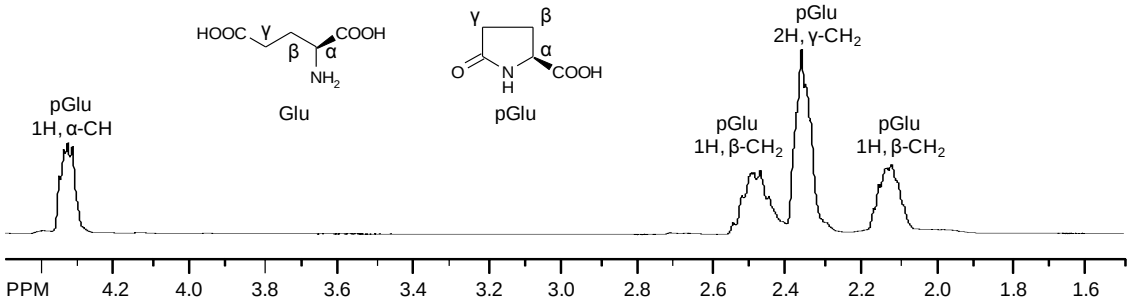
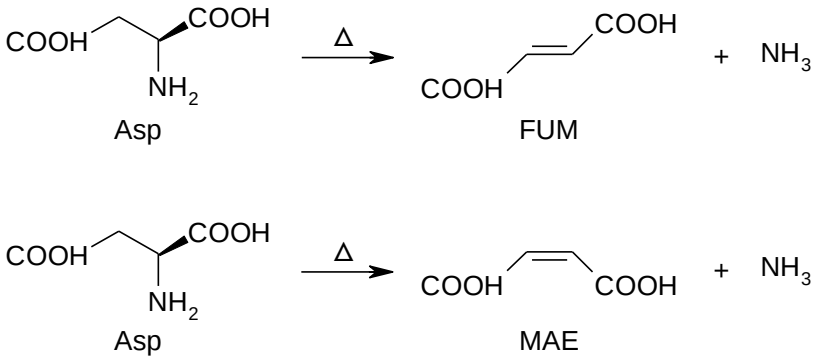


Fig. A2 ^{1}H-NMR spectrum of the product obtained from Glu to pGlu conversion under melt condition.

A3 Stability of Asp



Scheme A3 Thermal decomposition of Asp to FUM and MAE.

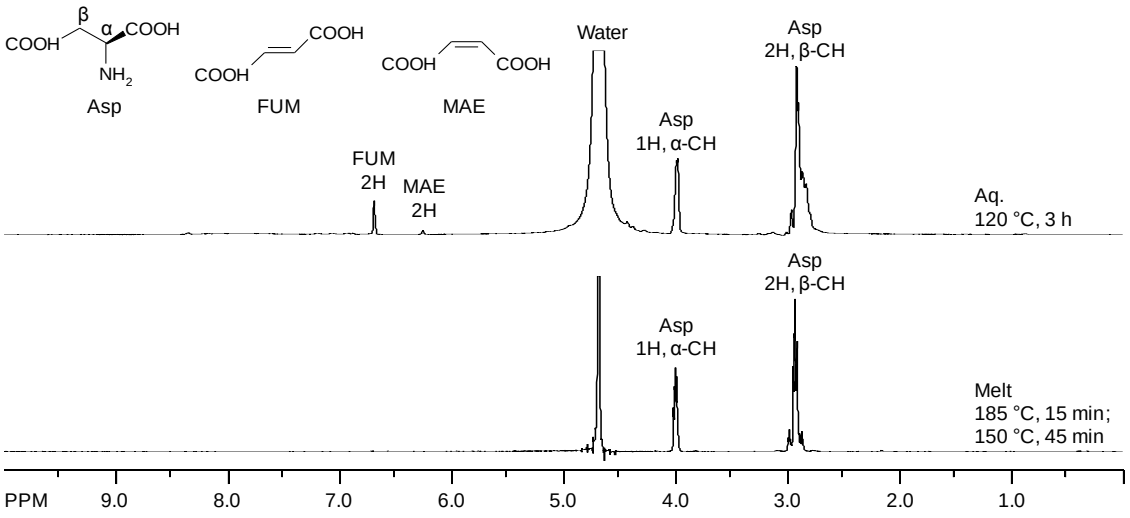
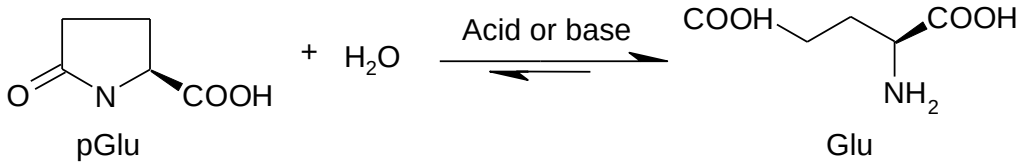


Fig. A3 ^{1}H-NMR spectra of the product obtained from Asp stability experiment under aqueous conditions (reacted at 120 °C for 3 hours) and melt conditions (incubated at 185 °C for 15 minutes and then at 150 °C for 45 minutes).

A4 Hydrolysis of pGlu to Glu



Scheme A4 Hydrolysis of pGlu to Glu under acidic or basic conditions

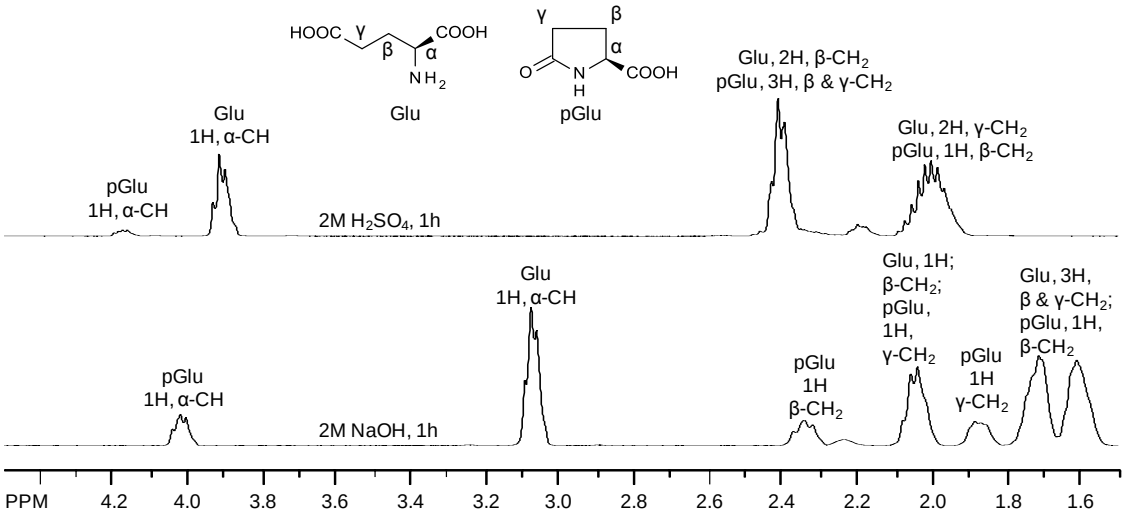


Fig. A4 ^{1}H-NMR spectra of product from pGlu hydrolysis under acidic conditions and basic conditions.

A5 Selective formation of pGlu as a means of separation (aqueous conditions)

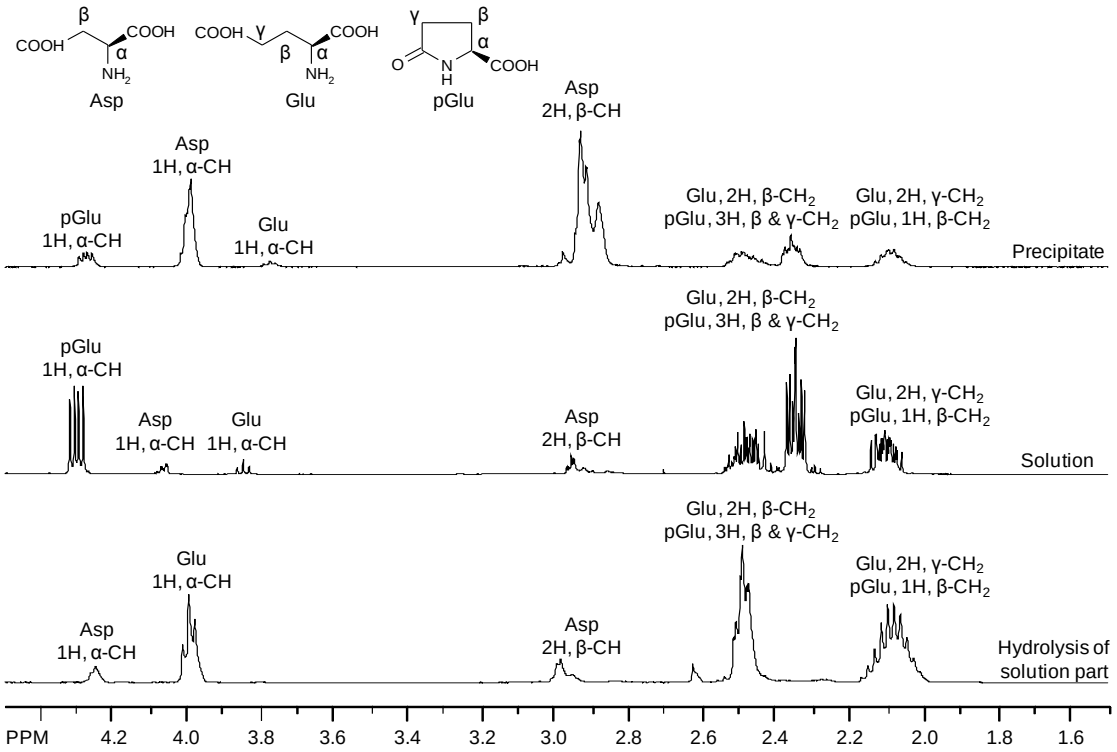


Fig. A5 ^{1}H-NMR spectra of the precipitate and solution part obtained from the separation of Asp and Glu aided by the formation of pGlu under aqueous conditions, and the hydrolysis product obtained from the solution part.

A6 Selective formation of pGlu as a means of separation (melt conditions)

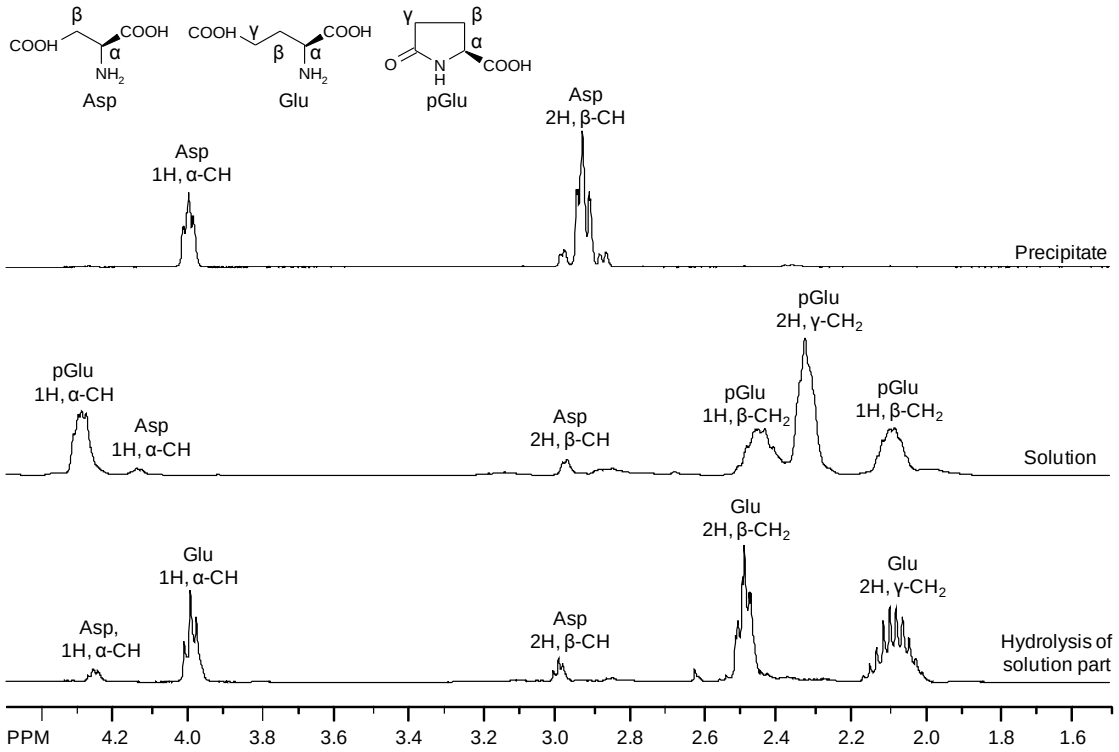


Fig. A6 ^{1}H-NMR spectra of the precipitate and solution part obtained from the separation of Asp and Glu aided by the formation of pGlu under melt conditions, and the hydrolysis product obtained from the solution part.

Appendix B

Supplementary material to chapter 4

Background

The enzymatic conversion of Glu GABA is assayed by pH-stat titration. Depending on the type of titration, Asp may present in the substrate solution, and $HCl_{(aq)}$, solution of Glu or the solution of Asp and Glu are used as the titrating solution. The acids used for the titrating solution have different dissociation properties. Also, as the titration was carried out at pH 4 which is very close to the pK_a values of Asp (3.71, side chain), Glu (4.15, side chain) and GABA (4.02, COOH),[3] the buffering effect of the reaction solution on the pH change should not be ignored. Thus in number of moles the amount of acid added does not equal to the amount of Glu converted, and correction is needed to know the later from the former.

The process of pH-stat titration can be done by simulating the titration process in software. For example, in case when enzymatic decarboxylation of Glu was titrated by HCl(aq), the three steps are:

1. The substrate solution was prepared by dissolving Glu (*k* M) and enough NaOH (*b* M) to reach pH 4.6
2. By decarboxylation, some of Glu was converted to eq. moles of GABA (*x* M) which leads to a pH increase.
3. HCl(aq) (*a* M) was added to bring the pH back to 4.6.

Thus in the reaction solution:

Before titration:	Glu (*k* M)	NaOH (*b* M)		
After titration:	Glu (*k* - *x* M)	NaOH (*b* M)	GABA (*x* M)	HCl (*a* M)

Since before and after the reaction the pH of the solution is at 4.6, if *k*, *x* and *b* are known, *a* can be calculated considering the charge balance and dissociation equilibrium.[1] Practically this pH calculation is done by software. If *a*/*x* (mole ratio of acid added to Glu converted) is a constant in practical concentration range, it can be used as a correction factor in pH-stat titration to calculate how much Glu is converted.

Calculate correction factors for pH-stat titration

All the pH values here are calculated in CurTiPot Version 3.5.4 for MS-Excel®, sheet "pH-calc".[2] The pH-stat titration is simulated in the software to study the pH changes and hence to calculate the correction factor for the titration.

1. Preparation of substrate solution was done by dissolve enough of Glu in water:

   [Glu] = 20 mM

   By loading the pK_a values of Glu and calculate the pH, it is found that:

   pH = 3.540

   This is lower than the pH needed for the reaction, so base should be added to bring up the pH. In software this was simulated by increase the concentration of OH^- gradually until pH = 4.6, giving:

   [Glu] = 20 mM, [OH^-] = 11.02 mM

2. Assuming that half of the Glu (10 mM) has been converted to GABA (pK_{a1} = 4.02, pK_{a2} = 10.35),[3] therefore:

   [Glu] = 10 mM, [OH^-] = 11.02 mM, [GABA] = 10 mM

   Giving:

   pH = 8.761

   This is higher than required by the enzymatic reaction, so extra acid needs to be added to bring the pH back to 4.6.

3. If $HCl_{(aq)}$ is added to adjust the pH, in software this was simulated by increase the concentration of H^+ gradually until pH = 4.6, giving:

   [Glu] = 10 mM, [OH^-] = 11.02 mM, [GABA] = 10 mM, [H^+] = 7.9 mM

   Correction factor = 7.9/10 ≈ 0.79

It is found that the correction factor is not changing with the amount of Glu converted, the start concentration of Glu, and the presence of Asp in the substrate solution. So this factor can be

used in the pH-stat titration to calculate how much Glu is converted from the amount of $HCl_{(aq)}$ added. In the similar way, the correction factors for other combination of substrate solution and titrating solution can be calculated.

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Summary

The current production of bulk chemicals is highly dependent on the use of fossil fuels as the carbon and energy sources. As discussed in **Chapter 1**, this leads to issues including the depletion of limited reserves, insecure supply and global warming. Thus biomass, which is considered to be sustainable and carbon neutral, becomes a promising substitute for fossil fuels in the production of fuels, chemicals and materials. Also the concept of biorefinery has been introduced to separate and convert biomass into marketable products. For the chemical industry biomass, which contains functionalities needed for bulk chemicals, can be used as a feedstock that replaces fossil fuels. Therefore providing benefits in saving process steps, extra chemical reagents, energy consumption and capital cost. For example, amino acids (AAs) are very interesting precursors for nitrogen (amine) containing chemicals. They can be derived as a mixture from the hydrolysis of potentially inexpensive proteins obtained from the byproducts of the biofuel production or agricultural and food waste streams. However, AAs derived from such sources are present as a mixture. Therefore separation is required to obtain individual AAs for subsequent transformations and applications. Electrodialysis (ED) is a promising separation method that can be carried out in continuous mode and large scale. Based on the difference in isoelectric points (IPs), ED separates AAs into acidic, basic and neutral streams. However further separation needs to be improved when AAs with similar IPs are present in the same stream. To aid the further separation inside the three streams by ED, specific conversion of AAs can be applied to give products with different charge or solubility behaviors and have industrial application. The aim of the research presented in this thesis is to explore and improve those specific conversions that can be used as a means to separate AAs for a biorefinery approach of bulk chemicals production.

Chapter 2 describes the study on the use of L-lysine decarboxylase (LDC) as a means to separate the basic AAs, L-arginine (Arg) and L-lysine (Lys). LDC is specific in the conversion of Lys (K_m = 0.78 mM) to 1,5-pentanediamine (PDA). PDA can be separated from the remaining Arg by an ED process at the IP of Arg (pH 10.8). PDA can be used as a monomer in the

production of nylon polymers. The storage and operational stability of LDC can be enhanced by entrapment in calcium alginate beads. Arg and NaCl have little effect on the activity of LDC until concentrations exceeding 30 mM are reached. The competitive inhibition caused by PDA (K_i = 9.68 mM) was observed, can be overcome by maintaining a sufficiently high concentration of Lys (*e.g.* at 20 mM). The presence of Arg caused a decline in operational stability, but this can be avoided by performing the reaction at a lower temperature (30 °C). Process design and cost estimation shows that immobilized LDC can be applied to aid the separation of AAs with the forming of useful chemical product.

Chapter 3 describes the study on the formation of L-pyroglutamic acid (pGlu) as a means to separate acidic AAs, L-aspartic acid (Asp) and L-glutamic acid (Glu), based on solubility difference. The formation of pGlu can be performed under aqueous or melt conditions. Under aqueous conditions, specificity was achieved by applying the optimal reaction conditions (120 °C, 3 hours, Glu : H_2O = 200 mg : 1 mL) . Under melt conditions, the specificity was achieved by melting Glu at 185 °C and reacting at 150 °C for 45 minutes, meanwhile Asp remained in the solid form without conversion. After the specific conversion of Glu, the highly water soluble pGlu formed was separated from Asp by solid-liquid separation. pGlu was then hydrolyzed to Glu under both acidic and basic conditions. Thus the separation of Asp and Glu was achieved. The synthesis of pGlu under aqueous and melt conditions, resulted in Glu being obtained with high and similar purity. However, under melt conditions more Asp and Glu were recovered and the Asp obtained was of higher purity.

Chapter 4 describes the study on the use of L-glutamate α-decarboxylase (GAD) as a means to separate acidic AAs (Asp and Glu). The assay of GAD was performed using a pH-stat method and this method was improved by introducing a correction factor to obtain the actual enzyme activity. GAD is specific for the conversion of Glu (K_m = 1.6 mM) to γ-aminobutyric acid (GABA). GABA has been shown to be a useful raw material for the industrial solvent *N*-methyl-pyrrolidone (NMP). The GABA formed can be separated from the remaining acidic AAs by an ED process at pH 3.5. Asp inhibits the enzyme competitively and reduces its operational stability, but this is not considered to be a problem as the inhibition is limited (K_i = 72.2 mM >> K_m) and can be overcome by maintaining a sufficiently high concentration of Glu (*e.g.* 20 mM) in the

reaction. GAD was immobilized by entrapment in calcium alginate without loss of activity, but its operational stability was not enhanced. To integrate the enzymatic reaction with the following ED separation of GABA, the reaction using immobilized GAD was also performed in continuous mode using a column reactor. Although the conversion is lower than expected, possibly due to diffusional limitations, recycling of the reaction mixture could lead to enhanced conversion.

Chapter 5 describes the study on the use of L-serine decarboxylase (SDC) and L-phenylalanine ammonia-lyase (PAL) as a means to separate three neutral AAs: L-serine (Ser), L-phenylalanine (Phe) and L-methionine (Met). The two enzymes were prepared and the activity of PAL in yeast was enhanced by permeabilization of the cells. The decarboxylation of Ser to ethanolamine (ETA) by SDC (K_m = 10.8 mM) and the deamination of Phe to *trans*-cinnamic acid (CA) by PAL (K_m = 23.2 mM) are both specific and not inhibited by Met. However, the activity of SDC is reduced by the increase in concentration of CA. To stabilize the reaction pH, the two enzymes were coupled in one reaction system to form both ETA and CA. This also allows the reaction products and Met to be separated from each other in a subsequent ED process.

Chapter 6 discusses the impact of the results as described in the former chapters, the overall route of the specific conversion and the ED separation of AAs. Suggestions for future improvement of this route were given mainly with regards to three aspects: other conversions that could be applied as a means to aid the ED separation of AAs, the integration of AA conversion and the following ED separation, and the possible methods for the preliminary separation of neutral AAs. Recommendations for future research are also given These include the use of novel or alternative enzymatic AA conversions, the development of more robust enzyme mutants, the issue of unwanted salts (or ions), the use of CO_2 for reversible pH adjustment, and the development of new applications for AA-based chemicals.

In conclusion, this thesis shows that specific conversion of AAs with similar charge behaviors can be used as a means to aid their separation by ED, offering the possibility to couple chemical production with product separation. Such a biorefinery route contributes to the use of biomass as the feedstocks for chemical production, bringing benefits in reducing the requirement of fossil fuels, avoiding the use of extreme reaction conditions, and reducing the need of extra chemicals and capital cost.

Samenvatting

De huidige productie van bulkchemicalien is zeer afhankelijk van het gebruik van fossiele brandstoffen als bron voor koolstof en energie. Zoals uiteengezet in **Hoofdstuk 1** leidt dit tot problemen omtrent het uitputten van gelimiteerde reserves, onzekere toevoer en het broeikaseffect. Hierdoor wordt biomassa, dat beschouwd wordt als duurzaam en CO_2 neutraal, een veelbelovend alternatief voor fossiele brandstoffen in de productie van brandstof, chemicaliën en materialen. Bovendien is het concept van de bioraffinage geïntroduceerd om biomassa te scheiden en om te zetten naar marktwaardige producten. Voor de chemische industrie kan biomassa, dat de noodzakelijke functionaliteiten voor bulkchemicaliën bevat, als grondstof gebruikt worden dat fossiele brandstoffen vervangt. Hierdoor levert het de voordelen op in het besparen van processtappen, extra chemische reagentia, energieconsumptie en kapitaalkosten. Aminozuren (AZ) zijn bijvoorbeeld zeer interessante uitgangspunten voor stikstofhoudende (amine) chemicaliën. Zij kunnen gewonnen worden als mengsel uit de hydrolyse van potentieel goedkope eiwitten verkregen van de bijproducten van de biobrandstofproductie of agrarische en voedselafvalstromen. Echter, AZ verkregen van zulke bronnen bevinden zich in een mengsel. Hierdoor is een scheiding nodig om de individuele AZ te verkrijgen voor vervolgomzettingen en toepassingen.

Electrodialyse (ED) is een veelbelovende scheidingsmethode die toegepast kan worden in continue modus en op grote schaal. Gebaseerd op het verschil in iso-elektrisch punt (IEP), scheidt ED AZ in zure, basische en neutrale stromen. Echter, verdere scheiding moet worden verbeterd wanneer AZ met vergelijkbare IEP aanwezig zijn in dezelfde stroom. Om verdere scheiding te bevorderen binnen de drie stromen van ED kan een specifieke omzetting van AZ toegepast worden om producten met verschillende lading of oplossingsgedrag te krijgen, die een industriële toepassing hebben. Het doel van het onderzoek dat in dit proefschrift is gepresenteerd is het verkennen en verbeteren van die specifieke omzettingen die gebruikt kunnen worden om AZ te scheiden voor een bioraffinage benadering van de productie van bulkchemicaliën.

Hoofdstuk 2 beschrijft de studie naar het gebruik van L-lysine decarboxylase (LDC) om de basische AZ L-arginine (Arg) en L-lysine (Lys) te scheiden. LDC is specifiek in de omzettingen van Lys (K_m = 0.78 mM) naar 1,5-pentaandiamine (PDA). PDA kan gescheiden worden van de overgebleven Arg door middel van een ED proces bij het IEP van Arg (pH 10.8). PDA kan gebruikt worden als monomeer in de productie van nylon polymeren. De opslag en operationele stabiliteit van LDC kan verbeterd worden door middel van het invangen in calciumalginaat korrels. Arg en NaCl hebben weinig effect op de activiteit van LDC tot concentraties boven 30 mM zijn bereikt. De competitieve inhibitie door PDA (K_i = 9.68 mM) die geobserveerd werd, kan overkomen worden door een voldoende hoge concentratie Lys (bijv. 20 mM) te behouden. De aanwezigheid van Arg veroorzaakte een afname in operationele stabiliteit, maar dit kan voorkomen worden door de reactie bij een lagere temperatuur (30 ^{o}C) uit te voeren. Procesontwerp en kostenschatting laten zien dat geïmmobiliseerde LDC toegepast kan worden om de scheiding van AZ te bevorderen door middel van de vorming van een nuttig chemisch product.

Hoofdstuk 3 beschrijft de studie naar de vorming van L-pyroglutaminezuur (pGlu) als een middel om de zure AZ L-asparaginezuur (Asp) en L-glutaminezuur (Glu) te scheiden op basis van verschil in oplosbaarheid. De vorming van pGlu kan uitgevoerd worden in waterige of smeltcondities. In waterige condities, specificiteit was bereikt door de optimale reactiecondities (120 °C, 3 uur, Glu : H_2O = 200 mg : 1 mL) toe te passen. In smeltcondities was de specificiteit bereikt door Glu te smelten bij 185 ^{o}C en te reageren onder 150 ^{o}C voor 45 minuten, ondertussen bleef Asp in vaste toestand zonder omzetting. Na de specifieke omzetting van Glu werd het gevormde pGlu, dat zeer goed in water oplost, gescheiden van Asp door een het scheiden van vaste stof en vloeistof. pGlu werd vervolgens gehydrolyseerd tot Glu onder zowel zure als basische condities. Hierdoor werd de scheiding van Asp en Glu bereikt. De synthese van pGlu in waterige en smeltcondities resulteerde in het verkrijgen van Glu in hoge en vergelijkbare zuiverheid. Echter, in smeltcondities werd meer Asp en Glu teruggewonnen en de verkregen Asp had een hogere zuiverheid.

Hoofdstuk 4 beschrijft de studie naar het gebruik van L-glutamine α-decarboxylase (GAD) als middel om de zure AZ (Asp en Glu) te scheiden. De analyse van GAD werd uitgevoerd met

behulp van een pH-stat methode en deze methode werd verbeterd door een correctiefactor te introduceren om de werkelijke enzymactiviteit te verkrijgen. GAD is specifiek voor de omzetting van Glu (K_m = 1.6 mM) naar γ-aminobutaanzuur (GABZ). Het is aangetoond dat GABZ een nuttige grondstof is voor het industriële oplosmiddel *N*-methylpyrrolidon (NMP). De gevormde GABZ kan gescheiden worden van de overige AZ door een ED proces bij pH 3.5. Asp inhibiteert het enzym competitief en reduceert zijn operationele stabiliteit, maar dit wordt niet als probleem beschouwd door de gelimiteerde inhibitie (K_i = 72.2 mM >> K_m) en kan overkomen worden door een voldoende hoge concentratie Glu (bijv. 20 mM) te behouden in de reactie. GAD was geïmmobiliseerd door het in te vangen in calciumalginaat zonder verlies in activiteit, maar de operationele stabiliteit werd niet verbeterd. Om de enzymatische reactie met de daarop volgende ED scheiding van GABZ te integreren, werd de reactie met geïmmobiliseerd GAD ook uitgevoerd in continue modus met behulp van een kolomreactor. Hoewel de omzetting lager is dan verwacht, mogelijk door diffusielimitaties, zou het recyclen van het reactiemengsel kunnen leiden tot verhoogde omzetting.

Hoofdstuk 5 beschrijft de studie naar het gebruik van L-serine decarboxylase (SDC) en L-phenylalanine ammonia-lyase (PAL) als middel om drie neutrale AZ te scheiden: L-serine (Ser), L-phenylalanine (Phe) en L-methionine (Met). De twee enzymen waren verkregen en de activiteit van PAL in gist was verbeterd door permeabilisatie van de cellen. De decarboxylering van Ser naar ethanolamine (ETA) door SDC (K_m = 10.8 mM) en de deaminering van Phe tot *trans*-kaneelzuur (KZ) door PAL (K_m = 23.2 mM) zijn beide specifiek en niet geïnhibiteerd door Met. Echter, de activiteit van SDC wordt verminderd door de toenemende concentratie KZ. Om de reactie pH te stabiliseren werden de twee enzymen gekoppeld in een reactiesysteem om zowel ETA als KZ te vormen. Dit maakt het ook mogelijk om de reactieproducten en Met van elkaar te scheiden in een volgend ED proces.

Hoofdstuk 6 beschrijft de impact van de resultaten zoals beschreven in de voorgaande hoofdstukken, de gehele route van de specifieke omzetting en de ED scheiding van AZ. Suggesties voor toekomstige verbeteringen van deze route werden gegeven voornamelijk met betrekking tot drie aspecten: andere omzettingen die toegepast zouden kunnen worden als middel om de ED scheiding van AZ te bevorderen, de integratie van AZ omzetting en de vervolg

scheiding met ED en de mogelijke methodes voor de voorlopige scheiding van neutrale AZ. Aanbevelingen voor vervolgstudies worden ook gegeven. Deze bevatten het gebruik van nieuwe of alternatieve enzymatische AZ omzettingen, het ontwikkelen van robuustere enzymmutanten, de kwestie van ongewenste zouten (of ionen), het gebruik van CO_2 voor reversibele pH correctie en de ontwikkeling van nieuwe toepassingen voor chemicaliën gebaseerd op AZ.

Concluderend, dit proefschrift toont dat specifieke omzettingen van AZ met vergelijkbaar ladingsgedrag gebruikt kan worden als middel om hun scheiding met ED te bevorderen, dit maakt het mogelijk om de chemische productie en productscheiding te koppelen. Een dergelijke bioraffinage draagt bij aan het gebruik van biomassa als grondstof voor chemicaliënproductie, dit levert als voordelen de vermindering in benodigde fossiele brandstoffen, het vermijden van het gebruik van extreme reactiecondities en de vermindering van de noodzaak van extra chemicaliën en kapitaalkosten.

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– “let the courage lead our way!”

Curriculum vitae

Yinglai Teng was born in January 1983, in Hunan, China. In 2005, he obtained his BSc degree of Material Science from Polymer and Material Science Department at Sun-Yet Sen University, China, after which he began to study Polymer Science in Chemistry Department at Groningen University, Groningen, The Netherlands. His study in Groningen was supported by DELTA scholarship. In 2007, he received his MSc degree and moved to his PhD program in the Chair of Biobased Commodity Chemicals at Wageningen University, Wageningen, the Netherlands. His PhD research was performed under the supervision of Prof. Johan P.M. Sanders and Dr. Elinor L. Scott. This research is part of the STW project "A novel process route for the production of building blocks for chemicals from protein sources using electrodialysis". In 2014, he completed his PhD thesis entitled "Specific modification of amino acids as a means for their separation".

List of publications

As the first author

1 **Teng Y**, Scott EL, van Zeeland ANT and Sanders JPM. The use of L-lysine decarboxylase as a means to separate amino acids by electrodialysis. *Green Chem* **13**:624-630 (2011).

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As the coauthor

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2 Cai Z, Wang Y, **Teng Y**, Chong K, Wang J, Zhang J and Yang D, A novel process of biodiesel production from waste cooking oil via recycling glycerol esterification. *Submitted*.

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1 Wan F, **Teng Y**, Wang Y, Li A, and Zhang N, Synthesis of highly pure oligomerized polyglycerol catalyzed by strongly acidic cationic exchange resin. *Submitted*.

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6 Du Z, Wang Y, **Teng Y**, Zhang N and Li A-J, The determination of PC, LPC and GPC by proton nuclear magnetic resonance spectroscopy. Submitted.

* English abstracts are available.

Overview of completed training activities

Discipline specific courses

Intensive Program "Renewable Bioresources and biorefinery", *Karl-Franzens University Graz, Austria*, 2008

Advanced Downstream Processing Course, *Institute Biotechnology Studies Delft Leiden*, 2009

Advanced Course Biocatalysis, *Institute Biotechnology Studies Delft Leiden,* 2011

General courses

Effective behaviour in your professional surroundings, *Wageningen Graduate Schools*, 2010

Career Perspectives, *Wageningen Graduate Schools*, 2011

Competence Assessment, *Wageningen Graduate Schools*, 2011

Teaching methodology and skills for PhD candidates, *Docenten Ondersteuning Wageningen University*, 2011

Techniques for Writing and Presenting Scientific Papers, *Wageningen Graduate Schools*, 2011

Presentation skills, *Wageningen UR Language Services*, 2011

Academic writing, *Wageningen UR Language Services*, 2011

Scientific Writing, *Wageningen UR Language Services*, 2011

Project and Time Management, *Wageningen Graduate Schools*, 2011

Optionals

Discussion group, *Varolisation of Plant Product Chains, Wageningen UR*, 2008-2012

STW users group meeting, *Technologiestichting STW, Utrecht, the Netherlands*, 2009- 2011

Renewable Resources for the Bulk Chemical Industry, *Wageningen University (ORC 90306)*, 2009

Excursion to Abengoa Rotterdam, *Organic Chemistry Department, Wageningen University*, 2011

Conferences & symposia

Netherlands Process Technology Symposium, *the Netherlands*, 2008-2011

International Conference on Renewable Resources & Biorefineries, *Rotterdam, the Netherlands & Gent, Belgium*, 2008 & 2009

STW Annual Congress, *Technologiestichting STW, Utrecht, the Netherlands*, 2010 & 2011

12th International congress on Amino acids, Peptides and Proteins, *Beijing, China*, 2011

Other training & teaching activities

Academic writing I, *Wageningen UR Language Services*, 2010-2011

Blusinstructie, *Berki advies & opleidingen B.V.*, 2011

Laboratory of organic chemistry (teaching), *Organic Chemistry Department, Wageningen University (WUR/ORC13803)*, 2010

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