Aqueous two-phase extraction of proteins and enzymes using tetraalkylammonium-based ionic liquids

Dissertation
zur
Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität Rostock

vorgelegt von

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Rostock 2008

urn:nbn:de:gbv:28-diss2009-0038-8

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Tag der Verteidigung:	27. Januar 2009

Die vorliegende Arbeit wurde im Zeitraum von Juli 2005 bis Oktober 2008 am Institut für Chemie der Universität Rostock angefertigt.

Danke

Mein großer Dank gilt Herrn Prof. Dr. Udo Kragl, der es mir durch eine interessante Themenstellung nicht nur ermöglicht hat, in der Arbeitsgruppe „Technische Chemie“ zu promovieren, sondern mir durch viele Anregungen und Diskussionen auch durch die Höhen und Tiefen der Arbeit geholfen hat. Weiterhin möchte ich mich für die zahlreichen Möglichkeiten bedanken, an Konferenzen und Tagungen im In- und Ausland teilzunehmen, die oft mit interessanten neuen Ideen und Erfahrungen verbunden waren.

Weiterhin möchte ich Herrn Prof. Dr. Uwe Bornscheuer und Herrn Prof. Dr. Jürgen Hubbuch für die freundliche Übernahme des Koreferates dieser Arbeit danken.

Mein Dank geht auch an Dr. Thomas Daußmann für die Bereitstellung der Alkohol-dehydrogenase aus *Lactobacillus brevis* und der T ADH, sowie an die Firma Solvent Innovation GmbH für interessante Anregungen, viele Informationen und die Proben der ionischen Flüssigkeiten. Dr. Thomas Schmidt danke ich für die Leihgabe des Viskosimeters und die Einführung in dessen Bedienung.

Ein großer Dank geht insbesondere an Herrn Dr. Ruth, Katja Neubauer und Christina Bathke, die mir in analytischen Fragen große Hilfestellungen und gute Ratschläge gegeben haben. Sebastian Trapp, Christian Westendorf, Juliana Ratzka, Florian Stein und Paulina Salim danke ich für die Unterstützung und interessanten Ergebnisse, die sie im Rahmen von ihren Tätigkeiten als studentische Hilfskraft bzw. wissenschaftliche Mitarbeiter zu dieser Arbeit beigetragen haben.

Weiterhin möchte ich der Degussa Stiftung sowohl für die finanzielle Unterstützung als auch die Einladungen zu den Stipendiaten-Treffen danken, die nicht nur interessant und informativ, sondern stets auch persönlich bereichernd waren. Dem Forschungsschwerpunkt SPP1191-Ionic Liquids danke ich ebenfalls für die finanzielle Unterstützung.

Danke, danke, danke! an die ganze Arbeitsgruppe „Technische Chemie”, die mich so herzlich in ihren Kreis aufgenommen und geduldig meine rheinländischen Eigenarten ertragen hat. Danke für die schöne, lustige und manchmal chaotische Arbeitsatmosphäre, die gemütlichen Tee- und Kuchenpausen, die Kicker-Spiele und die vielen Biologen-Witze! Besonders möchte ich mich noch einmal bei Julia, Katja, Daniela, Sandra, Vera, Annett und Jan bedanken, die mir nicht nur bei wissenschaftlichen, sondern auch persönlichen Fragen und Problemen immer zur Seite standen! Ein ganz großes „Danke“ auch an Frau Freitag, Frau Plagemann und Herrn Spallek für viele nette und aufmunternde Gespräche, Hilfestellung in allen Lebenslagen und die Leihgabe einer Latzhose.

Mein besonderer Dank geht an meine Großeltern, meine Eltern Eva und Hinnerk Dreyer, meine Schwestern Barbara und Pisa, und an Staffan. Danke für die Unterstützung und Hilfe in jeder Situation und Lebenslage. Danke, dass Ihr trotz der großen Entfernung immer da ward!

Für Eva, Hinnerk, Barbara und Pisa

De Möh von hück es de joot ahl Zick vun üvvermorje.
Our troubles of today are those ‘good old days’ of tomorrow.

Kölsche Weisheit

Abstract

To meet the challenges of a growing market of industrial biotechnology, the development of new and innovative downstream methods for proteins and enzymes is of great importance. Within the scope of this thesis, the application of ionic liquids for the generation of aqueous two-phase systems and their potential use as an alternative technique for the purification of catalytically active enzymes was evaluated.

The first objective comprised the characterisation of ionic liquid-based aqueous two-phase systems (IL-based ATPS). Several factors influencing the phase formation and composition were investigated, such as the type of phase-forming compounds, temperature and pH.

By studying the partitioning of different model proteins, correlations between the protein features and system properties could be found, thereby providing a deeper understanding of the mechanisms influencing the partition process. Based on these findings, electrostatic interaction has been identified as the main driving force of protein partitioning in IL-based ATPS. Moreover, a statistical model based on the model proteins' physico-chemical properties has been established and provides a good start for predicting the protein partitioning in IL-based ATPS.

With regard to the purification of enzymes, the main obstacle in developing a suitable extraction method represents the maintenance of the stability and the catalytic activity of the biocatalyst. Therefore, the application of IL-based ATPS for biocompatible extraction of enzymes from complex mixtures such as cell lysates was investigated. It was demonstrated that the ionic liquid Ammoeng^{TM} 110 in combination with an inorganic salt (K_2HPO_4/KH_2PO_4) can be used to establish an IL-based ATPS which provides the necessary conditions of pH and biocompatibility in order to maintain enzyme activity. Using efficient experimental design the partitioning of an ADH from *Lactobacillus brevis* (LB ADH) and an ADH from a thermophilic organism (T ADH) from a cell extract of *E. coli* was optimised with respect to the enrichment of the enzyme in the IL-containing phase. The specific enzyme activity in the upper phase could be increased to 206.7 ± 5.3 % for LB ADH and 403.4 ± 5.4 % for T ADH. The presence of ionic liquid within IL-based ATPS was also found to be advantageous thanks to its stability enhancing effect on both enzymes and its solubility enhancing effect on hydrophobic substrates. Thus, it could be shown that the IL-based ATPS offers the opportunity to combine the purification process of active biocatalysts with the performance of enzyme-catalysed reactions especially in case of the conversion of hydrophobic substrates. Advantages such as improved conversion and yield could be observed.

Parts of this thesis as well as further results not included in this work have already been presented, published and filed as a patent.

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Dreyer S. (16/11/2007). Ionic liquids for the purification and stabilisation of proteins. *Workshop Interaction of Ionic Liquids and Proteins*, Rostock

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Dreyer S. (03/09/2008). Ionic liquids for aqueous two phase extraction of enzymes: application and understanding. Presented by Julia Duwensee, *Biocat*, Hamburg

Poster presentations

Dreyer S, Trapp S, Kragl U. (30/05 01/06/2007). Stabilisation of two different alcohol dehydrogenases by a readily available ionic liquid. *European Bioperspectives*, Köln

Dreyer S, Kragl U. (05/08 10/08/2007). Ionic liquid-based aqueous two phase extraction of enzymes. *2nd International Congress on Ionic Liquids (COIL 2)*, Yokohama

Dreyer S, Kragl U. (09/10 - 11/10/2007). Ionische Flüssigkeiten für die Extraktion und Stabilisierung von Proteinen. *Biotechnica*, Hannover

Dreyer S, Kragl U. (15/11 16/11/2007). Ionic liquid-based aqueous two phase extraction of enzymes. *Workshop Interaction of Ionic Liquids and Proteins*, Rostock

Dreyer S, Kragl U. (27/03 - 29/03/2008). Protein distribution in ionic liquid-based aqueous two phase systems. *International 10th Frühjahrssymposium of GDCh JCF*, Rostock

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Abbreviations

A110	ionic liquid Ammoeng^{TM} 110
ADH	alcohol dehydrogenase
ASA	accessible surface area [Å^2]
ATPS	aqueous two-phase system
C_4mim	1-butyl-3-methylimidazolium
C_6mim	1-hexyl-3-methylimidazolium
C_8mim	1-octyl-3-methylimidazolium
EC	enzyme classification
E. coli	*Escherichia coli*
EINECS	European Inventory of Existing Commercial Chemical Substances
EO	ethylene oxide
HL ADH	alcohol dehydrogenase from horse liver
IEP	isoelectric point
IL	ionic liquid
IL-based ATPS	ionic liquid-based aqueous two-phase system
LB ADH	alcohol dehydrogenase from *Lactobacillus brevis*
MW	molecular weight [g mol ^{1}, kDa], (1 Da 1.6605388 · 10 ^{27} kg)
n. d.	not determined
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
PDB	protein data bank
PEG	polyethylene glycol
PO	propylene oxide
SDS PAGE	sodium dodecyl sulphate polyacrylamid gel electrophoresis
STY	space-time-yield [g L ^{1} d ^{1}]
T ADH	alcohol dehydrogenase from a thermophilic organism
TB ADH	alcohol dehydrogenase from *Thermoanaerobium brockii*
TE ADH	alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*
Tf_2N	bis(trifluoromethylsulfonyl)imide
TSCA	Toxic Substances Control Act Inventory List
Y ADH	alcohol dehydrogenase from yeast

Symbols

c	concentration [g L^{-1}, mol L^{-1}]
d	diameter [cm]
D	droplet diameter [mm]
F	sphericity factor [-]
G	molar Gibbs free energy [kJ mol^{-1}]
G_{hyd}	Gibbs free energy of hydration [kJ mol^{-1}]
g	gravitational acceleration [9.810 m s^{-2}]
H	molar enthalpy [kJ mol^{-1}]
h	height [cm]
K	partition coefficient [-]
R	universal gas constant [8.314472 J mol^{-1} K^{-1}]
r_i	relative surface area [-]
S	entropy [J K^{-1}]
S	surface area [$Å^2$]
T	temperature [°C, K]
V	volume [mL, cm^3, m^3]
V_s	droplet rise/fall velocity [m s^{-1}]
x	concentration of phase-forming compounds [wt %]
Y	specific enzyme activity [U μg^{-1} protein]
ε	extinction coefficient [mL μmol^{-1} cm^{-1}]
η	dynamic viscosity [kg s^{-1} m^{-1}]
ν	kinematic viscosity [m^2 s^{-1}]
ρ	density [kg m^{-3}, g cm^{-3}]
σ	interfacial tension [N m^{-1}]
x	mole fraction [-]

1 Introduction

Industrial biotechnology products are defined as chemical products relying on biobased feedstocks, fermentation, enzymatic conversion or some combination of the above. In 2000, the biobased products market was valued to € 53 billion, representing 5 % of all chemicals sold worldwide. In 2005, the market had grown to € 77 billion (7 % of total chemicals produced) and expectations for 2010 assume a market growth to € 125 billion, or 10 % of the chemicals market (Figure 1-1) (Caesar 2008). The four specific drivers of growth in industrial biotechnology have recently been reviewed by McKinsey & Co and were identified as: (1) high crude oil prices, (2) the demand of end-consumers for 'green' biotech products which in turn creates a 'pull' for manufacturers to adopt biobased processes and products instead of crude oil-based materials, (3) concerns about greenhouse gas emissions and the cost of CO_2 and (4) scientific progress (e. g. advancements in synthetic biology, pathway engineering, directed evolution) which allow achievements that were not possible in the past (Caesar 2008).

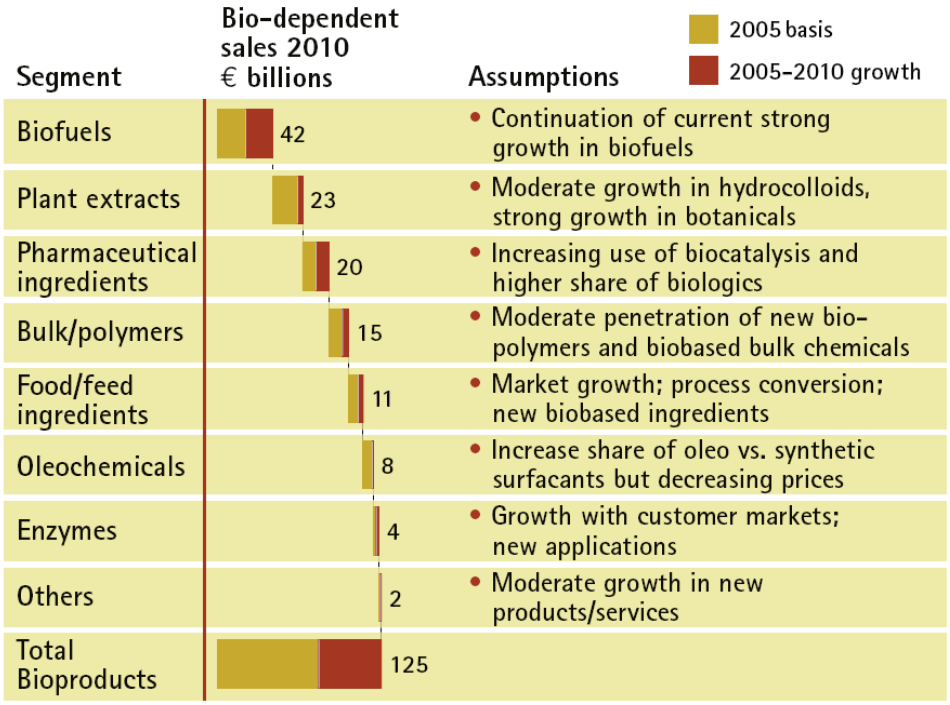


Figure 1-1: Segments in industrial biotechnology and their sales as published by McKinsey & Co (Caesar 2008). Source: SRI, FO Licht, Frost and Sullivan, Press clippings, McKinsey.

Among the industrial biotechnology market the biopharmaceutical industry is one of the fastest growing sectors in global economy (BioPortfolio 2005; Pavlou and Reichert 2004). Over the last 25 years the production of biologically active proteins has dramatically increased due to the advancements in molecular engineering and protein design. Nowadays, the large-scale production of virtually any protein by fermentation routes at increased titers is feasible. The number of recombinant proteins for pharmaceutical and diagnostic use as well as for food and biotechnology applications is growing rapidly (Headon and Walsh 1994;

Walsh 2000). In parallel with this development the bottleneck in biopharmaceutical process development is shifting towards the purification of protein products from biological feed materials (Smith 2005; Thiel 2004). The demand for protein-separation technology is increasing as the isolation and purification of proteins from the contaminated complex mixture accounts for 30-80 % of the production costs (Table 1-1) (Ghosh 2003; Pietruszka et al. 2000).

Table 1-1: Protein products as well as estimated costs for their bioseparation as proposed by Gosh with some modifications (Ghosh 2003).

Protein product	Examples	Approximate relative price	Bioseparation cost as % of total cost of production
Food / Additives	Egg albumin, Casein, Alpha lactalbumin, Lysozyme	1	10-30
Nutraceuticals	Soy bean proteins, Lupin proteins	2-10	30-50
Industrial enzymes	Hemicellulase, Glucose isomerase, Penicillin G acylase, Alkaline proteases, Detergent enzymes, Digestive enzymes	5-10	30-50
Diagnostic enzymes	Peroxidase, Glucose oxidase	50-100	50-70
Therapeutic enzymes and proteins	Urokinase, Streptokinase, Monoclonal antibodies, Serum immunoglobulins, Factor VIII	50-500	60-80

Manufacturing of bioproducts typically involves the processing of large, dilute, multiphase fermentation broths which require a significant number of downstream separation trains to obtain a marketable concentration. Traditional methods to purify biomolecules involve several steps, such as dialysis, ionic and affinity chromatography and are therefore time consuming, costly and not applicable for large-scale operations (Scopes 1994). Additionally, the composition and volume of the material handled during a given protein purification process continuously change and therefore require changing purification steps. In order to combine several features of the early processing steps, liquid-liquid extractions have evolved as an interesting purification alternative. Advantages lie in the simplicity, the low costs and the ease of scale up of these systems. Nevertheless, conventional liquid-liquid extraction processes employed in industry consist of water-organic solvent two-phase systems which are generally not suitable for the purification of biomolecules due to problems such as protein denaturation and loss of enzymatic activity. Therefore, the development of new separation technologies which will decrease enzyme purification costs and improve yield is an indispensable prerequisite to expand the market for industrial enzymes and proteins.

Partitioning in aqueous two-phase systems (ATPS) represents an emerging technique which has found applications in purification of various proteins and enzymes. An aqueous two-phase

system is formed when two structurally different polymers are solubilised in water above certain concentrations. Due to the high water content within the phases (70-90 %) and the low surface tension between the phases, ATPS offer a mild and biocompatible method for the purification of proteins and enzymes (Albertsson 1986; Walter and Johansson 1994). The technique is inexpensive and meets requirements of purity and selectivity. The simplicity of the process and the low cost of phase-forming materials make it feasible for large-scale protein purification using appropriate scale-up techniques (Hustedt et al. 1985; Hustedt et al. 1988). However, several drawbacks have prevented the extensive use of ATPS in chemical and biotechnological industry. The limited application has been attributed to the necessary time involved in the learning process of the technique and the poor understanding of the mechanisms governing partition of solutes between the phases (Benavides and Rito-Palomares 2008). Moreover, the aqueous nature of ATPS often prevents a combination of the purification step with an enzyme-catalysed reaction due to reasons such as low substrate solubility, restricted enzyme stability and activity or the occurrence of side reactions. Therefore, there is a rising demand for new and innovative simple separation techniques.

As a result of their unique chemical and physical properties, ionic liquids (ILs) have aroused increasing interest for their promising role as alternative media in biocatalysis, synthesis, separation and electrochemistry (Earle and Seddon 2000; Huddleston et al. 1998b; Klembt et al. 2007; Olivier-Bourbigou and Magna 2002; Plechkova and Seddon 2008; Sheldon 2001; Visser et al. 2001; Wasserscheid and Keim 2000; Welton 1999; Weyershausen and Lehmann 2005). ILs are defined as molten salts which are liquid at temperatures below 100°C and have been found to exhibit several advantages compared to aqueous or organic solvents such as their strong solubility power, low volatility and flammability and their stabilising and activating effects on proteins and enzymes (Wasserscheid 2007). The application of ionic liquids for the formation of aqueous two-phase systems (IL-based ATPS) has first been reported by Rogers and coworkers in 2003. Compared with conventional ATPS, the method provides many advantages such as low viscosity, quick phase separation and high extraction efficiency. Nevertheless, investigations on IL-based ATPS have been mainly focused on analytical applications such as the extraction of testosterone and epitestosterone in human urine and the partition of opium alkaloids in [C_4mim][Cl] / salt ATPS (He et al. 2005; Li et al. 2005). However, due to the reason that IL-based ATPS share the advantages of ionic liquids and conventional ATPS, they hold a great potential for the establishment of a new and innovative downstream processing method for catalytically active proteins. The great potential of IL-based ATPS arises from the opportunity to combine the purification process of active biocatalysts with the performance of enzyme-catalysed reactions by simply employing the IL-enriched, enzyme-containing upper phase within biotransformation processes. Therefore, this work focuses on the investigation of ionic liquids for the formation of IL-based ATPS, their applicability for the purification of catalytically active enzymes as well as the understanding and modelling of the partitioning behaviour of biomolecules between the phases.

2 Scope of work

In order evaluate the potential of ionic liquids for the formation IL-based ATPS with respect to their application in downstream processing of enzymes, a special class of ionic liquids was investigated. These ionic liquids are called 'Ammoeng™' ionic liquids and represent acyclic ammonium salts which contain cations with oligoethylene glycol units of different chain length. Due to the fact that Ammoeng™-ILs are already listed (TSCA and EINECS) and available in technical quantities for reasonable prices, they represent an interesting alternative to 'conventional' ionic liquids and are therefore of particular interest for industrial applications (Weyershausen and Lehmann 2005).

To outline the scope of this work, Figure 2-1 roughly depictures the milestones in the development of an optimised IL-based ATPS process. Initial investigations have to focus on assessing the applicability of different ILs and inorganic salts for the formation of IL-based ATPS. Factors influencing the composition and formation of IL-based ATPS need to be identified and characterised. At this point, the thesis can be devided into two major parts that are closely related with each other: (1) Understanding the driving forces of protein partitioning between the phases of IL-based ATPS and (2) the use of IL-based ATPS for the purification of catalytically active enzymes.

(1) The successful application of IL-based ATPS in downstream processing is clearly influenced by the ability to understand, or even predict, the partitioning of a target molecule between the phases. Therefore, the first objective aims to develop correlations and models which allow understanding how physico-chemical properties of proteins and their interaction with the properties of the surrounding IL and salt phases affect partitioning in IL-based ATPS. By investigating the partitioning of different model proteins which are available in high purity, well characterised and structurally determined, correlations between the protein and system properties can be found and thereby reveal the main driving forces of the partition process. In consequence, based on a protein's fundamental properties, the optimisation of a suitable IL-based ATPS would be within reach and significantly facilitate the selection process and expenditure of time when applying IL-based ATPS for biomolecule purification.

(2) The second objective comprises the application of IL-based ATPS for the biocompatible extraction of enzymes from complex mixtures such as cell lysates. With regard to the special requirements associated with the purification of catalytically active biomolecules, a suitable type of IL-based ATPS has to be chosen from the pool of available IL-based ATPS and optimised in its system characteristics. Ideally, the target enzyme should get enriched in the IL-rich phase which then in turn can be applied for the performance of an enzyme-catalysed reaction. Thereby, the special properties of the IL (e. g. solubility and stability enhancing effect) as well as of the ATPS (biocompatibility, gentle extraction method) can be used to full capacity. The purification of two different alcohol dehydrogenases (ADH) from crude cell extracts of *E. coli* was chosen to exemplify the purification of biomolecules by IL-based ATPS. In this regard, it is also important to identify a suitable method for optimising the composition of an IL-based ATPS when the physico-chemical properties of a target enzyme are mostly unknown.

Finally, a general consideration of the competitive capacity of IL-based ATPS with already industrially applied downstream methods will close the thesis.

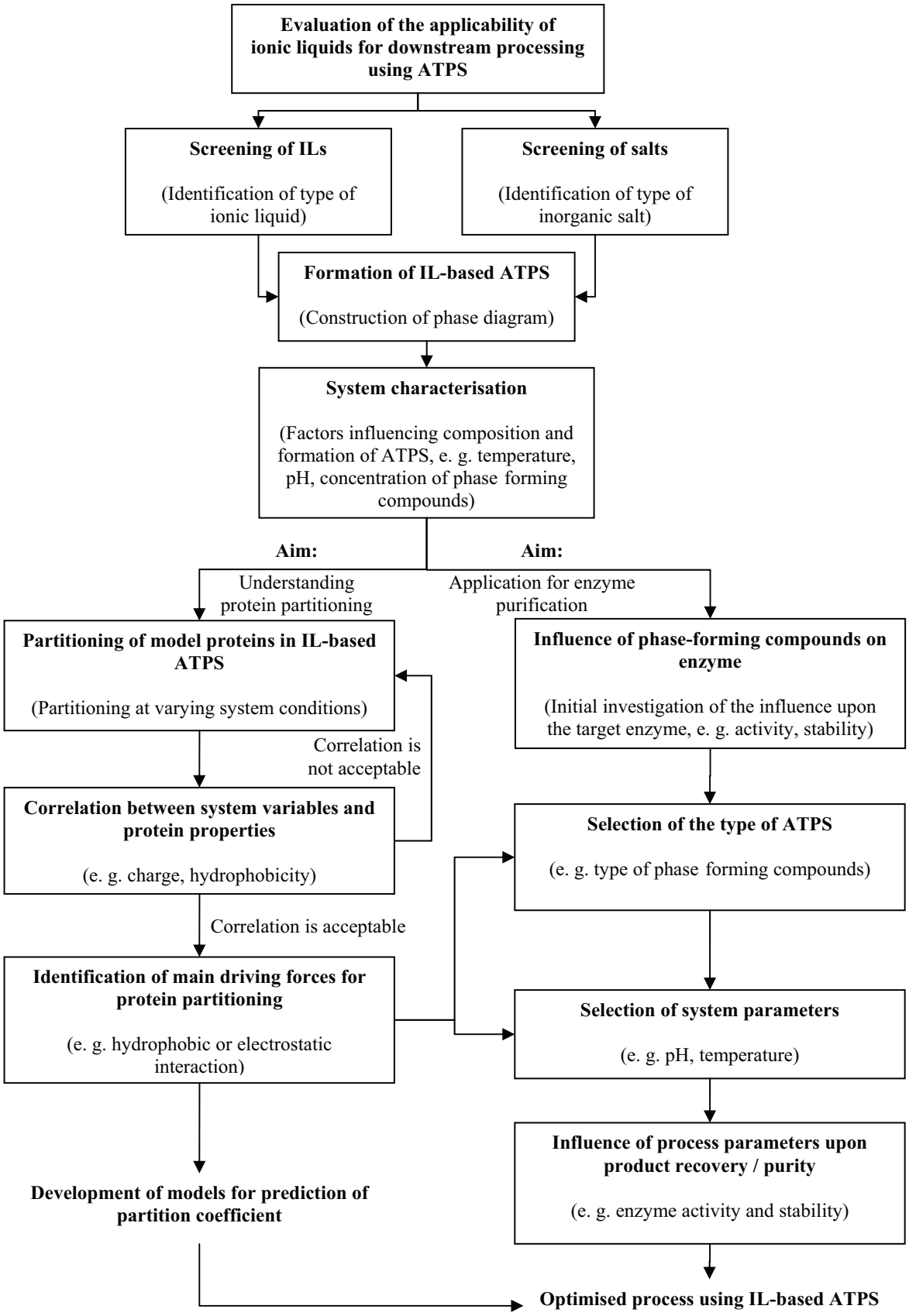


Figure 2-1: Schematic overview on the main topics of this thesis.

3 Literature review

3.1 Purification of proteins and enzymes

Biotechnological processes for chemical, pharmaceutical and food industries are mostly conducted in aqueous media. Hence, the product to be prepared is present in dilute form in an aqueous solution and has to be concentrated, isolated and purified from other constituents of the batch, such as remaining substrates, by-products, proteins and cells. To reach the required quality standards of the products an efficient and selective downstream processing is essential. The different stages of the purification process as well as the challenges to be met will be pointed out and discussed in the following.

3.1.1 Protein purification processes

In general, protein purification processes can be divided into four different stages based on the main objectives at each process step in order to facilitate the process design. For illustrative purposes, Figure 3-1 shows a block scheme of the downstream process as it has been proposed by Petrides et al. and modified by Nfor et al. (Nfor et al. 2008; Petrides et al. 1989). The four different sections represent the different process stages: (1) recovery, (2) concentration, (3) purification and (4) product formulation. Each stage again consists of a number of well-defined process steps depending on the localisation (intracellular or extracellular) and the required purity and final form of the product. The process steps exploit physical and molecular property differences between the components to be separated. Examples for typical industrially applied unit operations within the different process steps are depicted in Figure 3-1 (e. g. cell harvesting, cell disruption, cell debris removal, biomass removal).

(1) The first step of recovery aims to separate the protein in native or denatured form from non-protein components of the fermentation broth. Depending on the localisation of the target protein (intracellular or extracellular) different process steps have to be performed. Both physical methods (e. g. filtration, centrifugation) and direct extraction of the product from cells or cell debris can be applied to accomplish this first clarification step (Figure 3-1). Using the latter, partial purification of the product may be achieved depending on the selectivity of the technique employed. If, however, the target protein is present as insoluble inclusion bodies, an additional process step is needed in order to solubilise and refold the protein (Cabrita and Bottomley 2004; Choe et al. 2006; Clark 1998; Freydell et al. 2007).

(2) After recovery, a dilute or concentrated solution of the target protein in a complex mixture of contaminant proteins which is virtually free of particulate material is obtained. In the case of sufficient concentration, the solution can directly be further processed in the purification section. A dilute protein solution, however, needs to be further concentrated e. g. by ultrafiltration or extraction.

(3) In general, the purification section consists of three main steps: The ‘capture’ step involves isolation, concentration and volume reduction, the ‘intermediate purification’ step includes the removal of bulk impurities or main protein contaminants and the ‘polishing’ step is applied to remove trace impurities, closely related contaminants and protein aggregates.

Commonly employed purification steps involve chromatographic operations due to their high resolving power. Nevertheless, alternative unit operations such as membrane filtration or aqueous two-phase extraction have been applied in order to offer better throughputs and lower material costs (Przybycien et al. 2004; Thommes and Etzel 2007).

(4) The main objective of the product formulation stage is to get the protein product in the form desired by the final consumer or costumer. If necessary, additives are also added at this stage in order to meet the requirements of the final application or to improve the stability and shelf life of the protein product (Sellers and Maa 2005).

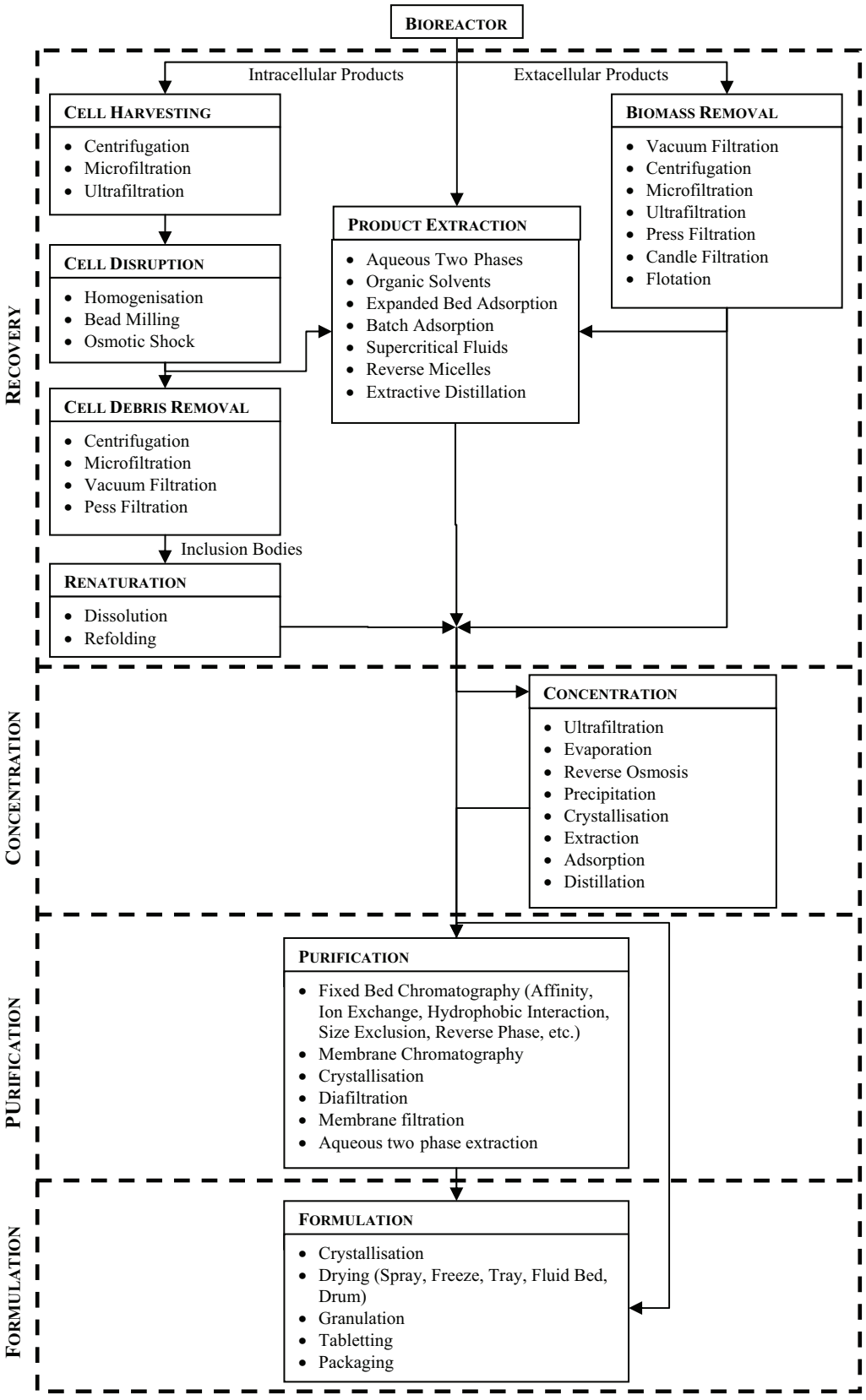


Figure 3-1: Block scheme for the illustration of protein downstream processing (Nfor et al. 2008; Petrides et al. 1989).

3.1.2 Challenges in protein purification

Ideally, every protein purification process should be designed in order to fulfil the requirements of efficient and economical purification in combination with sufficient purity and quantity. Depending on the end-use and application of the protein product, the requirements and thus the extent of purification need to be individually defined and investigated. In general, commercially available proteins can be classified into the following broad categories: food and nutritional products, pharmaceutical and therapeutic products, industrial enzymes and diagnostic enzymes (for examples see Table 1-1). The purity requirements vary significantly between the four groups with therapeutic proteins destined for human application generally having the most stringent purity requirements (exceeding 99 %) compared to the other categories (Headon and Walsh 1994). The main challenges to be addressed in protein purification directly result from the process steps pointed out in chapter 3.1.1 and include the following:

- *Low feed concentrations*: Biological feed streams are generally very dilute with respect to the target protein product.
- *Complex feed mixtures*: The products are often present in complex mixtures of a large number of impurities, some of which may have properties very similar to the product itself and thereby complicate the separation process.
- *Presence of critical contaminants*: It is important to differentiate between impurities which must be removed, so-called critical contaminants, and those which can be tolerated. With regard to enzymes, catalysis other than the type desired as well as catalysis poisoning has to be prevented by removing toxic or inhibitory substances and/or undesired enzymes.
- *Poor feed characterisation*: The crude mixture containing the product is often poorly characterised with respect to its physicochemical- (e. g. molecular weight, charge, hydrophobicity), thermodynamic- (e. g. solubility) and flow-properties (e. g. viscosity, diffusivities).
- *Inclusion bodies renaturation*: The protein product may be present in soluble form or as insoluble inclusion bodies, which need to be renaturated.
- *Product stability*: Most protein products are thermolabile and sensitive to extreme pH values and to chemical substances such as surfactants and organic solvents.
- *Strict product requirements*: The products often have strict requirements such as purity, biological activity and quantity.
- *Process conditions optimisation problem*: There may be several operating conditions for a given unit operation or process from which the best one has to be selected, giving rise to an optimisation problem.

3.2 Aqueous two-phase extraction

Among the numerous protein downstream processes described above, liquid-liquid extraction has been established as an interesting alternative purification method since several features of the early processing steps (recovery, concentration and purification) can be combined into a single operation (Mazzola et al. 2008). Generally, liquid-liquid extraction is defined as the transfer of certain components from one phase to another when immiscible or partially soluble liquid phases are brought into contact with each other. Due to its simplicity, low costs and ease of scale-up this process has widely been employed in chemical industry. Compared to other separation techniques, extraction is considered to have special advantages when handling labile substances or when distillation is impossible for economic reasons or product properties. Nevertheless, liquid-liquid extraction systems applied in industry usually involve the use of organic solvents which are not suitable for protein recovery as most proteins are either insoluble in organic solvents or are denatured irreversibly (Kula et al. 1982). Therefore, the production of recombinant proteins and enzymes requires the development of an adequate separation technique at reasonable cost.

Liquid-liquid binary systems can also be formed using two polymers or polymer/salt solutions. These so-called aqueous two-phase systems (ATPS) were first discovered in 1896 by the Dutch microbiologist M. Beijernick who noticed the separation of two phases when solubilising gelatine and agar or starch in water (Beijerinck 1896). However, the first report of ATPS remained unnoticed until 1956 when a Swedish biochemist, P. A. Albertsson, rediscovered the phenomenon and developed the phase separation technique (Albertsson 1956). Since then, aqueous two-phase systems (ATPS) have found widespread application. They have proven to be highly suitable for the gentle separation of cell membranes and organelles from crude cell lysates as well as for the selective purification of proteins and enzymes from protein mixtures or cell extracts (Agasoster 1998; Albertsson 1956; Albertsson 1986; Gunduz 2000; Rito-Palomares 2004; Roobol-Boza et al. 2004; Walter and Johansson 1994).

In general, ATPS are composed of either two incompatible polymers (e. g. polyethylene glycol (PEG) and dextran) or one polymer and a salt (e. g. PEG and a phosphate salt) in aqueous solution. If these phase-forming compounds are solubilised above a critical concentration in aqueous solution, the separation of two phases occurs. Each phase contains predominantly water (70-90 %) and is enriched with regard to one of the phase-forming components. Both polymer/polymer and polymer/salt ATPS provide advantages over conventional extraction methods using organic solvents. Due to the aqueous nature of the phases and the low interfacial tension (between 0.0001 and 0.1 dyne cm^{-1}), ATPS allow one phase to disperse into the other and thus create a high interfacial contact area for efficient mass transfer. Moreover, the polymers have been reported to exhibit stabilising effects on the biological activity and structure of proteins and enzymes (Albertsson 1986; Kaul 2000). The mild process conditions as well as the fast phase separation allow the purification of biomolecules by providing a biocompatible environment and reducing protein denaturation and enzyme inactivation.

3.2.1 Phase diagram

Phase diagram data are commonly used in order to delineate the potential working area for a particular two-phase system. The phase diagram provides information about (1) the concentration of the phase-forming components necessary to form a system with two phases which are in equilibrium, (2) the subsequent concentration of phase components in the top and bottom phase, and (3) the ratio of phase volumes. This information can be drawn from the binodal curve and the tie-lines which are characteristic for a phase diagram of an investigated ATPS (Figure 3-2). The top phase 'polymer 1' is plotted on the abscissa and the bottom phase 'salt or polymer 2' is plotted on the ordinate. Usually, the axes are labelled with polymer and salt concentrations, in the units of weight of the substance per 100 weight units of the mixture (percent by weight; wt %). The binodal curve separates the one-phase region, located below the binodal, from the two-phase region, above the curve. Component concentrations from the region above the binodal curve will form two immiscible aqueous phases, while those from below the binodal will provide only a one-phase system. Information about the final concentration of phase components in the top and bottom phases of a generated ATPS can be drawn from the tie-line that connects two nodes (T and B in Figure 3-2) on the binodal. The ratio of the segments PB (top phase) and PT (bottom phase) can be approximated graphically by the volume ratio of the two phases. The points P1, P2 and P3 represent the total compositions of three systems lying on the same tie-line with different volume ratios. ATPS prepared by component concentrations along the tie-line will provide systems with the same final concentration of phase components in the top and bottom phases, but a differing total composition and volume ratio between the phases. Just above the so-called critical point (C_P) the composition and volume of both phases are almost equal.

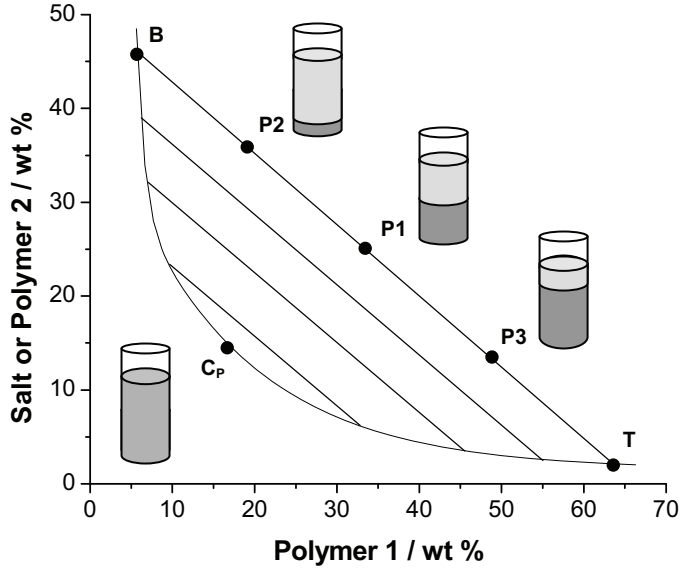


Figure 3-2: Schematic illustration of a phase diagramm for an aqueous two-phase system.

3.2.2 Process of phase formation

The state of knowledge on the theory of phase formation in aqueous two-phase systems is still characterised by a huge variety of ideas, models and methods (Cabezas 1996). This fact is

reflecting the relatively poor understanding of the process itself. Still, for polymer/polymer ATPS, phase formation has been generally attributed to the hydrated surfaces of each species which are sufficiently incompatible to generate phase separation (Albertsson 1986; Rito-Palomares 2004). From a thermodynamical point of view, phase separation can be explained by consideration of the Gibbs free energy. A closed aqueous two-phase system at constant temperature and pressure reaches equilibrium when the total Gibbs free energy is at minimum. The change of Gibb's free energy is defined by ΔH_m (enthalpy change), ΔS_m (entropy change) and T (absolute temperature) according to Equation 3-1.

$$\Delta G_m \quad \Delta H_m - T\Delta S_m$$

Equation 3-1: Calculation of Gibbs free energy.

The value of ΔG_m must be negative for mixing of components to occur and consists of an entropy and an enthalpy term. The entropy term, ΔS_m, is a measure of the degree of disorder in the system as a result of the mixing process. Summation of individual net enthalpy changes associated with the interaction of unlike components defines the enthalpy of mixing, ΔH_m. Phase separation occurs, when the interaction energy between unlike components becomes slightly positive (ΔH_m increases). For ATPS, the large size of the polymer reduces the entropy of mixing and results in the decrease of the term $T\Delta S_m$. Hence, the enthalpy of mixing dominates the Gibbs free energy, resulting in a positive change of ΔG_m. As a consequence, the system becomes unstable and the formation of two phases takes place due to a reduction of interactions between unlike molecules which is needed to reach equilibrium.

Phase separation in polymer/salt systems remains largely unclear. It was initially proposed to be associated with differing interactions with the ether dipoles of the polymer chain but this theory has not been widely adopted (Huddleston et al. 1991). However, the relative effectiveness of various salts in promoting phase separation is seen to follow the Hofmeister series (see also chapter 3.2.3 and 3.2.4).

3.2.3 System variables influencing partitioning

Several factors are known to influence the properties of aqueous two-phase systems and partitioning of biomolecules therein. In this regard, system variables affecting the composition of ATPS, and therefore partitioning are discussed in this section. These have to be distinguished from protein-related properties which also exhibit a strong influence on the partition behaviour (chapter 3.2.4).

Partitioning between the phases of ATPS is dependent on the chemical difference between the phases and thus the polymer concentrations. In general, with increasing polymer concentration, the partitioning of a substance towards the polymer-enriched phase will be enhanced due to an increased chemical difference between the phases (Albertsson 1986; Johansson et al. 1998; Walter and Johansson 1994). Additionally, the molecular weight of the polymer used influences ATPS formation; the greater the molecular weight, the lower the concentration required for phase separation. This effect has been attributed to the interaction of the PEG chain with water molecules. For low molecular weights only tightly bound water molecules are associated with the PEG chain. Increasing the molecular weight, however, results in the chain beginning to fold on itself (adopting a secondary structure), as it shares

loosely bound water between adjacent segments (Harris 1992). As a consequence the interaction reduces the contact of the PEG chain with water molecules and phase formation is facilitated (Zaslavsky 1995).

The most commonly used polymers for the formation of ATPS are polyethylene glycol (PEG) and dextran. Since both of these polymers are non-toxic and have been intensively tested for food and pharmaceutical use, they are also classified as safe for the recovery of therapeutic proteins. A wide variety of other polymers have been investigated for the formation of ATPS, including e. g. random copolymers of ethylene oxide (EO) and propylene oxide (PO) or ficoll (Alred et al. 1994; Alred et al. 1993; Berggren et al. 1995; Harris et al. 1991; Johansson et al. 1996).

Studies on PEG/salt ATPS have revealed that the location of the binodal and tie-lines for a particular pair of components depends on the system's temperature. Since PEG possesses a low cloud point of approximately 100°C in water, a raise of temperature above 100°C results in insolubility of PEG and the formation of two phases. Moreover, it has been reported that the cloud point temperature can be changed by the addition of salt to the PEG solution. With increasing salt concentration, the cloud point temperature is decreased, resulting in the fact that lower concentrations of PEG and salt are needed to obtain phase separation (Albertsson and Tjerneld 1994; Harris 1992). The relative effectiveness of salts in promoting phase separation has been reported to follow the Hofmeister series, which is a classification of ions based on their salting-out ability (see also chapter 3.2.4).

3.2.4 Protein properties affecting partitioning

The basis of aqueous two-phase extraction is the selective distribution of substances between the two phases. The partition coefficient (K) of a protein is defined as the concentration of the biomolecules in the top phase divided by the concentration in the bottom phase (Equation 3-2).

$$K \quad C_{Upper\ phase}/C_{Lower\ phase}$$

Equation 3-2: Calculation of the partition coefficient K.

In general, protein partitioning is dependent on the properties of the protein as well as on the two aqueous phases. Factors contributing to the partition behaviour of biomolecules include protein size, charge and hydrophobicity, polymer molecular weight, concentration of phase-forming compounds, pH and temperature. Hence, the partition coefficient can be described by a set of contributing terms (Equation 3-3).

$$K \quad K^{0} + K_{el} + K_{hphob} + K_{biosp} + K_{size} + \ldots$$

Equation 3-3: Terms related to protein properties contributing to the partition coefficient K.

The different terms correspond to electrochemical (K_{el}), hydrophobic (K_{hphob}) and biospecific (K_{biosp}) interactions as well as the biomolecule size (K_{bioesp}). K^{0} includes factors such as molecular weight and concentration or temperature.

Protein surface properties

Generally, protein partitioning in ATPS is a process wherein the exposed groups on a protein surface come into contact with the phase components and therefore represents a surface-dependent phenomenon (Albertsson 1986; Andrews and Asenjo 1989; Lamarca et al. 1990). It can be proposed that the distribution of hydrophobic and hydrophilic residues in combination with charged and other polar groups on a protein's surface determine protein solubility in the aqueous phases and partitioning therein.

The discovery that the surface of globular proteins contains non-polar hydrophobic groups has led to increasing interest in the involvement of hydrophobic interaction during protein partitioning in ATPS (Hachem et al. 1996; Lee and Richards 1971). Several approaches have been used in order to study the effect of hydrophobicity on partitioning in ATPS. Examples comprise the coupling of hydrophobic tails to PEG in order to increase the hydrophobicity of the phase rich in that polymer or the hydrophobic modification of proteins that were partitioned in a number of ATPS to investigate the effect of hydrophobicity as a single property on partitioning (Franco et al. 1996b; Shanbhag and Axelsson 1975). In general, high correlations could be found between the surface hydrophobicity of proteins and their partition coefficient in PEG/salt ATPS as well as PEG/dextran ATPS.

Besides protein hydrophobicity, the protein surface charge plays a distinctive role in the partition process. The solubility of proteins in aqueous solutions is determined by a combination of different interactions such as polar interactions with the solvent, ionic interactions with the salt present and repulsive electrostatic forces between molecules of the same charge (Scopes 1994). Since proteins are composed of sequences of amino acids that carry charged groups (depending upon their acidic or basic character), the net electric charge on a protein surface represents the sum of all electric charges present on the amino acids. At the so-called 'isoelectric point' the net surface charge of a protein is neutral. If the pH value of an aqueous solution containing protein is above the isoelectric point, proteins carry a negative net charge while below the isoelectric point their charge is positive. Hence, the pH value within an ATPS directly influences the charge at a protein's surface. A general rule of thumb recommends to select a system pH above the isoelectric point (pI) or pK_a of the target compound. As a consequence, an electrochemical affinity is generated between the negatively charged product and PEG, which has a positive dipolar momentum due to its terminal hydroxyl groups (Benavides and Rito-Palomares 2008; Nerli et al. 2001). However, the pH value should not be increased unnecessarily (e. g. 4-5 units above the pI) since an increasing amount of contaminants will also obtain electrochemical affinity towards the recovering phase.

Furthermore, partitioning in ATPS has been reported to be dependent on the biomolecules' molecular weight. Generally, small molecules such as amino acids tend to distribute evenly between the phases, while larger particles such as proteins or DNA get enriched in one of the phases. Even larger biomolecules like whole cells generally distribute between the interface and one of the two phases or collect entirely at the interface between the phases (Albertsson 1986; Sasakawa and Walter 1972). These observations can be explained by the greater contact area between high molecular weight biomolecules and the system components. According to the biomolecules' characteristics and the kind of interaction, partitioning will tend preferentially to one of the phases.

Finally, another factor to be considered is the temperature during the partition process. The exposure of proteins to increasing temperature results in the weakening of a number of bonds in the protein molecule. The long range interactions which are necessary for the presence of tertiary structure are affected first. As these bonds are weakened and then broken, the protein obtains a more flexible structure and the groups are exposed to the solvent. If heating ceases at this stage the protein sometimes is able to refold back to the native structure. A further increase in temperature, however, would result in breaking some of the cooperative hydrogen bonds which stabilise the helical structure of the protein. Water can interact and form new hydrogen bonds with the amide nitrogen and carbonyl oxygens of the peptide bonds. The presence of water further weakens nearby hydrogen bonds by causing an increase in the effective dielectric constant near them. As the helical structure is broken, hydrophobic groups are exposed to the solvent. As a result, losses in solubility or enzymatic activity of the proteins are observed. Therefore, special care has to be taken in order to prevent thermal denaturation when selecting the temperature of aqueous two-phase extraction.

Interaction of proteins and phase-forming compounds

The influence of molecular weight of PEG is related to the molecular weight and hydrophobic character of the target compound. Therefore, its selection must be done according to two physicochemical properties: (1) It has been reported that the purification of hydrophilic high molecular weight compounds (>10000 g mol^{-1}) is favoured by the application of low molecular weight PEG (<4000 g mol^{-1}) within ATPS. (2) On the other hand, the recovery of hydrophilic compounds of low molecular weight (<10000 g mol^{-1}) is improved by applying high molecular weight PEG (>4000 g mol^{-1}). This behaviour is related to the free volume available within the top and bottom phase (Cabezas 1996).

An effect of salts on proteins has already been reported more than a century ago by Franz Hofmeister, who noticed that ions exhibited various abilities of precipitating proteins (Hofmeister 1888). The so-called Hofmeister series classifies ions in order of their ability to change water structure and their effect on the stability of the secondary and tertiary structure of proteins. Ions which exhibit strong interactions with water molecules and thereby increase the structuring of water are called ‘kosmotrope’, while ions that decrease the structuring of water are named ‘chaotropes’. Kosmotropic salts are usually small and highly charged, while chaotropic salts are large and low charged (Zhao 2006). Nevertheless, the mechanism of the Hofmeister series is not entirely clear. Recent reports postulate that the mechanism does not result from changes in general water structure but instead from more specific interactions between ions and proteins and ions and the water molecules directly contacting the proteins (Zhang and Cremer 2006).

The typical ordering of the ion Hofmeister series and some of its related properties are summarised in Figure 3-3. While early members of the series increase solvent surface tension and decrease the solubility of nonpolar molecules (salting-out), later salts in the series act contrariwise by increasing the solubility of nonpolar molecules (salting-in) and increasing the order in water. In effect, they weaken the hydrophobic effect. The salts also interact directly with proteins, which are charged and have strong dipole moments, and may even bind specifically (e. g. phosphate and sulfate binding to ribonuclease A). Ions which have a strong ‘salting-in’ effect, such as I^- and SCN^-, salt-in the peptide group of proteins and thus interact much more strongly with the unfolded form of a protein than with its native state.

Consequently, they shift the chemical equilibrium of the unfolding reaction towards the unfolded protein state and act as strong denaturants (Baldwin 1996).

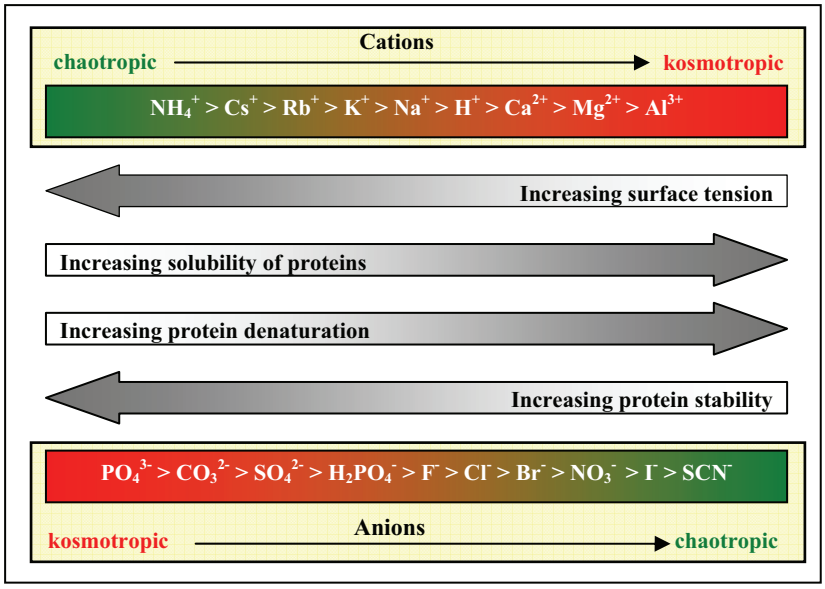


Figure 3-3: The Hofmeister series as an order of cations and anions from kosmotropic to chaotropic.

3.2.5 Application of ATPS

Aqueous two-phase systems have been applied in two main areas: as an analytical tool and for product recovery. These applications as well as selected examples thereof will be discussed in more detail in the following two sections.

Analytical application

The dependence of partitioning in ATPS from surface properties and conformation of proteins has rendered the technique a useful tool for a variety of analytical applications. Beside the sensitivity and the rapidity of the method, a particular advantage lies in the possibility to analyse both macromolecular and cellular structures. Basically, analytical applications of ATPS can be divided in two main categories, (1) interactions between biomolecules and (2) studies of protein surface properties.

(1) When biomolecules interact with each other, their partitioning behaviour in ATPS is affected due to changes in properties such as size, net surface charge and hydrophobicity (Lundberg and Backman 1994; Middaugh and Lawson 1980; Walter et al. 1985). Mattiasson and coworkers have exploited this concept in order to develop an immunoassay format in ATPS called ‘partition affinity ligand assay’ for rapid quantification of antigen (Mattiasson 1986). The concept is based on the change in partitioning of one of the reactants as a function of increasing concentration of the other and can be used for the calculation of dissociation constants. Moreover, it has been shown that interactions between molecules can be used to separate enantiomers in ATPS. The preferential partitioning of bovine serum albumin towards the bottom phase in PEG/dextran systems could be used to separate L- and D-tryptophan due to the fact that the protein binds selectively to one of the enantiomeric forms (L-tryptophan) of the racemic mixture (Ekberg et al. 1985).

(2) Aqueous two-phase systems have also been applied for studying changes on protein surfaces. Examples comprise e. g. the estimation of surface charges and the isoelectric point of proteins by varying conditions of pH and salt during partitioning in ATPS, or the determination of surface hydrophobicity and conformational changes in proteins by partitioning in the presence of hydrophobic and affinity ligands (Walter and Johansson 1994). Moreover, changes in the protein surface as a result of point mutations were reported to be predictable from partition coefficients of peptides (Berggren et al. 2000).

Product recovery in biotechnology

Representing a technically simple, easily scalable, energy-efficient and mild separation technique ATPS provide a useful tool for product recovery in biotechnology. They are utilised predominantly as primary recovery steps in purification processes. Since most biotechnological products are typically manufactured in large, dilute, multiphase fermentation broths, the first desirable step for their recovery is concentration. Aqueous two-phase systems are particularly suitable for this application due to the biocompatible environment and the possibility to include cells or cell debris in the extraction. The usage of ATPS for protein downstream processes is not only restricted to intracellular enzymes from microbial cells but also proteins from more complex raw materials like animal tissues and mucilaginous plant material (Jordan and Vilter 1991). Moreover, ATPS have been applied for the isolation of membrane proteins which normally are rather difficult and time-consuming to purify, the isolation of DNA and the extraction of small molecular weight compounds such as amino acids (Cole 1991; Ramelmeier et al. 1991; Sanchez-Ferrer et al. 1989; Sikdar et al. 1991).

Further interesting applications of ATPS include the so-called ‘extractive bioconversion’. Since the productivity of biotechnological processes is often very low due to either inhibition and toxicity of the product to the producer-organism or the instability of the product itself, ATPS can be applied to combine product removal and bioconversion. While the bioconversion takes place in one phase, the product is extracted to the other (Kuboi et al. 1995; Kwon et al. 1996; Zijlstra et al. 1996). Additional alternative applications of ATPS are exemplarily summarised in Table 3-1.

The purification of proteins using ATPS has been successfully carried out in large-scale for more than a decade. Scaling up an ATPS process is relatively straightforward since the same partitioning can be obtained both in small laboratory scale and in large-scale extractions. One major advantage lies in the fact that the conventional extraction equipment as used for organic-aqueous extraction can be applied in chemical industry (Hart et al. 1994; Kula 1990; Kula and Selber 1999; Raghavarao et al. 1995; Strandberg et al. 1991). Examples for large-scale protein extractions include among others the application of PEG/salt systems for purification of recombinant proteins from *E. coli* and the purification of cholesterol oxidase by surfactant-based cloud point extraction systems (Minuth et al. 1997; Minuth et al. 1996; Strandberg et al. 1991). However, limitations in the industrial application of ATPS arise from the incomplete theoretical understanding of phase equilibrium and protein partitioning, as well as the cost of polymers and the isolation of protein from the phase-forming compounds. The latter can be achieved by ion-exchange chromatography or back-extraction though (Asenjo et al. 1994b; Johansson 1994). Additionally, ultrafiltration can be used to recycle

both polymer and salt thereby rendering the process itself more economical (Hustedt 1986; Veide et al. 1989).

Table 3-1: Alternative applications of aqueous two-phase systems.

ATPS	New application	Reference
PEG/dextran	Separation of polymerase chain reaction (PCR)-inhibitory substances from bacterial cells	(Lantz et al. 1996)
PEG/phosphate	Recovery of viral coat proteins from recombinant *E. coli*	(Rito-Palomares and Middelberg 2002)
	Preparation of highly purified fractions of small inclusion bodies	(Walker and Lyddiatt 1998)
	Recovery of aroma compounds under product inhibition conditions	(Rito-Palomares et al. 2000)
PEG/sulphate	Drowning-out crystallisation of sodium sulphate	(Taboada et al. 2000)
	Recovery of metal ions from aqueous solutions	(Rogers et al. 1996)
	Recovery of food coloring dyes from textile plant wastes	(Huddleston et al. 1998a)
	Partition of small organic molecules	(Rogers et al. 1998)

3.2.6 Limitations in aqueous two-phase extraction

Despite the versatile advantages of ATPS, the aqueous nature of these systems entails some drawbacks with regard to the purification of industrially interesting enzymes. The occurrence of side reactions as well as the low solubility of numerous pharmaceutical substrates in aqueous media often prevents the combination of enzyme purification with enzyme-catalysed reactions. Many enzymes are known to be very sensitive and instable, thus losing activity during the purification process. Moreover, the phase-forming compounds within the phase, which contains the enzyme, might interfere with the enzyme-catalysed reaction. Therefore, in many cases subsequent purification steps are needed in order to separate the phase-forming compounds from solution containing the enzyme (Rito-Palomares 2004). Additionally, the successful exploitation of ATPS in industrial biotechnology is restricted due to several factors such as a lack of knowledge of the technique and poor understanding of the mechanism governing phase formation and solute partition. Hence, the factors which impose limitations on the commercial adoption of the technique need to be addressed.

3.3 Ionic liquids in biocatalysis

Ionic liquids (ILs) are gaining increasing importance as a new class of solvents for organic synthesis in general and catalytic processes in particular (Earle and Seddon 2000). Over the last three years alone, more than 4000 publications on room-temperature ionic liquids have been published (MacFarlane and Seddon 2007). The increasing interest in ILs, both by academia and industry, is given by their special properties. Basically and essentially, ILs represent purely ionic, salt-like materials which are composed of organic cations and various anions (Figure 3-4).

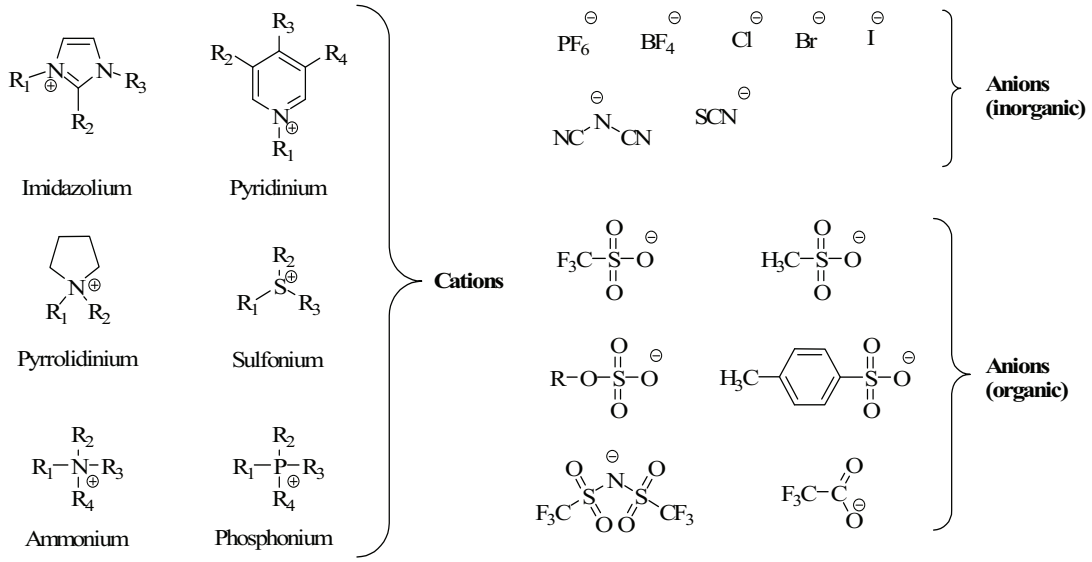


Figure 3-4: Typical cations and anions within ionic liquids.

The properties of an IL can be varied by changing the nature of the cation/anion combination. Therefore, ionic liquids are also referred to as designer solvents. Compared to water and organic solvents, they exhibit a negligible volatility, non-flammability, a wide electrochemical window, high thermal and chemical stability and strong solubility power (Dupont et al. 2002). Table 3-2 gives a brief comparison of typical properties of organic solvents and ionic liquids (Plechkova and Seddon 2008). Still it has to be emphasized that this summary is not comprehensive but rather aims to provide a general overview. The chirality of ionic liquids, for example, is not yet as common as proposed and organic solvents are known to exhibit strong solvating power on many compounds.

The special features of ILs render them suitable for applications in various fields of chemistry (Anderson et al. 2006; Favre et al. 2001; Fukumoto et al. 2005; Leone et al. 2001; Song and Roh 2000). Several comprehensive reviews on different aspects of ILs such as their use as general (co)solvents for reactions and catalysis, their use as chiral solvents and reagents in synthesis and their chemical properties can be found in the literature (Anderson et al. 2002; Baudequin et al. 2005; Chowdhury et al. 2007; Durand et al. 2007; Hunt et al. 2007; Izgorodina et al. 2007; Lee 2006; Parvulescu and Hardacre 2007; Sheldon 2005; Wilkes 2004; Zhang 2006).

Table 3-2: Comparison of organic solvents with ionic liquids as published by Plechkova and Seddon (2008).

Property	Organic solvent	Ionic liquids
Number of solvents	> 1000	> 1000000
Applicability	Single function	Multifunction
Catalytic ability	Rare	Common and tuneable
Chirality	Rare	Common and tuneable
Vapour pressure	Obeys the Clausius-Clapeyron equation	Negligible vapour pressure under normal conditions
Flammability	Usually flammable	Usually non-flammable
Solvation	Weakly solvating	Strongly solvating
Polarity	Conventional polarity concepts apply	Polarity concept questionable
Tuneability	Limited range of solvents available	Virtually unlimited range means 'designer solvents'
Cost	Normally cheap	Typically between 2 and 100 times the cost of organic solvents
Recyclability	Green imperative	Economic imperative
Viscosity / cP	0.2-100	22-40000
Density / g cm^{-3}	0.6-1.7	0.8-3.3
Refractive index	1.3-1.6	1.5-2.2

Due to the need of establishing environmentally friendly alternative syntheses in chemical industry, the application of enzymes and whole cells for catalysis is of increasing importance in order to compete with economically well-established chemical processes (Liese et al. 2006; Schoemaker et al. 2003; Thayer 2006). Throughout the history of biocatalysis, alternative reaction conditions have been investigated to overcome problems such as low substrate solubility, selectivity, yield or catalyst stability. Advancements have been made by the use of organic solvents, the addition of high salt concentrations, the use of microemulsions or supercritical fluids (Cabral et al. 1997; Carrea and Riva 2000; Hartmann et al. 2000; Khmelnitsky et al. 1994; Orlich and Schomaecker 1999). Thus, it is not surprising that researchers in the field of biocatalysis focused on ionic liquids as novel solvents to find new solutions to known problems.

Indeed, during the last years, several ILs have been applied for biotransformation processes and advantages such as improvement of enzyme stability, substrate and/or product selectivity and suppression of side reactions could be observed (Klembt et al. 2007; Kragl et al. 2002; Park and Kazlauskas 2003; van Rantwijk et al. 2003; van Rantwijk and Sheldon 2007; Welton 1999). Some examples for biocatalysis using ionic liquids are summarised in Table 3-3. The use of ILs as solvents for chemical reactions also revealed their excellent physico-chemical properties: they dissolve both polar and non-polar organic, inorganic and polymeric materials

(Dupont et al. 2002; Earle and Seddon 2000; Holbrey and Seddon 1999; Olivier-Bourbigou and Magna 2002; Sheldon 2001; Wasserscheid and Keim 2000; Welton 1999). In consequence, the solubility-related effect of ILs can be utilised within the field of biocatalysis as well. To cite an example, the ionic liquids $[C_4mim][BF_4]$ and $[C_4mim][PF_6]$ have successfully been applied to increase the low solubility of sugars and to maintain the enzymatic activity in lipase-catalysed synthesis of fatty acid sugar esters (Ganske and Bornscheuer 2005a; Ganske and Bornscheuer 2005b).

Table 3-3: Examples of biocatalysts applied in ionic liquids.

Biocatalyst	Ionic liquid	Reaction system	Reference
Alkaline phosphatase *E.coli*	Ethylammonium nitrate	Enzyme activity and stability assayed by hydrolysis of *p*-nitrophenol phosphate	(Magnuson et al. 1984)
Esterases	1-butyl-3-methylimidazolium hexafluorophosphate	Transesterification of 1-phenylethanol	(Persson and Bornscheuer 2003)
Hen egg white lysozyme	Ethylammonium nitrate	Protein renaturation	(Summers and Flowers 2000)
Thermolysine	1-butyl-3-methylimidazolium hexafluorophosphate	Synthesis of *Z*-aspartame	(Erbeldinger et al. 2000)
β-galactosidase	1-butyl-3-methylimidazolium tetrafluoroborate	Hydrolytic activity	(Husum et al. 2001)
Subtilisin	1,3-dimethylimidazolium methylsulfate	Synthesis of *N*-acetyllactosamine	(Kragl et al. 2001)
Peptide amidase	1,3-dimethylimidazolium methylsulfate,	Amidation of H-Ala-Phe-OH	(Kaftzik et al. 2003)
	1-butyl-3-methylimidazolium methylsulfate		
Protease *α*-chymotrypsin	1-ethyl-3-methylimidazolium bis(triflouromethylsulfonyl)imide	Stability investigations	(De Diego et al. 2004)
	1-butyl-3-methylimidazolium hexafluorophosphate,	Transesterification of *N*-acetyl-L-phenylalanine ethyl ester	(Laszlo and Compton 2001)
	1-octyl-3-methylimidazolium hexafluorophosphate		
	1-butyl-3-methylimidazolium hexafluorophosphate,	Transesterification of *N*-acetyl-L-tyrosin ethyl ester	(Lozano et al. 2001)
	1-butyl-3-methylimidazolium tetrafluoroborate,		
	1-ethyl-3-methylimidazolium bis(triflouromethylsulfonyl)imide,		
	1-ethyl-3-methylimidazolium tetrafluoroborate,		
	methyltrioctylammonium bis(triflouromethylsulfonyl)imide		
Papain	1-butyl-3-methylimidazolium tetrafluoroborate	Resolution of racemic amino acid derivatives	(Lou et al. 2004)
Formate dehydrogenase	1,3-dimethylimidazolium methylsulfate	Regeneration of NADH	(N. Kaftzik, unpublished results)

Table 3-3: Examples of biocatalysts applied in ionic liquids. Continued.

Biocatalyst	Ionic liquid	Reaction system	Reference
Morphine dehydrogenase	1-(3-hydroxy-propyl)-3-methylimidazolium glycolate	Synthesis of the drug oxycodone	(Walker and Bruce 2004a; Walker and Bruce 2004b)
Alcohol dehydrogenase	1-butyl-3-methylimidazolium bis (triflouromethylsulfonyl)imide	Enantioselective reduction of 2-octanone in a biphasic system	(Eckstein et al. 2004)
Horseradish peroxidase	1-butyl-3-methylimidazolium tetrafluoroborate	Enzyme immobilization in IL	(Liu et al. 2005)
Candida antarctica Lipase *B*	1-butyl-3-methylimidazolium bis(triflouromethylsulfonyl)imide	Acylation of octan-1-ol and kinetic resolution of 1-phenylethanol using IL/scCO2	(Reetz et al. 2002)
Pseudomonas cepacia lipase	1-butyl-3-methylimidazolium hexafluorophosphate, 1-ethyl-3-methylimidazolium hexafluorophosphate	Kinetic resolution of sec. alcohols	(Kim et al. 2001)
Pseudomonas sp. lipase	1-butyl-3-methylimidazolium bis(triflouromethylsulfonyl)imide	Kinetic resolution of (*R,S*)-1-phenylethanol	(Goderis et al. 1987; Kragl et al. 2001)
Candida rugosa lipase	1-butyl-3-methylimidazolium hexafluorophosphate, 1-butyl-3-methylimidazolium bis(triflouromethylsulfonyl)imide, 1-butyl-3-methylimidazolium tetrafluoroborate, 1-butyl-3-methylimidazolium trifluoromethanesulfonate, 1-butyl-3-methylimidazolium hexafluoroantimonate	Kinetic resolution of allylic alcohols	(Itoh et al. 2001)

3.4 Ionic liquids for the extraction of biomolecules

Their special properties render ILs suitable or even superior solvents for liquid-liquid extraction processes. In particular, hydrophobic ILs have been applied as a replacement for volatile organic solvents by generating two-phase ionic liquid-water systems. Huddelston et al. were the first to investigate the potential of these novel liquid-liquid extraction systems (Huddleston et al. 1998b). They found the partitioning of simple, substituted-benzene derivatives between water and $[C_4mim][PF_6]$ to be dependent on the solutes' charge and relative hydrophobicity. Since then the extraction of metal ions, organic and aromatic compounds by water immiscible ionic liquids with high selectivities have been reported (Holbrey et al. 2003; Visser et al. 2001; Wei et al. 2003). Moreover, by using imidazolium based ionic liquids, biological molecules such as DNA and amino acids were found to be selectively extracted from aqueous media into hydrophobic ILs (Cheng et al. 2007; Wang et

al. 2005). To state an example, Wang et al. achieved the selective recovery of some amino acids from aqueous solution into the hydrophobic ILs [C_4mim][PF_6], [C_6mim][PF_6], [C_6mim][BF_4] and [C_8mim][BF_4] with varying partition coefficients (Wang et al. 2005). They propose the hydrophobicity of the amino acids as well as electrostatic interactions between the anion of the ILs and the cationic form of the amino acids to be the main driving forces of the extraction process.

However, with regard to the extraction of proteins and enzymes, only few reports can be found in literature due to the fact that proteins are either insoluble or their solubility in ILs is very low (Fujita et al. 2006; Fujita et al. 2005; Lau et al. 2004; Sheldon et al. 2002). One approach to enhance protein solubility within ILs is the addition of a small amount of water. The formation of aqueous microemulsion droplets in a hydrophobic IL stabilised by a layer of anionic surfactant allowed the solubilisation of active enzymes (Moniruzzaman et al. 2008). Another approach comprises the chemical modification of enzymes or proteins themselves (Laszlo and Compton 2001; Maruyama et al. 2002; Maruyama et al. 2004; Ohno et al. 2003). Nakashima et al. reported about an improved protein solubility of Subtilisin Carlsberg in [C_4mim][PF_6], [C_2mim][Tf_2N], [C_2OHmim][Tf_2N] and [C_2OC_1mim][Tf_2N] by conjugation of the enzyme with comb-shaped polyethylene glycol PM_{13} (Nakashima et al. 2005). Nevertheless, usually attempts to dissolve enzymes in ILs result in a reduced catalytic activity due to conformational changes (Lau et al. 2004; Turner et al. 2003).

In order to overcome the limitations of low protein solubility and enzyme activity, recently the use of hydrophilic ionic liquids for the formation of aqueous two-phase systems has been reported, abbreviated as IL-based ATPS (ionic liquid-based aqueous two-phase systems) (Gutowski et al. 2003; He et al. 2005; Li et al. 2005; Ruiz-Angel et al. 2007). Rogers and coworkers were the first to report the phase diagrams of IL-based ATPS (Gutowski et al. 2003). They investigated the ability of kosmotropic salts (e. g. K_3PO_4) to salt-out hydrophilic ILs such as [C_4mim][Cl] and [N_{4444}][Cl]. First IL-based ATPS have been applied for the extraction of testosterone and epitestosterone in human urine or the extraction of major opium alkaloids in *Pericarpium papaveris* using [C_4mim][Cl] / K_2HPO_4 systems (He et al. 2005; Li et al. 2005). The extraction of proteins by IL-based ATPS was first achieved by Du et al. who extracted proteins from human body fluids by employing a [C_4mim][Cl] / K_2HPO_4 system (Du et al. 2007). However, the application of IL-based ATPS for the purification of catalytically active biomolecules (enzymes) has not been investigated up to this time. Potential advantages of such a system would lie in the combination of a purification process with the performance of enzyme-catalysed reactions. The presence of ionic liquid within the system could further be advantageous with regard to stability and solubility enhancing effects. Thus, the system would take advantage of both the selective, gentle purification process of an aqueous two-phase extraction and the special properties of the applied ionic liquid.

4 Results and discussion part I: Properties and characterisation of ionic liquid-based aqueous two-phase systems[1]

4.1 Screening of suitable ionic liquids

In 2004, the company Solvent Innovation (Cologne, Germany) introduced a new ionic liquid product line called Ammoeng™ in collaboration with Degussa (since 2007: Evonik Industries AG). This ionic liquid family comprises ethylene glycol functionalised ammonium cations in combination with various anions and can also be found in literature as TEGO®-ILs. Due to the fact that Ammoeng™-ILs are already listed (TSCA: 'Toxic Substances Control Act' and EINECS: 'European Inventory of Existing Commercial Chemical Substances') and available in technical quantities for reasonable prices, they represent an interesting alternative to 'conventional', imidazolium-based ionic liquids (Weyershausen and Lehmann 2005). First industrial applications involve the use of these ILs as secondary dispersing agents thereby enabling the application of universal, water-based pigment pastes for all types of paints and coatings (Weyershausen and Lehmann 2005).

The incorporation of ethylene glycol units into the IL cation, and hence the structural similarity to the phase-forming compound polyethylene glycol, suggests applicability of Ammoeng™-ILs for the generation of aqueous two-phase systems. In first evaluation experiments, the Ammoeng™ family was screened for the individual potential of each IL to form two immiscible aqueous phases by using a potassium phosphate salt as second phase-forming compound (Table 4-1). The inorganic potassium salt was selected in order to ensure the required biocompatibility of the resulting two-phase system. Furthermore, literature confirms multivalent anions like HPO_4^{2-} and SO_4^{2-} to be the most effective in inducing phase separation within polyethylene glycol-based ATPS (Hatti-Kaul 2001). A general scheme to visualise the formation of an ionic liquid-based aqueous two-phase system (IL-based ATPS) is given in Figure 4-1.

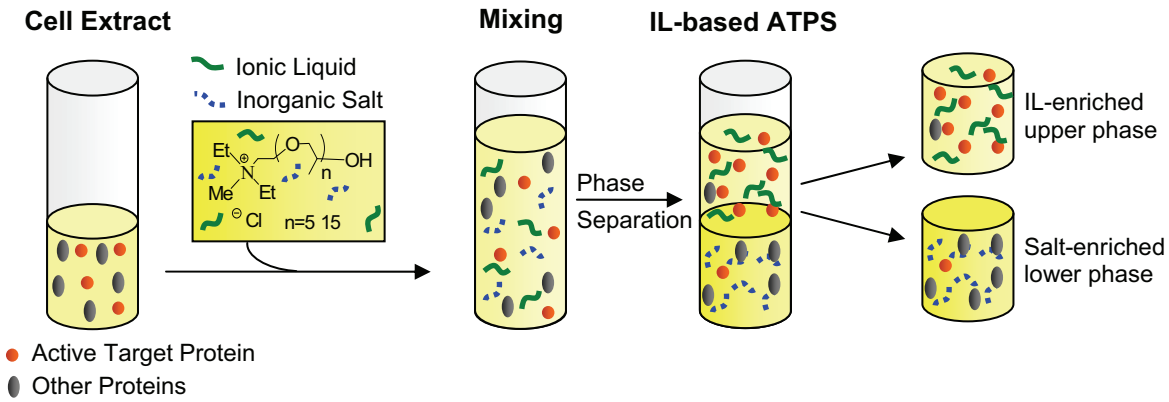
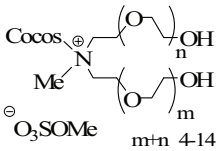
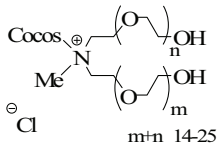
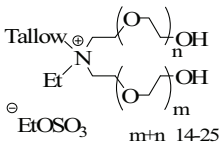
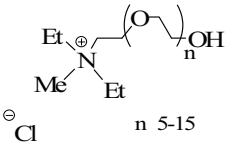
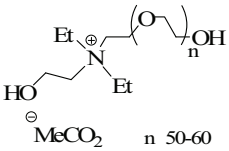


Figure 4-1: Schematic illustration of the formation of aqueous two-phase systems based on ionic liquids.

[1]Parts of this chapter were published in: Dreyer S, Kragl U. (**2008**). Ionic liquids for aqueous two-phase extraction and stabilization of enzymes. *Biotechnology and Bioengineering* 99(6):1416-1424.

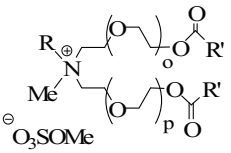
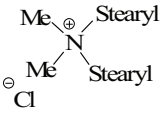
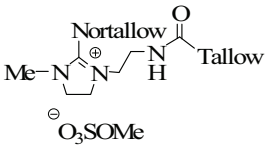
As shown in Table 4-1, the formation of an ionic liquid-based aqueous two-phase system could be achieved by three ionic liquids: Ammoeng™ 100, Ammoeng™ 101 and Ammoeng™ 110. Above critical concentrations of the ILs and the potassium salt in aqueous solution, phase separation takes place resulting in the formation of an IL-enriched, inorganic salt-poor upper phase which coexists with a salt-enriched, IL-poor lower phase (Figure 4-1). Naturally, the most important property of an ionic liquid in order to generate an ATPS is a sufficiently high water solubility. With regard to the chemical structure of Ammoeng™ 102, Ammoeng™ 111, Ammoeng™ 120, Ammoeng™ 130 and Ammoeng™ 520, the non-ability to generate an IL-based ATPS can be ascribed to their limited solubility in aqueous solution. The length of ethylene glycol chains within Ammoeng™ 111, as well as the fatty acid nature of the tallow and stearyl side chains within Ammoeng™ 102, Ammoeng™ 130 and Ammoeng™ 520 restricts their solubility to concentrations below the critical phase-forming point of ATPS. Still, the first screening experiments revealed three different ionic liquids of the Ammoeng™ family to be applicable for the establishment of an aqueous two-phase system. Therefore, these ILs were further characterised and their potential application as a phase-forming compound within aqueous two-phase extraction processes was investigated.

Table 4-1: Screening of the Ammoeng™-IL-family with regard to the application as phase-forming compound within aqueous two-phase systems using potassium phosphate salts.

Ionic liquid		Melting point[2] / °C	Density at 25°C[1] / g cm^{-3}	Viscosity at 25°C[1] / mPas	Formation of aqueous two-phase system Yes	No
Ammoeng™ 100 / TEGO® IL K5MS	 m+n 4-14	≤ (-65)	1.0875	1664.63	X	
Ammoeng™ 101 / TEGO® IL K5	 m+n 14-25	10-12	1.0623	1359.68	X	
Ammoeng™ 102 / TEGO® IL T16ES	 m+n 14-25	10-15	1.0782	420.56		X
Ammoeng™ 110 / TEGO® IL P9	 n 5-15	≤ (-65)	1.0282	494.77	X	
Ammoeng™ 111 / TEGO® IL P54A	 n 50-60	≤ (-65)	1.0485	617		X

[2] Information obtained from Solvent Innovation (Cologne, Germany)

Table 1-1: Screening of the Ammoeng™-IL-family with regard to the application as phase-forming compound within aqueous two-phase systems using potassium phosphate salts. Continued.

Ionic liquid		Melting point[1] / °C	Density at 25°C[1] / g cm^{-3}	Viscosity at 25°C[i] / mPas	Formation of aqueous two-phase system Yes	No
Ammoeng™ 120 / TEGO® IL ZTI		8-10	0.9946	807.93		X
Ammoeng™ 130 / TEGO® IL DS		95-100	-	-		X
Ammoeng™ 520 / TEGO® IL IM90		40-50	-	-		X

4.2 Identification of characteristic system properties

In general, phase separation in solutions containing polymer and salt mixtures is a very complex phenomenon. An understanding in terms of fundamental molecular properties, molecular parameters and interactions is still underway. Therefore, practical strategies to design ATPS extraction processes are needed to overcome the poor understanding of the molecular mechanism governing the behaviour of solutes in ATPS. A general strategy has been proposed by Rito-Palomares and is presented in Figure 4-2 (Rito-Palomares 2004). Roughly following the depictured procedure, the first step in evaluating the potential of Ammoeng™-ionic liquids for aqueous two-phase extraction focuses on the formation and characterisation of the system itself.

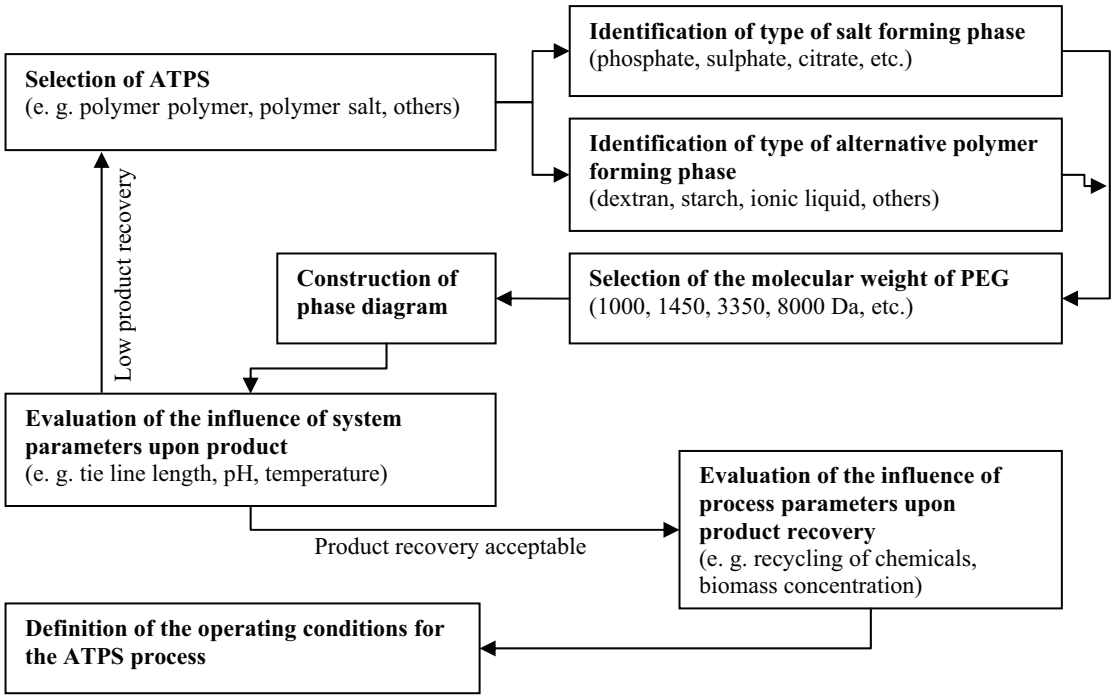


Figure 4-2: Practical strategies to ATPS process development for recovery of biological products (Rito-Palomares 2004).

The fact, that two incompatible polymers or one polymer and a salt in aqueous solution promote the separation of two aqueous phases has been well studied for several decades (Albertsson 1986). In order to characterise these systems and apply them for purification processes, knowledge of the underlying phase equilibrium is fundamental and can be provided by the phase diagrams which delineate the potential working area for a particular two-phase system.

4.2.1 Effect of the type of ionic liquid

As pointed out above, the construction of a binodal curve is essential for evaluating the applicability of ionic liquids for the generation of IL-based ATPS. Since first screening experiments confirmed the general potential of Ammoeng^{TM} 100, Ammoeng^{TM} 101 and Ammoeng^{TM} 110 to form an IL-based ATPS, binodal curves of these systems were prepared by applying the cloud point method (Figure 4-3). As indicated by the location of the binodal curves, the ability for phase separation varies among the applied ILs and follows the order Ammoeng^{TM} 101 > Ammoeng^{TM} 110 > Ammoeng^{TM} 100. In other words, to induce phase separation, a lower concentration of Ammoeng^{TM}101 is needed than for Ammoeng^{TM} 110 and Ammoeng^{TM} 100 as can be seen from a closer location of the binodal curve towards the origin.

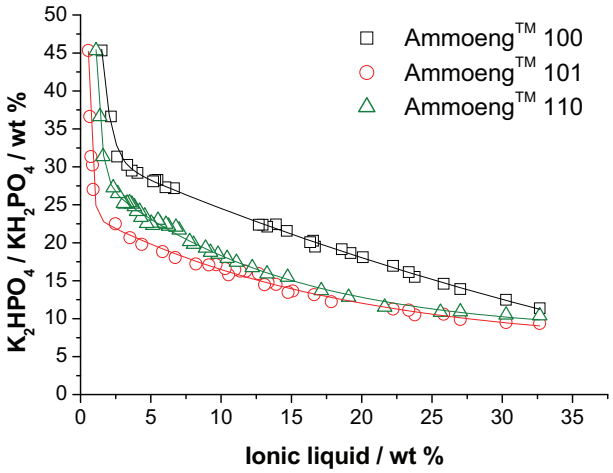


Figure 4-3: Binodal curves of the ionic liquids Ammoeng™ 100, Ammoeng™ 101 and Ammoeng™ 110 and the inorganic salt mixture K_2HPO_4/KH_2PO_4 at 25°C as determined by the cloud point method (Albertsson 1986). The compound which is enriched in the upper phase (Ammoeng™ ILs) is plotted on the abscissa and the compound enriched in the lower phase (K_2HPO_4/KH_2PO_4) is plotted on the ordinate.

A structural comparison of the three tetraammonium-based ILs is given in Table 4-2 and can be used to interpret the observed differences in phase formation. Although the ionic liquids Ammoeng™ 100 and Ammoeng™ 101 posses the same cation, they differ in the type of anion and the length of functional groups. Ammoeng™ 110 shares the chloride anion with Ammoeng™ 101 but holds a cation with only one ethylene glycol-chain of shorter length. This structural distinction considerably influences the location of the binodal curves and is reflected in the closest location of Ammoeng™ 101 (higher chain length of functional groups) towards the origin. In traditional PEG/salt ATPS, it has been reported that the binodal curves shift towards the origin with an increase in molecular weight of PEG. This effect has been attributed to the more hydrophobic character of PEG with higher molecular weight. In agreement with this theory, the phase-forming ability of the Ammoeng™-ionic liquids is in accordance with the order of their functional group chain length (Ammoeng™ 101 > Ammoeng™ 110 > Ammoeng™ 100).

Table 4-2: Structural comparison of the Ammoeng™ ionic liquids.

	Cation (R_4N^+)		**Anion**
Trade name	**Alkyl or Acyl groups**	**Functional groups**	
Ammoeng™ 100	Me-, Cocos-(C_{14} alkyl)	$-CH_2CH_2(OCH_2CH_2)_mOH$, $-CH_2CH_2(OCH_2CH_2)_nOH$, ($m + n$ = 4-14)	$MeSO_4^-$
Ammoeng™ 101	Me-, Cocos-(C_{14} alkyl)	$-CH_2CH_2(OCH_2CH_2)_mOH$, $-CH_2CH_2(OCH_2CH_2)_nOH$, ($m + n$ = 14-25)	Cl^-
Ammoeng™ 110	Et-, Et-, Me-	$-CH_2CH_2(OCH_2CH(CH_3))_nOH$, ($n$ = 5-15)	Cl^-

With regard to the impact of the IL anion, a closer look has to be taken at the phase formation process itself. The effect of the addition of salts on the miscibility of a given solute (in this case also of ionic nature) in an aqueous solution is very complex, preliminary because a large number of different types of intermolecular interactions have to be taken into account. Various theories have been proposed to explain the mechanisms of salting-out, stressing the importance of different interactions between the added ions and the solvent, the added ions and the solute ions, and the solute ions and the solvent (Marcus 1986; Marcus and Loewenschuss 1984). Some of the most common theories involve the concepts of structure-forming (kosmotropic) salts which promote a water-structuring effect around them. The kosmotropicity concept (see also Hofmeister series, chapter 3.2.4) is used as an indicator for the salting-out ability of a salt (Zhao 2006). Kosmotropic ions exhibit stronger interaction with water molecules and are beneficial for the formation of aqueous two-phase systems. Since all ionic liquids were salted out by the same K_2HPO_4/KH_2PO_4 salt-mixture, the kosmotropic effects of HPO_4^{2-} and $H_2PO_4^-$ can be neglected. The salting-out ability of the IL anions can further be supported by a thermodynamic approach which enables the quantification of the Hofmeister series by utilising the Gibbs free energy of hydration, ΔG_{hyd} (Marcus 1991; Marcus 1997). ΔG_{hyd} is defined as the change in free energy from an isolated naked ion in the ideal gas phase to the aqueous solvated ion in solution. Kosmotropic ions have a large negative ΔG_{hyd}, due to the resulting structured water 'lattice' around the ion (Johansson et al. 1993), while chaotropic ions have smaller negative or even positive values for ΔG_{hyd} (Marcus 1997). The values of ΔG_{hyd} for Cl^- and SO_4^{2-} are -340 and -1080 kJ mol^{-1}, respectively (Marcus 1991). Consequently, the IL with methyl sulfate anion (Ammoeng™ 100) hydrates more water molecules than those with chloride anions (Ammoeng™ 101 and Ammoeng™ 110), resulting in a hindered phase formation as potassium salt was added.

Following the argumentation above, the phase formation ability and thus the location of binodal curves for the investigated IL-based ATPS can be explained by a combination of two different effects which are related to the IL cation and anion properties. (1) An increase of the functional group's chain length of the IL cation will result in a more hydrophobic character of the IL and facilitate phase formation, while (2) a more kosmotropic character of the anion will reduce the phase formation ability due to hydration competition phenomena with the phase-forming inorganic potassium salt. These results are in good agreement with previously reported phase diagrams of imidazolium based ionic liquids and salts (Pei et al. 2007). Pei et al. (2007) found a shift of the binodal curves towards the origin with an increase in alkyl chain length and ILs with a Br^- anion were more suitable to form two phases compared to those with Cl^- anions (ΔG_{hyd} for Cl^- and Br^- are -340 and -315 kJ mol^{-1}). However, it has to be emphasized that the proposed effects can explain the observed tendencies of the location of binodal curves only in a semi-quantitative way due to the intrinsic system complexity of aqueous solutions and ionic liquids.

4.2.2 Effect of the type of inorganic salt

The application of Ammoeng™-ILs for the formation of IL-based ATPS opens up an enormous variety of feasible IL-based ATPS. By variation of the ionic liquid as well as the inorganic salt (as the second phase-forming compound), the development of a huge set of new IL-based ATPS is rendered possible. However, with regard to the application of these systems

for biotechnological downstream processes and in order to get a deeper insight into the phenomena underlying the phase formation and partitioning within these systems, further investigations had to be focused on the examination of one exemplary ionic liquid for the purpose of limiting the number of experiments while extracting as much information as possible. The IL Ammoeng^{TM} 110 was chosen according to the most important system requirements: (1) biocompatibility and (2) possibility of protein quantification.

As already mentioned above, the relative effectiveness of various inorganic salts in promoting phase separation within PEG-based ATPS has been reported to follow the Hofmeister series, which originally was applied to order the ions' abilities of stabilising or destabilising proteins and membranes (Hofmeister 1888; Kunz et al. 2004). Usually, kosmotropic salts are small and highly charged, while chaotropic salts are large and low charged (Zhao 2006). It has been reported that the contribution of the anion for the formation of ATPS is generally more pronounced than that of the cation in determining the effectiveness of a particular salt. Additionally, multivalent anions are the most effective in inducing phase formation, and monovalent anions least effective (Huddleston et al. 1991). The typical ordering of the ion series and some of its related properties has been reported in chapter 3.2.3.

The formation of IL-based ATPS using the IL Ammoeng^{TM} 110 and a variety of kosmotropic inorganic salts is shown in Figure 4-4. The observed shifts in the binodals follow the Hofmeister series for the strength of kosmotropic salts: K_2HPO_4 > K_2HPO_4/KH_2PO_4 > KOH. Hence, it can be concluded that strongly kosmotropic ions favour IL-based ATPS formation, as it has also been observed for PEG-based ATPS. This observation can further be supported by utilising the Gibbs free energy of hydration, ΔG_{hyd}, as has been discussed above. The more negative the Gibbs free energy of hydration, the greater the salting-out effect of an ion for PEG or rather the ionic liquid (Table 4-3).

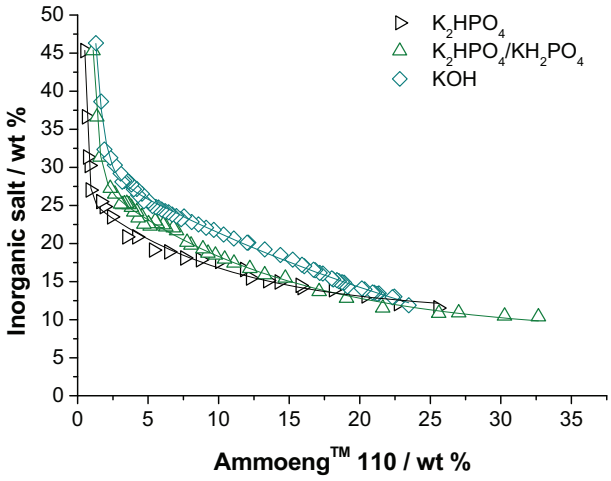


Figure 4-4: Binodal curves of Ammoeng^{TM} 110 with different inorganic salts as second phase-forming compound at 25°C.

However, the mechanism through which the salt influences phase separation is still poorly understood. A qualitative explanation for phase separation in IL-based ATPS relates the observed behaviour to the tendency of chaotropic salts to be salted-out by kosmotropic salts.

Since ILs are designed to have depressed melting points, a result of low symmetry ions, delocalized charge, and weak directional intermolecular interactions, most ILs would be classified as chaotropic salts (Bridges et al. 2007). Hence, the phase separation process can be pictured as follows: When an inorganic salt is added to a solution of an ionic liquid, the ions will compete with each other for the solvent molecules. The competition is won by the more kosmotropic inorganic ions with a stronger affinity for the solvent. Consequently, a 'migration' of solvent molecules away from the ions of the IL to those of the inorganic salt takes place, which in turn decreases the hydration and hence the solubility of the ions of the IL. As a consequence, a phase rich in IL separates from the rest of the solution. Therefore, the salting-out effect is directly correlated to the hydration strength of the different ions of the inorganic salt (Trindade et al. 2007).

Table 4-3: A selected list of ΔG_{hyd} of cations and anions illustrating the kosmotropic and chaotropic salts (Marcus 1991).

Cation	ΔG_{hyd} / kJ mol^{-1}	Anion	ΔG_{hyd} / kJ mol^{-1}
Al^{3+}	-4525	PO_4^{3-}	-2765
H^+	-1050	CO_3^{2-}	-1315
Na^+	-365	SO_3^{2-}	-1295
K^+	-295	SO_4^{2-}	-1080
NH_4^+	-285	$H_2PO_4^-$	-465
Rb^+	-275	F^-	-465
Cs^+	-250	OH^-	-430
$(CH_3)_4N^+$	-160	Cl^-	-340
$(C_2H_5)_4N^+$	0	Br^-	-315
$(C_6H_5)_4As^+$	50	BF_4^-	-190

4.3 Influence of system parameters

Several factors like type and concentration of the phase-forming compounds, pH and temperature are known to influence the nature of PEG-based aqueous two-phase systems (Albertsson 1986; Walter and Johansson 1994). In order to investigate the influence of these system parameters, the IL-based ATPS using Ammoeng^{TM} 110 and the salt mixture K_2HPO_4/KH_2PO_4 (ratio of 1.00:1.82) as phase-forming compounds was chosen as a model system. This selection was done according to the required system characteristics which are needed to ensure biocompatibility of the system. The inorganic salt mixture allows the adjustment of a physiological pH and therefore contributes to the stabilisation of proteins and maintenance of enzyme activity. These criteria are of major importance with regard to the target application of IL-based ATPS for enzyme purification.

4.3.1 Concentration of phase-forming compounds

Variation of the concentration of phase-forming compounds exhibits a direct influence on the phase composition and volume of IL-based ATPS. An investigation of the phase volumes revealed that with increasing amount of IL within the system, the volume of the upper phase increases, while the volume of the lower phase decreases. Using a fixed amount of IL, an increase of the inorganic salt concentration will result in an increased volume of the lower phase (Figure 4-5). This observation again confirms the assumed competition of ions for solvent molecules. The more inorganic salt added, the more solvent molecules are withdrawn from the IL-containing phase, thereby decreasing the IL-phase volume. On the other hand, an increase in IL concentration at a fixed amount of inorganic salt will nevertheless reduce the availability of solvent molecules accessible for the inorganic salt and thus decrease the volume of the lower phase.

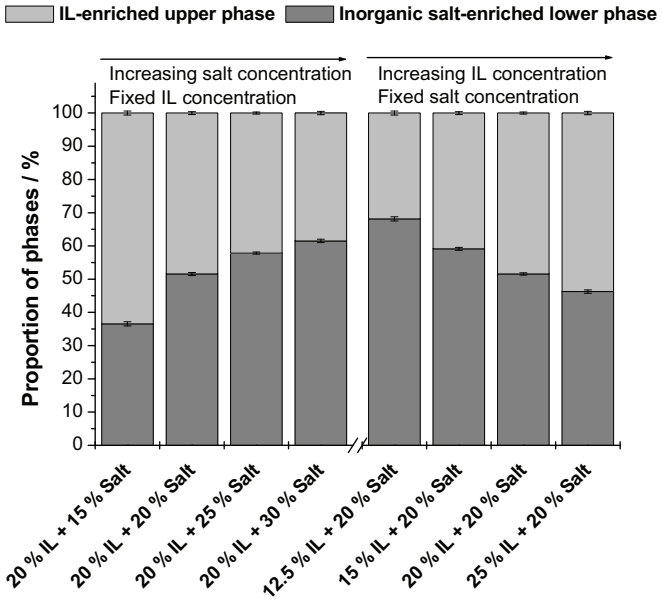


Figure 4-5: Proportion of phase volumes within IL-based ATPS while changing the concentration of the two phase-forming compounds Ammoeng^{TM} 110 and K_2HPO_4/KH_2PO_4 at 4°C.

At the same time it was found that with increasing amount of IL and constant concentration of inorganic salt, the amount of inorganic salt in the IL-containing upper phase decreases and vice versa. This observation is illustrated and confirmed by the tie-lines within the binodal curve of the investigated IL-based ATPS (Figure 4-6).

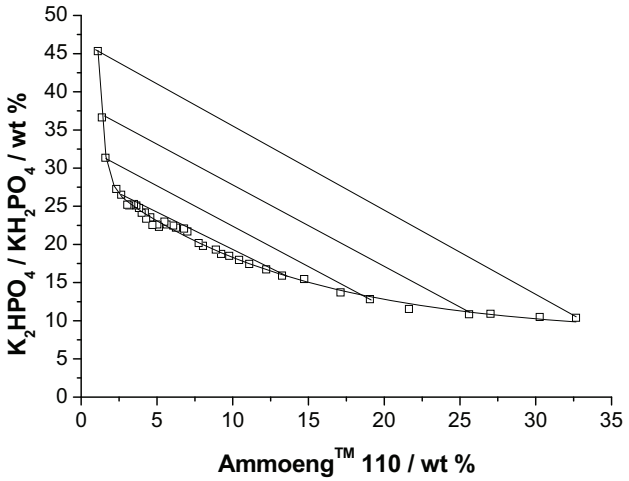


Figure 4-6: Phase diagram of the IL-based ATPS Ammoeng^{TM}110 and K_2HPO_4/KH_2PO_4 with tie lines at 25°C. The compound which is enriched in the upper phase (Ammoeng^{TM} 110) is plotted on the abscissa and the compound enriched in the lower phase (K_2HPO_4/KH_2PO_4) is plotted on the ordinate. Information about the final concentration of phase components in the top and bottom phases of the IL-based ATPS can be drawn from the tie-lines that connect two nodes on the binodal.

4.3.2 Temperature

The influence of temperature on the location of the binodal curve of the model IL-based ATPS is shown in Figure 4-7. It has been reported, that a decrease in temperature can be used to inhibit phase separation of PEG/salt-based ATPS if their composition is close to the binodal curve (Albertsson 1986; Brooks et al. 1985). In other words, as temperature increases, the solubility of PEG in water decreases and lower concentrations of PEG and salt are needed to obtain phase separation (Albertsson and Tjerneld 1994). However, the IL-based ATPS shows the opposing behaviour. As temperature increases, higher concentrations of IL and inorganic salt are needed to obtain phase separation, while a decrease in temperature results in a shift of the binodal curve towards the origin, thus enabling phase separation at lower concentrations of the phase-forming compounds. This unusual observation was also reported for other IL-based ATPS. Du et al. (2007) found an increase of the area of two-phase coexistence with decreasing temperature (from 60°C to 25°C) for [C_4mim][Cl] / K_2HPO_4 systems (Du et al. 2007). Following the argumentation of the salting-out ability being related to the hydration strength of ions, the reason for this unusual observation could be found in a small decrease of the Gibbs free energy of hydration for the IL ions with decreasing temperature. Hence the solubility of the ions of the IL will decrease and phase separation is facilitated. However, the binodal curves obtained for a system based on [C_4mim][Br] / K_2HPO_4 were not sensitive to an increase of temperature from 25°C to 45°C (Pei et al. 2007). Therefore, all in all it must be concluded that the complicated structure of aqueous solutions and the complex nature of interactions of the IL ions (in particular bulky cations with hydrophobic side chains) will introduce perturbations to the general trends that will always be hard to quantify and warrant further studies on this subject.

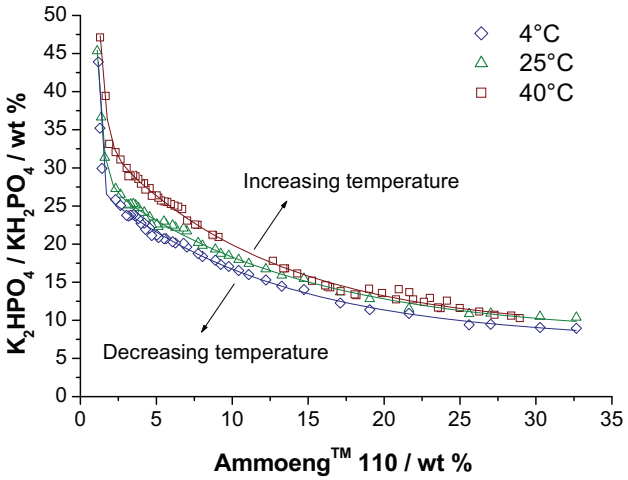


Figure 4-7: Phase diagram of the ionic liquid Ammoeng™ 110 and the inorganic salt K_2HPO_4/KH_2PO_4 in water determined by the cloud point method (Albertsson 1986) at 4°C (rhomb), 25°C (triangle) and 40°C (square) (see also chapter 9.2.2).

4.3.3 pH value

In order to complete the factors influencing the composition of ATPS, the effect of varying pH values has to be investigated. Therefore, the pH of IL-based ATPS was adjusted by variation of the ratio of potassium salts which were applied as phase-forming compounds. The influence of pH on the volume of phases is shown in Figure 4-8. From the constant volumes of phases over the whole pH range it can be concluded that the pH value does not have a great impact on the phase composition of IL-based ATPS. However, it has to be emphasized that the pH plays a decisive role for the partitioning of proteins in ATPS due to its direct influence on protein charge and conformation.

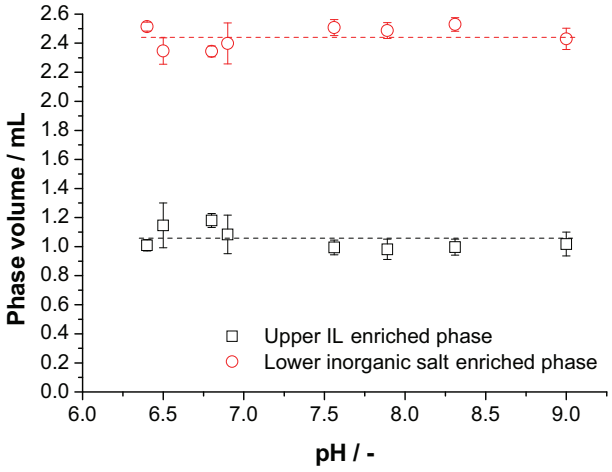


Figure 4-8: Influence of pH on the volume of phases in an IL-based ATPS composed of 12 wt % Ammoeng™ 110 and 20 wt % inorganic salt at 20°C. Auxiliary lines (dashed red and black lines) are added in order to guide the eye.

4.3.4 Time of phase separation

Commonly the use of aqueous two-phase systems involves two unit operations: equilibration and phase separation. Equilibration is easily and rapidly achieved by mixing the components which constitute the phase system and dispersing the phases to obtain equilibrium of phase compositions. This procedure is followed by a separation of the liquid phases. The phase separation of PEG-based ATPS under gravity is not as rapid as in water-organic solvent systems, varying from a few minutes to a few hours because of the rather low differences in the densities of the two phases (about 0.05-0.15 g cm^3) (Hustedt et al. 1985), their high viscosities and the time required by the small droplets, formed during mixing, into larger droplets (Walter et al. 1985). Centrifugation at low speed is commonly used to fasten the process. For the investigated IL-based ATPS phase separation under gravity is completed within a couple of seconds up to a few minutes (Figure 4-9 A and B) thus rendering the application of energy (e. g. by centrifugation) unnecessary. The time of separation is dependent on the amount of phase-forming compounds and can be accelerated by increasing the concentration of IL (Figure 4-9 A) or inorganic salt (Figure 4-9 B).

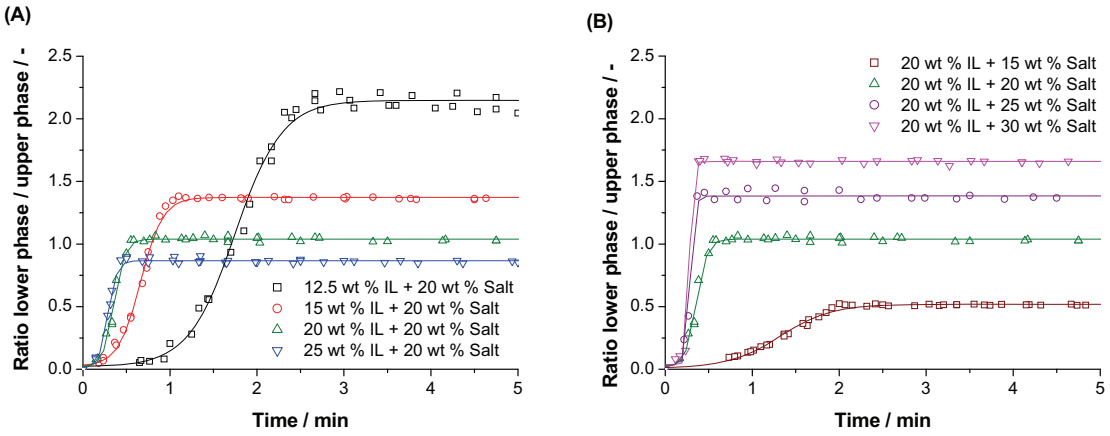


Figure 4-9: Effect of the concentration of phase-forming compound on the phase separation under gravity within IL-based ATPS at 20°C. (A) Phase separation with increasing amount of IL Ammoeng™ 110, (B) Phase separation with increasing amount of salt mixture K_2HPO_4/KH_2PO_4.

In general, phase separation as a function of time can be described as being dependent on several physico-chemical properties of the phases. Among them, the density difference $\Delta\rho$, the viscosity difference $\Delta\eta$ and the interfacial tension σ between the phases play a decisive role (Asenjo et al. 2002). It has been proposed that the Stoke's law can be used to approximately describe phase demixing under gravity, with D representing the droplet diameter, η_c the dynamic viscosity of the continuous phase, g the acceleration due to gravity and V_S the droplet rise/fall velocity (Nagaraj et al. 2002):

$$V_S = \frac{D^2 \Delta\rho g}{18\eta_c}$$

Equation 4-1: Stoke's law for the description of phase demixing under gravity

As can be seen from Table 4-4, an increase in both the concentration of ionic liquid and inorganic salt will result in an increased difference of density and viscosity between the phases, thus explaining the observed acceleration of phase separation time. On a general basis, it was found that the density of the IL-enriched upper phase is lower than the density of the salt-enriched bottom phase, the difference between both phases varying between 0.09 0.28 g cm ^{3}. Hence, in comparison to PEG-based ATPS, the density difference is relatively high. Regarding the viscosity of the IL-enriched upper and the salt-enriched lower phase, it was found that the top phase exhibits a three to nine times higher viscosity than the bottom phase. Regarding a PEG-based ATPS composed of 20 wt % PEG4000 and 10 wt % K_2HPO_4, the kinematic viscosities of the upper and lower phases are around $32 \cdot 10^{6}$ and $3 \cdot 10^{6}$ $m^2 s^{1}$, respectively (Asenjo et al. 2002). In comparison to these systems, the viscosities of IL-based ATPS are relatively low. Based on the assumption that the dense bottom phase is dispersed in the continuous top phase, Stoke's law can be used to explain the fast phase separation of these systems as compared to PEG-based ATPS: due to a bigger density differences between the phases and a smaller dynamic viscosity of the top phase, the droplet rise/fall velocity V_S and hence phase separation is enhanced.

Table 4-4: System parameters of the IL-based ATPS (Ammoeng^{TM} 110 / K_2HPO_4/KH_2PO_4) at varying phase-forming concentrations as measured at 20°C.

IL / wt %	**Inorganic salt / wt %**	**Density ρ upper phase / kg m^{-3}**	**Density ρ lower phase / kg m^{-3}**	**Kinematic viscosity ν upper phase (20°C) / m^2 s^{-1}**	**Kinematic viscosity ν lower phase (20°C) / m^2 s^{-1}**	**Dynamic viscosity η upper phase (20°C) / kg s^{-1} m^{-1}**	**Dynamic viscosity η lower phase (20°C) / kg s^{-1} m^{-1}**
12	20	$1.095 \cdot 10^3$	$1.226 \cdot 10^3$	$6.179 \cdot 10^{-6}$	$2.100 \cdot 10^{-6}$	0.0068	0.0026
12	25	$1.064 \cdot 10^3$	$1.348 \cdot 10^3$	$11.745 \cdot 10^{-6}$	$2.321 \cdot 10^{-6}$	0.0125	0.0031
12	30	$1.069 \cdot 10^3$	$1.285 \cdot 10^3$	$18.858 \cdot 10^{-6}$	$2.833 \cdot 10^{-6}$	0.0202	0.0036
10	20	$1.107 \cdot 10^3$	$1.194 \cdot 10^3$	$5.7467 \cdot 10^{-6}$	$2.321 \cdot 10^{-6}$	0.0064	0.0028
14	20	$1.078 \cdot 10^3$	$1.237 \cdot 10^3$	$8.067 \cdot 10^{-6}$	$2.100 \cdot 10^{-6}$	0.0087	0.0026

4.4 Interims summary

First screening experiments revealed the general ability of three ILs out of the Ammoeng™ series, namely Ammoeng™ 100, Ammoeng™ 101 and Ammoeng™ 110, to form an aqueous two-phase system by using an inorganic potassium salt as second phase-forming compound. To further characterise these systems and to provide a starting point for the subsequent application within protein and enzyme extraction, the binodal curves of these IL-based ATPS were determined and factors influencing the phase formation and composition were investigated. The main findings can be summed up by the following points:

- The generation of an IL-based ATPS is mainly depending on the tendency of **chaotropic** salts to be salted-out by **kosmotropic** salts.
- Generally, the ability for phase formation and thus the location of binodal curves can be explained by a combination of two different effects which are related to the **ionic liquid** cation- and anion-properties: (1) An increase of the functional group's chain length of the **IL cation** results in a more hydrophobic character of the IL and facilitates phase formation, while (2) a more kosmotropic character of the **IL anion** reduces the phase formation ability due to hydration competition phenomena with the phase-forming inorganic potassium salt.
- With regard to the selection of an **inorganic salt** as second phase-forming compound, it can be concluded that strongly kosmotropic ions favour IL-based ATPS formation by salting-out the more chaotropic IL.
- Further investigations revealed that **temperature** plays an important role in the phase formation process as well. As temperature increases, higher concentrations of IL and inorganic salt are needed to obtain phase separation, while a decrease in temperature results in a shift of the binodal curve towards the origin.
- Additional interesting properties of the IL-based ATPS were found by studying some of their physical properties. The **phase separation** of these systems under gravity is completed within a couple of seconds up to a few minutes thus rendering the application of energy unnecessary. The time of separation is dependent on the amount of phase-forming compounds and can be accelerated by increasing the concentration of IL or inorganic salt. An increase in both the concentration of ionic liquid and inorganic salt results in an increased difference of density and viscosity between the phases, thus explaining the observed acceleration of phase separation time.
- Generally, it was found that the **density** of the IL-enriched upper phase is lower than the density of the salt-enriched bottom phase, the difference between both phases varying between 0.09 0.28 g cm ^{3}. Regarding the **viscosity** of the IL-enriched upper and the salt-enriched lower phase, it was found that the top phase exhibits a three to nine times higher viscosity than the bottom phase.

All in all, the general applicability of the investigated ionic liquids for the formation of aqueous two-phase systems has been proven within this chapter. The characterisation of IL-based ATPS has revealed a number of parameters influencing the system formation and composition. Generally, it has to be kept in mind that the type and concentration of phase-forming compounds and the temperature exhibit an effect on IL-based ATPS, while the pH did not significantly change the system properties.

5 Results and discussion part II: Protein distribution in IL-based ATPS[3]

The development of new IL-based ATPS is targeted to the application of these systems within biocompatible downstream processes. Since proteins are usually produced by fermentation in aqueous solutions, downstream processing must take into account the special properties of proteins such as a restricted stability in aqueous media within narrow limits of parameters like temperature, pH and ionic strength. Therefore, extraction by an organic solvent is rarely an appropriate process for concentration and purification of proteins. However, extraction using aqueous two-phase systems allows adjusting the process conditions in such a way that product loss through protein denaturation can be minimized.

Generally, several factors like type and concentration of the phase-forming compounds, pH and temperature have been reported to influence the nature of and partitioning within PEG-based aqueous two-phase systems (Albertsson 1986; Sasakawa and Walter 1972). However, the driving forces for the unequal distribution of proteins and enzymes between the phases are not yet understood. Studies on the partitioning of model proteins in PEG-based ATPS have revealed a number of properties which influence the partitioning behaviour, such as protein hydrophobicity, net charge and molecular weight (Andrews and Asenjo 1996; Asenjo et al. 1994a; Franco et al. 1996a; Franco et al. 1996b; Hachem et al. 1996; Schmidt et al. 1996). Therefore, the potential driving forces of protein partitioning were investigated by means of the distribution of four model proteins at varying system characteristics of IL-based ATPS. The four model proteins are structurally determined with an available structure file on the RCSB protein data bank (http://www.rcsb.org/) and were selected in order to cover a range of physico-chemical properties (Table 5-1).

Table 5-1: Proteins applied for studying the driving forces of protein partitioning in IL-based ATPS.

Protein	Source	EC	PDB Code	Isoelectric point (IEP)	Molecular weight / kDa
Myoglobin	Equine heart		1ymb	6.8-7.2	16.983[c]
Trypsin	Bovine pancreas	3.4.21.4	2g55	10.1-10.5	23.8[d]
Lysozyme	Hen egg white	3.2.1.17	1dpx	11.35[a]	14.3[e]
BSA	Bovine serum		1ao6	4.7-4.9[b]	66.4[f]

[a](Wetter and Deutsch 1951), [b](Malamud and Drysdale 1978; Righetti and Caravaggio 1976), [c](Andrews and Asenjo 1996), [d](Sigma Aldrich), [e](Canfield 1963), [f](Hirayama et al. 1990)

According to Albertsson, protein properties determining the partitioning can formally be represented by several factors contributing to the partition coefficient *K* (Albertsson 1986). In logarithmic terms, the contribution of these factors to log *K* can be summed up by Equation

[3] Parts of this chapter have been submitted for publication in *Biochemical Engineering Journal*: Dreyer S, Salim P, Kragl U. (**2008**). Driving forces of protein partitioning in an ionic liquid-based aqueous two-phase system.

5-1, where *el*, *hphob*, *size* and *biosp* stand for electrochemical, hydrophobic, size and biospecific contributions to the partition coefficient, and K^0 includes other factors.

$$log\ K \quad log\ K_0 + log\ K_{el} + log\ K_{hphob} + log\ K_{size} + log\ K_{biosep}$$

Equation 5-1: Factors contributing to the calculation of logarithm of partition coefficient *K*.

To gain an insight into the driving forces of protein partitioning in IL-based ATPS, several factors which are known to have an effect on protein properties (such as temperature and pH) were varied for the purpose of relating the observed tendencies with features of the proteins. In order to exclude the influence of varying phase compositions on protein partitioning, the system composition was fixed to 12 wt % Ammoeng^{TM} 110 and 20 wt % potassium salt (K_2HPO_4/KH_2PO_4).

5.1 Partitioning of model proteins in PEG-based ATPS

In order to point out the special features of IL-based ATPS and for better comparison of results, partitioning experiments of the four model proteins within a conventional ATPS composed of 12 wt % PEG6000 and 20 wt % K_2HPO_4/KH_2PO_4 in aqueous solution were performed. The system composition was selected in order to provide comparable concentrations and conditions as for the IL-based ATPS investigated in the following chapters. As can be seen from Figure 5-1, all four model proteins preferably partition to the salt-rich lower phase. Hence, only faint protein bands are visible on the gel for the upper phases of PEG-based ATPS containing trypsin and lysozyme (line 6 and 8) while no protein could be detected for myoglobin and BSA (line 3 and 10). Partition coefficients (log *K*) were -1.82 for myoglobin, 0.14 for trypsin, -0.75 for lysozyme and 1.20 for BSA, respectively.

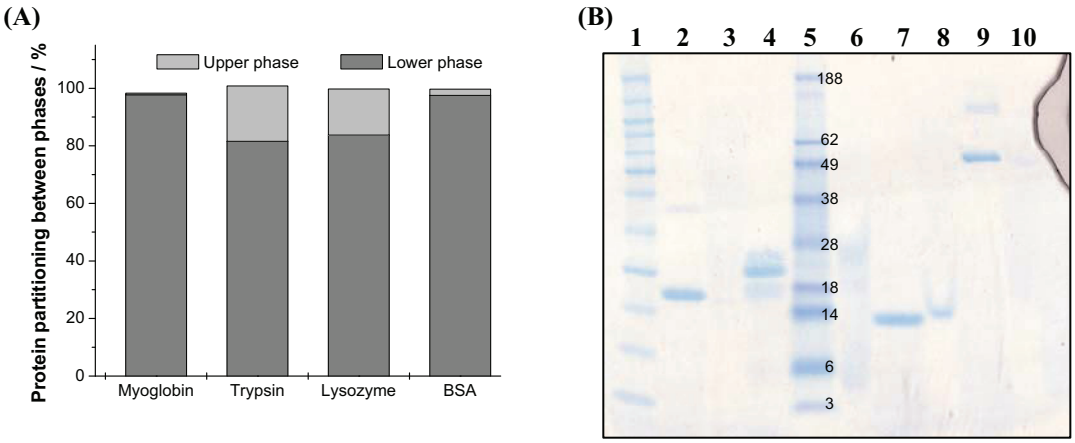


Figure 5-1: (A) Partitioning of the model proteins within a PEG6000/potassium salt ATPS composed of 12 wt % PEG6000 and 20 wt % K_2HPO_4/KH_2PO_4, (B) SDS PAGE of samples taken from the protein solutions and upper phases of PEG6000/salt ATPS. Line 1: protein standard unstained, 2: myoglobin (1.6 mg mL^{-1} in *A.dest*), 3: myoglobin upper phase, 4: trypsin (3 mg mL^{-1} in *A. dest*), 5: protein standard stained, 6: trypsin upper phase, 7: lysozyme (2 mg mL^{-1} in *A.dest*), 8: lysozyme upper phase, 9: BSA (1.4 mg mL^{-1} in *A. dest*), 10: BSA upper phase.

It has to be emphasized though, that the preferential enrichment of proteins within the salt-rich lower phase applies only for the investigated system and represents no general observation in PEG-based ATPS. A variation of the system composition (e. g. reduction of the molecular weight of PEG) could be used to direct the protein partitioning towards the PEG-rich upper phase (see also chapter 3.2.3).

5.2 Effect of temperature

In general, temperature effects on protein partitioning in ATPS can be of varying nature. Temperature can alter the shape of the binodal curve, lead to changes in protein structure or even result in protein denaturation (Baskir et al. 1989). Therefore, the effect of temperature on protein partitioning in IL-based ATPS was investigated on two levels. First, the protein solutions themselves were incubated at 4°C and 70°C for one hour and were afterwards partitioned in an IL-based ATPS at 4°C. Usually, the conformation of proteins is stable at low temperatures (4°C). An increase of temperature can result in a conformational change thereby rendering the surface properties of proteins. Denatured proteins generally partition differently in ATPS than their native state due to a significantly greater and more hydrophobic exposed surface area (Albertsson 1986). However, as pointed out in Figure 5-2, no significant difference of partitioning between the thermally treated proteins and the ones stored at 4°C could be observed as indicated by an unchanged extraction efficiency of the protein to the IL-enriched upper phase. Even though BSA started to precipitate at 65°C, no significant difference in extraction efficiency between the native and the denatured state was found. Nevertheless, a comparison of the four model proteins with each other reveals a significant variation of extraction efficiencies. While myoglobin partitions more or less equally between the phases, trypsin, lysozyme and BSA clearly get enriched in the upper IL-containing phase with extraction efficiencies between 83 100 %. This behaviour is also interesting if compared to the partitioning within PEG-based ATPS. For these systems a preferential enrichment of the proteins within the salt-rich lower phase was observed (see chapter 5.1).

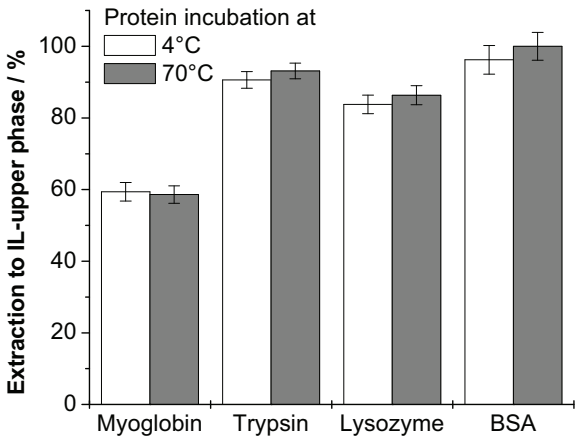


Figure 5-2: Partitioning of model proteins in an IL-based ATPS at 4°C and pH 7 after thermal treatment of the protein solutions at 4°C and 70°C for 1h. The diagram illustrates the extraction efficiency of protein to the upper IL-enriched phase.

As a second level, the effect of temperature during the phase separation process was investigated. For this purpose the model proteins were added to the IL-based ATPS, the phases were dispersed and phase formation was induced by storing the systems at selected temperatures between 4°C up to 70°C (Figure 5-3). The partition coefficients of the model proteins display a very diverse behaviour. While the extraction of trypsin and lysozyme decreases strongly at increasing temperature, the partitioning of myoglobin shows only a slightly decreasing slope up to 70°C. Nevertheless, a general trend of decreasing log *K* values with increasing temperature could be observed for all proteins except BSA, which is completely extracted to the upper phase at all temperatures. Interestingly, Du et al. also reported about a virtually unchanged extraction efficiency of more than 90 % for BSA in a [C_4mim][Cl] / K_2HPO_4 system at temperatures below 60°C (Du et al. 2007). At 70°C though, they found a significant drop of BSA extraction efficiency to about 20 % due to thermal protein denaturation. With regard to the Ammoeng^{TM} 110 / salt system, no denaturation of BSA could be observed and extraction efficiency remained high even at 70°C. Although BSA in aqueous solution is known to undergo two structural changes during heating, for the IL-based ATPS neither at 65°C (first, reversible stage of structural changes of BSA) nor at 70°C (second stage of irreversible denaturation) thermal denaturation could be observed (Kuznetsov et al. 1975; Laurie and Oakes 1976; Wetzel et al. 1980). These results indicate a stabilising effect of the ionic liquid on the protein BSA by preventing denaturation.

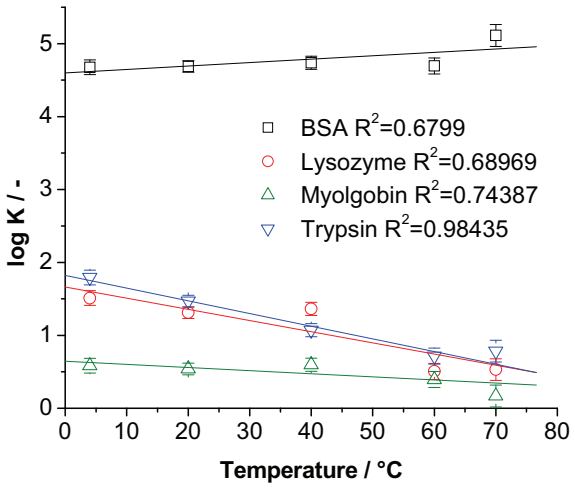


Figure 5-3: Effect of temperature on the log *K* of the model proteins within IL-based ATPS. Phase separation was carried out at different temperatures in an IL-based ATPS consisting of 12 wt % Ammoeng^{TM} 110 and 20 wt % K_2HPO_4/KH_2PO_4 at pH 7.

The partitioning of proteins within IL-based ATPS can also be described thermodynamically as the transfer from the salt-rich lower to the IL-rich upper phase. At a given temperature, the free energy change of the protein extraction process is related to the partition coefficient *K* by Equation 5-2:

$$\Delta G_T^0 \quad -RT \ln K$$

Equation 5-2: Calculation of free energy change (Gibbs free energy).

Moreover, the enthalpy and entropy change associated with protein partitioning can be calculated from the slope and intercept of a ln K vs. T^{1} plot by applying Equation 5-3.

$$\ln K \quad -\Delta H_T^0 R^{-1} T^1 + \Delta S_T^0 R^1$$

Equation 5-3: Calculation of enthalpy and entropy change.

The values for ΔG_T^0, ΔH_T^0 and $T\Delta S_T^0$ as obtained by linear least-square analysis for partitioning of the four model proteins within IL-based ATPS are summarised in Table 5-2 and Figure 5-4.

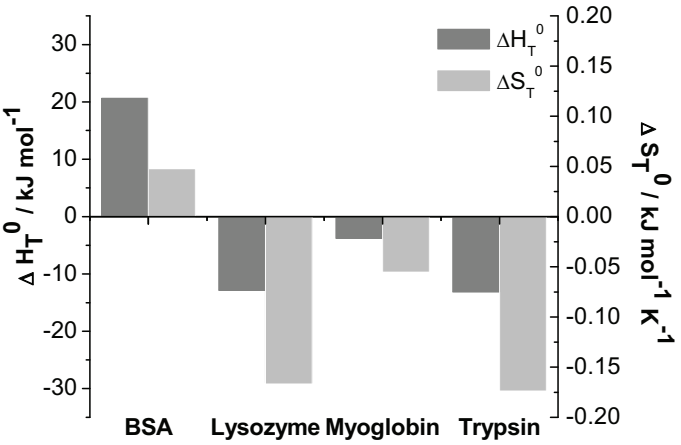


Figure 5-4: Entropy and enthalpy change for the extraction of the four model proteins within IL-based ATPS at pH 7.

All resultant ΔG_T^0 values are negative, indicating that the extraction of all model proteins is spontaneous. Since a linear correlation was found between ln K and T^{1} within the temperature range of 4-70°C, the enthalpy change ΔH_T^0 is constant over the investigated temperature range. However, significant differences become evident from the changes of entropy and enthalpy. While the entropy and enthalpy change for the extraction of BSA is characterised by positive values, and therefore endothermic and entropically driven, partitioning of the other three model proteins is marked by negative values for ΔH_T^0 and $T\Delta S_T^0$ with the enthalpy change being greater in value than the entropy change. Thus it can be concluded that partitioning of these proteins involves an exothermic, spontaneous process. However, since no concordant trend between temperature and partition coefficients could be observed, further driving forces must be involved in the partitioning of the four model proteins. Bearing in mind the effect of temperature on the location of the binodal curve of IL-based ATPS and the related change of system composition (chapter 4.3.2), a direct interaction between the phase-forming components and the proteins has to be considered.

Table 5-2: The transfer thermodynamic properties for the model proteins from the inorganic salt-rich phase to the IL-rich phase in ATPS at pH 7 and 4°C.

Protein	Gibbs energy change ΔG_T^O / kJ mol^{-1}	Entropy change $T\Delta S_T^O$/ kJ mol^{-1}	Enthalpy change ΔH_T^O / kJ mol^{-1}
Myoglobin	-3.1	-6.1	-9.5
Trypsin	-9.5	-20.8	-30.2
Lysozyme	-8.0	-20.4	-29.0
BSA	-24.8	32.7	8.2

5.3 Effect of 'simplified' protein properties

Since the partitioning experiments described above revealed varying protein extraction efficiencies which cannot be explained by the system properties alone, partitioning must be related to the proteins' characteristics. Olivera-Nappa et al. found a good correlation of the partition coefficient of several proteins within PEG/phosphate-ATPS by including a molecular weight and sphericity factor into their model (Olivera-Nappa et al. 2004). The sphericity factor F is defined by Equation 5-4, where S is the surface area and V is the volume of the protein molecule. The sphericity factor has a minimum value of 1, if the molecule is a perfect sphere and increases if the surface-volume ratio increases.

$$F = \frac{S^3}{36\pi V^2}$$

Equation 5-4: Calculation of the sphericity factor of proteins.

In order to determine how well protein partitioning in IL-based ATPS could be correlated to simplified representations of protein properties, Figure 5-5 shows the partition coefficients plotted against the proteins' molecular weight, sphericity factor, volume and surface area. For PEG-based ATPS it has been reported that small molecules usually partition evenly between the phases while larger proteins tend to partition more unevenly and high molecular weight compounds such as DNA and viruses partition almost entirely into one phase (Albertsson 1986; Sasakawa and Walter 1972). An increase of protein size or of polymer chain length usually decreases the partition coefficient (enrichment of protein in the lower salt phase) due to excluded volume effects (Hachem et al. 1996). Surprisingly, Figure 5-5 (A-D) demonstrates the opposing behaviour within the IL-based ATPS. The partition coefficients tend to increase with increasing values of the four simplified protein descriptors, the correlation coefficients (R^2) between each protein property and the partition coefficient ranging between 0.95 and 0.98. Due to the fact that all investigated representations of the model proteins' properties are closely related to the proteins' size, it can be stated that an increase of this parameter clearly enhances the extraction of proteins to the IL-enriched upper phase. Since excluded volume effects are generally attributed to the increasing volume of

PEG molecules which are excluded from the surface of larger proteins and therefore 'push' the protein to the lower phase, it is assumed that the ethylene glycol-chain length (5 - 15) within the IL cation in the upper phase does not exhibit any protein exclusion effects. Contrariwise, the results indicate an interaction of the ionic liquid with the proteins which increases with the proteins' molecular weight and is in accordance with an enhanced total accessible surface area. Additionally, a direct interaction of ionic liquid and protein could also explain the increased thermostability of BSA (see chapter 5.2). If the IL-molecule interacts more strongly with the native than the denatured protein state, the protein conformation is stabilised. On the other hand, an alternative explanation relates the high concentrations of IL within the upper phase (approximately 20 wt %) to the increased thermostability of BSA. Since proteins exist in two thermodynamic states which are at equilibrium, the native and the denatured state, the high concentration of IL might cause a densely packed solution which favours the more compact native protein state over the denatured, unfolded state.

However, although a general correlation between the ‚simplified' protein properties and the partition coefficients was found, these parameters alone cannot be used to sufficiently explain the partitioning behaviour of proteins in IL-based ATPS. In particular with regard to the extraction of myoglobin (which represents an 'outliner' within Figure 5-5), further driving forces are suggested to be involved in the extraction process.

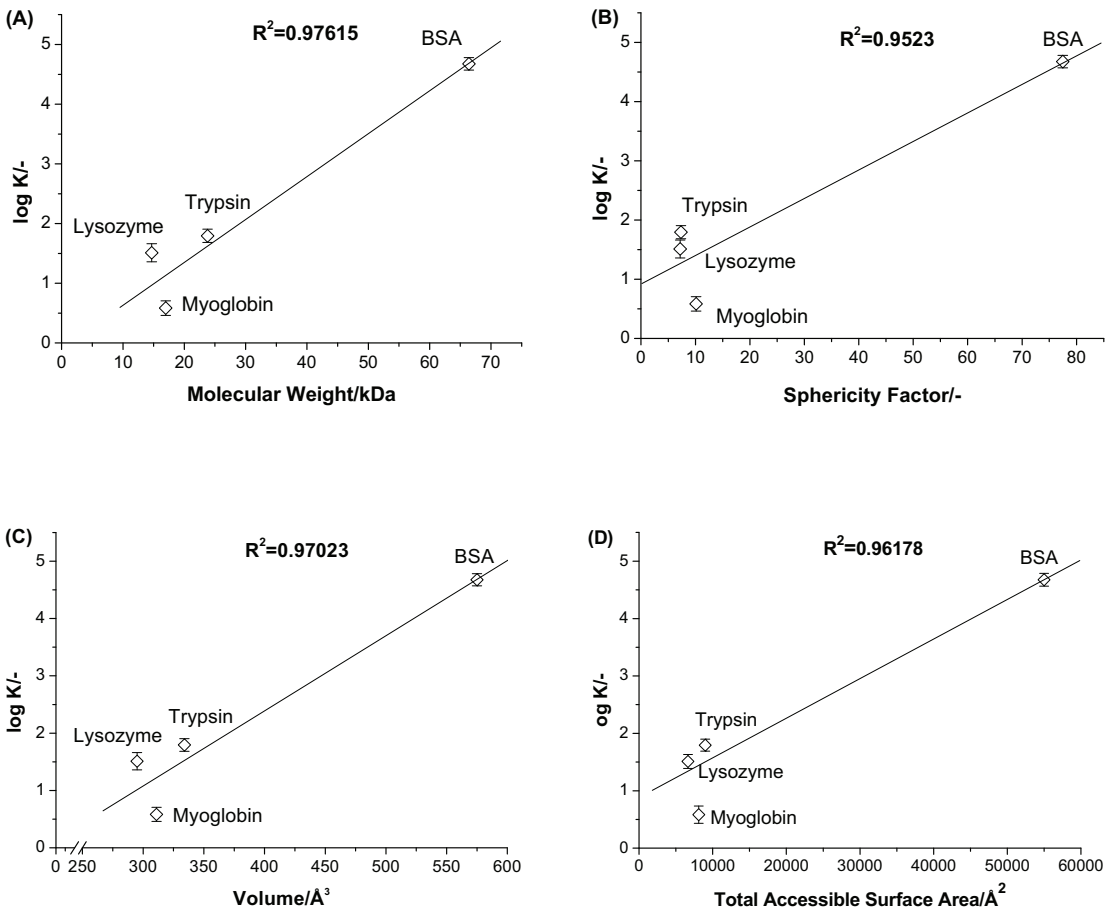


Figure 5-5: The logarithm of partition coefficients of the model proteins within IL-based ATPS at 4°C and pH 7 plotted against simplified representations of protein properties: (A) molecular weight, (B) sphericity factor, (C) volume and (D) accessible surface area (ASA).

5.4 Effect of hydrophobicity

During partitioning the exposed groups of proteins come into contact with the phase components and therefore determine the partitioning behaviour. This surface-dependent phenomenon is very complex since a protein can interact with the surrounding molecules within a phase via hydrogen bonding, charge interactions, van der Waals forces, hydrophobic interactions and steric effects. The net effect of these interactions is likely to be different in the two phases and the protein will partition preferentially into one phase (Franco et al. 1996b).

Studies of separation mechanisms in PEG-based ATPS revealed hydrophobic forces to be of major importance because hydrophobic interaction between the proteins and water can provide net factorable free energy for the formation of the native structure in an aqueous solution (Chu and Chen 2000). Surface exposed amino acid residues of a protein determine the protein surface properties and thus the partition behaviour. In order to visualize the protein surfaces and to calculate the solvent accessible surface areas of the 20 different amino acid residues in the model proteins, the computer program GETAREA 1.1 was used. To facilitate a comparison between the different proteins, Table 5-3 summarises the relative amino acid surface areas of each protein by classifying three different groups: (1) charged amino acid residues, (2) aromatic residues and (3) long aliphatic residues with intermediate character. The relative surface areas occupied by charged and aromatic residues were found to vary considerably between the investigated proteins. Myoglobin and BSA had the highest relative surface exposure of charged residues and a low exposure of aromatic residues, while lysozyme and trypsin were found to have a low exposure of charged residues in combination with a higher exposure of aromatic residues. However, these results could not be used to explain the observed partition coefficients of the model proteins that follow the order BSA > trypsin > lysozyme > myoglobin.

Table 5-3: The exposed relative surface areas ($r = \Sigma r_i$) for charged, aromatic and intermediate amino acid residues as calculated by GETAREA 1.1. Please note that the sum of all listed residues does not equal 1 since only selected amino acids were taken into account.

Protein	***r* for charged residues (Arg, Asp, Glu, His, Lys)**	***r* for aromatic residues (Phe, Trp, Tyr)**	***r* for intermediate residues (Asn, Gln, Ile, Leu, Val)**
Myoglobin	0.549	0.023	0.156
Trypsin	0.398	0.067	0.260
Lysozyme	0.204	0.093	0.258
BSA	0.570	0.032	0.152

Subsequently, six different hydrophobicity scales were applied for the calculation of hydrophobicity. These scales can roughly be devided into three categories. (1) Two of the scales are based on the partitioning between organic and water phases (octanol → water, cyclohexane → water) (Radzicka and Wolfenden 1988), (2) two hydrophobicity scales are based on the amino acid residue distribution between the surface and interior of a number of monomeric proteins (surface → interior, hydropathy) (Janin 1979; Kyte and Doolittle 1982)

and (3) two scales are based on the partitioning of amino acid-peptides in an ATPS composed of 7.1 % dextran and 6.8 % EO30PO70 and 9 % dextran and 9 % EO30PO70 (System 1 and 2), respectively (Berggren et al. 2002). The applied EO-PO-systems were composed of a copolymer of 30 % ethylene oxide and 70 % propylene oxide (EO30PO70) and their properties have been reported to be similar to those of PEG/dextran systems, although a larger hydrophobicity difference between the phases has been found.

Additionally, the surface hydrophobicity of the model proteins was experimentally determined using the procedure described by Hachem et al. who introduced the parameter 1/m* as a good measure of protein hydrophobicity with regard to predicting protein partitioning in PEG-based ATPS (Figure 5-6). Using ammonium sulphate as precipitating agent, a small increase in the concentration of salt transfers the protein from being relatively soluble to being largely precipitated. This discontinuity point m* is reported to be a good reference for measuring the solubility and thus hydrophilicity of a protein (Hachem et al. 1996; Przybycien and Bailey 1989). Since the point of discontinuity m* represents an indicator of hydrophilicity, Hachem et al. suggested to use its inverse - the parameter 1/m* - as a measure of protein hydrophobicity in solution. They found very good linear correlations between 1/m* and the partition coefficient of different model proteins in PEG/PO_4 and PEG/dextran systems (Hachem et al. 1996). With regard to the four model proteins investigated in this thesis, precipitation of proteins occurs at relatively high salt concentrations above 2.4 M (Figure 5-6). These results are in good agreement with previously reported solubility curves (Hachem et al. 1996).

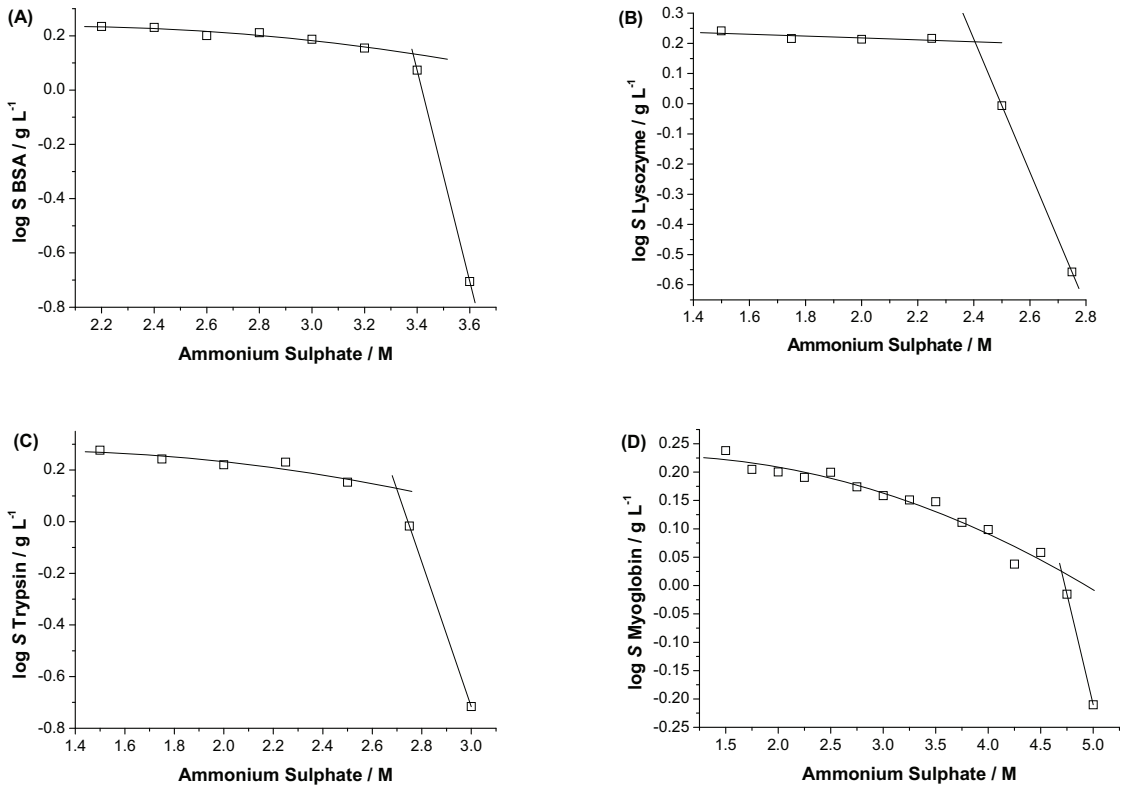


Figure 5-6: Determination of protein solubility at 25°C by ammonium sulphate precipitation. The logarithm of protein solubility is plotted against ammonium sulphate concentration for (A) BSA, (B) lysozyme, (C) trypsin and (D) myoglobin. The point of discontinuity m* is defined as the point of intersection of the two curves (Hachem et al. 1996).

The calculated and experimentally determined hydrophobicity values of the four model proteins used within this work are summarised in Table 5-4. Generally, the correlation between the hydrophobicity values and the protein partition coefficient log *K* within IL-based ATPS is very poor, as can be seen from the correlation coefficient R^2 in Figure 5-7. Applying the four commonly used hydrophobicity scales (H_1 H_4), correlation coefficients between 0.14 and 0.37 were found. The hydrophobicity scales obtained from peptide slopes in ATPS describe the partitioning with correlation coefficients of around 0.1. This result is in contrast to reports from Berggren et al. who found high correlations of 0.961 and 0.949 for the partitioning of eight monomeric model proteins in EO30PO70 systems using these hydrophobicity scales (Berggren et al. 2002). Moreover, also the experimentally determined hydrophobicity values represented by 1/m* gave very poor correlations with the partition coefficient (R^2 0.15).

Table 5-4: Hydrophobicity values of the four model proteins as calculated by the different hydrophobicity scales. [a] H_1 = Surface → Interior (Janin 1979), [b] H_2 = Octanol → Water (Radzicka and Wolfenden 1988), [c] H_3 = Hydropathy (Kyte and Doolittle 1982; Radzicka and Wolfenden 1988), [d] H_4 = Cyclohexane → Water (Radzicka and Wolfenden 1988), [e] H_5 = Peptide Slope System 1 (Berggren et al. 2002), [f] H_6 = Peptide Slope System 2 (Berggren et al. 2002), [g] H_7 = 1/m* (Hachem et al. 1996).

Protein	**H_1[a]**	**H_2[b]**	**H_3[c]**	**H_4[d]**	**H_5[e]**	**H_6[f]**	**H_7[g]**
Myoglobin	-0.57	0.16	-1.91	-3.50	0.004	0.011	0.213
Trypsin	-0.53	-0.03	-2.02	-5.38	0.020	0.038	0.416
Lysozyme	-0.33	0.35	-1.20	-2.39	0.033	0.048	0.371
BSA	-0.61	0.03	-1.96	-4.09	0.008	0.017	0.295

All in all, the results obtained for IL-based ATPS clearly demonstrate that the partitioning of model proteins within IL-based ATPS can neither be described by differences in the hydrophobicity of surface exposed amino acids nor by the experimentally determined parameter 1/m*. Therefore, as opposed to reports of the main driving forces within PEG-based ATPS, it is concluded that hydrophobic interaction does not represent a main driving force of protein partitioning in IL-based ATPS. This observation is further supported by the observation that BSA partitioning remains unchanged even if the protein is denatured and therefore reveals a more hydrophobic surface (chapter 5.2). Since hydrophobic interaction within PEG-based ATPS is commonly traced back to differences in hydrophobicity between the more hydrophobic PEG-enriched upper phase and the less hydrophobic salt-enriched lower phase, we assume that in IL-based ATPS the discrepancy of hydrophobic properties between the two phases is not big enough to exhibit any hydrophobic interaction-related effects on the partition coefficient.

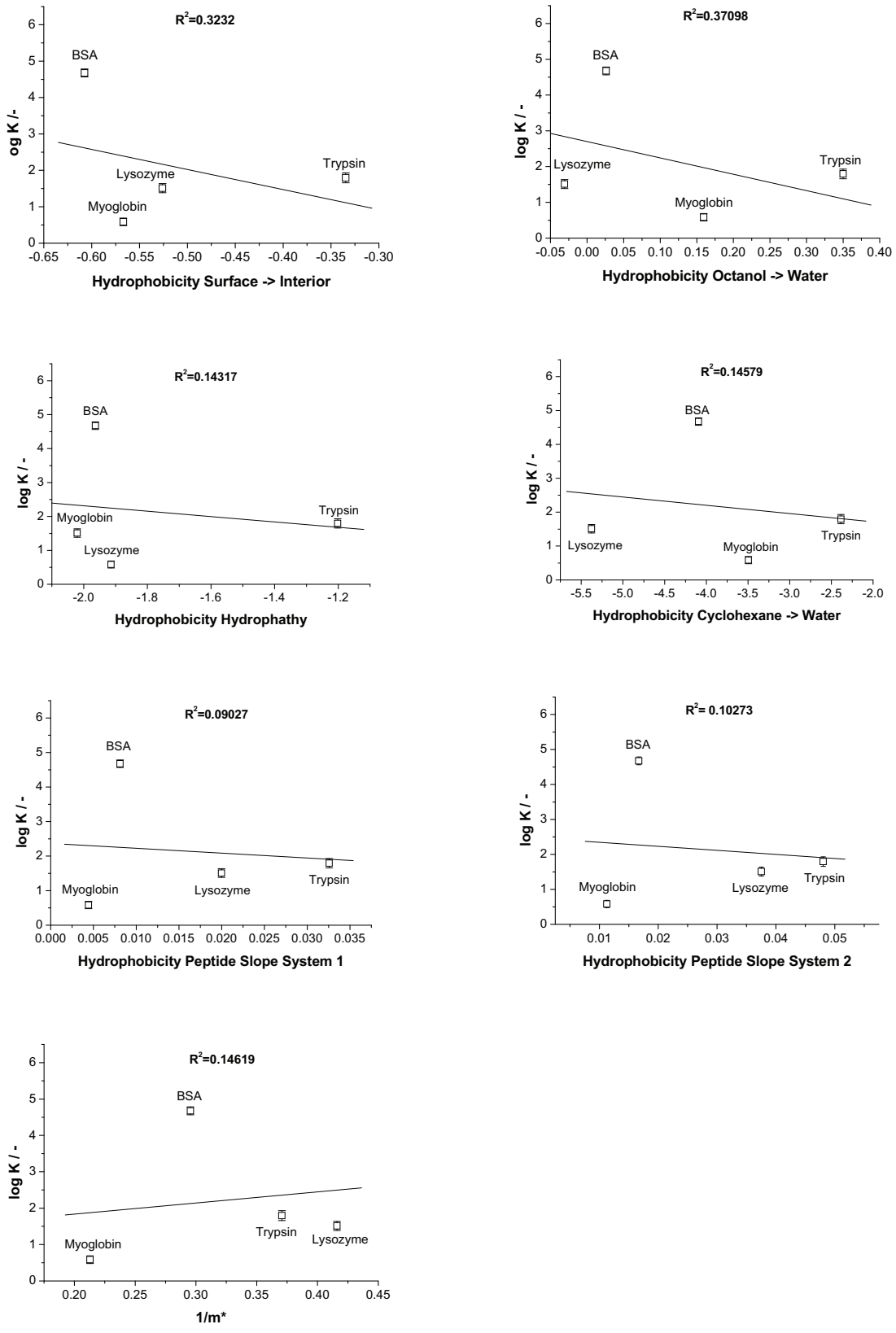


Figure 5-7: Logarithm of partition coefficients of model proteins in IL-based ATPS as plotted against the calculated and experimentally determined hydrophobicity values.

5.5 Effect of pH and charge

Besides hydrophobicity, the different types of amino acids on a protein surface also determine the protein's charge. Therefore, the effect of electrostatic interaction in IL-based ATPS was investigated by changing the pH value of the IL-based ATPS. Since the isoelectric points of the four model proteins are 4.9 for BSA, 7.0 for myoglobin, 10.3 for trypsin and 11.4 for lysozyme (Table 5-1), the net charge of proteins within the IL-based ATPS varies in dependence of the system pH. The total charge of proteins at a given pH of the system was calculated by the computer program H++ and results are summarised in Table 5-5. Naturally, the proteins' net charge becomes more negative with an increase of pH, but due to the different isoelectric points, the total charges vary strongly between the proteins. BSA is negatively charged at all investigated pH values, while lysozyme and trypsin always exhibit a positive charge. However, for myoglobin the total charge drops to negative values at pH 9.

Table 5-5: Total charge of model proteins at different pH values as calculated by the computer programm H++.

Protein	**Total Charge at pH**					
	6.4	**6.9**	**7.56**	**7.89**	**8.31**	**9**
Myoglobin	4.0	1.0	0	0	0	-1.0
Trypsin	9.0	9.0	7.0	7.0	6.0	6.0
Lysozyme	9.0	9.0	8.0	8.0	8.0	8.0
BSA	-6.0	-11.0	-25.0	-28.0	-29.0	-38.1

Figure 5-8 A demonstrates the effect of system pH on the partition coefficient of the four model proteins. Obviously, an increase in system pH 'drives' the proteins to the IL-enriched upper phase as indicated by a strong increase of partition coefficients. This observation is true for all proteins except BSA which shows nearly 100 % extraction efficiency already at pH 6.4. Plotting the total protein charge against the log *K* value of model proteins, Figure 5-8 B illustrates an increasing partition coefficient with decreasing total protein charge of myoglobin, lysozyme and trypsin. However, the net value of charge alone does not allow a prediction of partition behaviour within IL-based ATPS since for instance trypsin is enriched in the upper phase to nearly 100 % at a charge of +6 while myoglobin at a charge of +4 is only extracted to about 60 %.

Obviously, although no direct correlation between the total protein charge and the partition coefficient log *K* can be found, negatively charged characteristics of the proteins contribute to an enrichment in the IL-containing upper phase. This observation could be explained by electrostatic interactions between the exposed groups of the proteins and the phase-forming compounds. Investigations of ATPS based on polyethyleneglycol and potassium salts have revealed a similar behaviour: the partition coefficient of a protein increased as it became more negatively charged (higher pH) (Asenjo et al. 1994a).

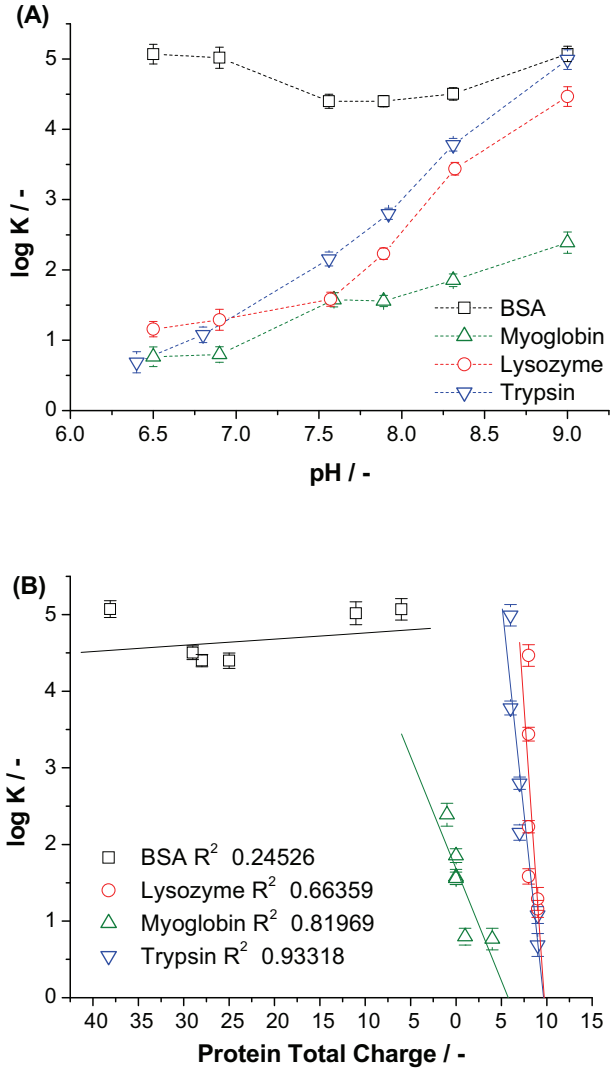


Figure 5-8: (A) Protein partition coefficient at different pH values in an IL-based ATPS consisting of 12 wt % Ammoeng^{TM} 110 and 20 wt % inorganic salt at 4°C. Dashed lines are added to guide the eye and do not represent experimental results. (B) Correlation between protein total charge and partition coefficient log *K*. A log *K* value above 1 represents the extraction of more than 80 % of protein to the upper phase of IL-based ATPS.

Cammarata et al. have reported that ILs in aqueous solutions tend to dissociate into free ions due to a disruption of hydrogen bonds between the cation and anion (Cammarata et al. 2001). However, this general statement has been revised during the last years and led to a more detailed understanding of the solution process of ILs in water. Recently, Zhang et al. have proposed that with increasing water content in [C_2mim][BF_4]/water mixtures, the three-dimensional network structure of pure ILs is first destroyed gradually into ionic clusters and then the clusters are further dissociated into water-surrounded ionic pairs (Zhang et al. 2008). On the other hand, thermodynamic studies of imidazolium-based IL/water-systems have revealed that below the mole fraction of IL (x_{IL} 0.013-0.015), the [C_4mim] cation and its counterion seem to be completely dissociated (Katayanagi et al. 2004; Miki et al. 2005). Unfortunately, studies concerning the solvation process of ionic liquids from the Ammoeng^{TM} series are not available at present. Still, due to the high amount of water present within the IL-based ATPS, a complete dissociation of ions seems to be likely.

Following this train of thought, the enrichment of proteins at higher pH values could depend on electrostatic interactions with the ionic liquid. In this context, two different mechanisms regarding the interaction of proteins and the phase-forming compounds of the IL-based ATPS are proposed: (1) the negatively charged residues of proteins interact with the positively charged ammonium cation and (2) the positively charged amino acids on a proteins' surface interact with the inorganic salts' anions $H_2PO_4^-$ and HPO_4^{2-} which in turn again allow the interaction with the positively charged IL-cation (Figure 5-9).

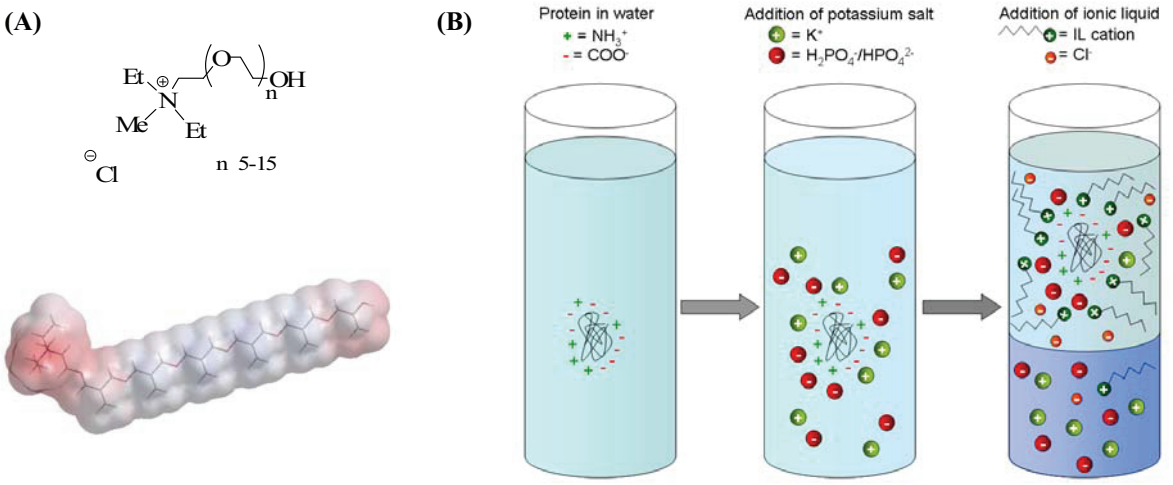


Figure 5-9: Schematic illustration of electrostatic interaction between a protein and cations and anions of the phase-forming compounds within IL-based ATPS. (A) Rough illustration of the charge distribution within the IL-cation as obtained by ChemDraw (red colour represents positive charge, blue colour negative charge), (B) schematic illustration of the partitioning of proteins within IL-based ATPS as driven by electrostatic interaction.

The proposed mechanisms can also be used to explain the observed differences in the partition coefficients in relation to the proteins' total charge. Since BSA is negatively charged at all pH values, it is completely extracted to the upper phase due to the direct electrostatic interaction with the Ammoeng™-cation. With regard to the positively charged proteins lysozyme and trypsin it is proposed that positively charged amino acids on the proteins' surface will interact with $H_2PO_4^-$ and HPO_4^{2-} anions of the inorganic salt. With increasing pH, a higher number of HPO_4^{2-} anions (and lower number of $H_2PO_4^-$ anions) will interact with the protein surface, thereby compensating the positive surface charge and allowing stronger electrostatic interaction with the IL-cation resulting in a higher partition coefficient. Myoglobin possesses only very small charges in comparison to the other three model proteins (Figure 5-10 and Table 5-5). Therefore, its interaction with the IL cations and HPO_4^{2-} anions is weaker and the partition coefficients remain lower. Please note that the colouring of charges within Figure 5-9 and Figure 5-10 are inverted meaning that the red areas within the ionic liquid cation (positive charges) will interact with the red area within the proteins' surface (negative charge).

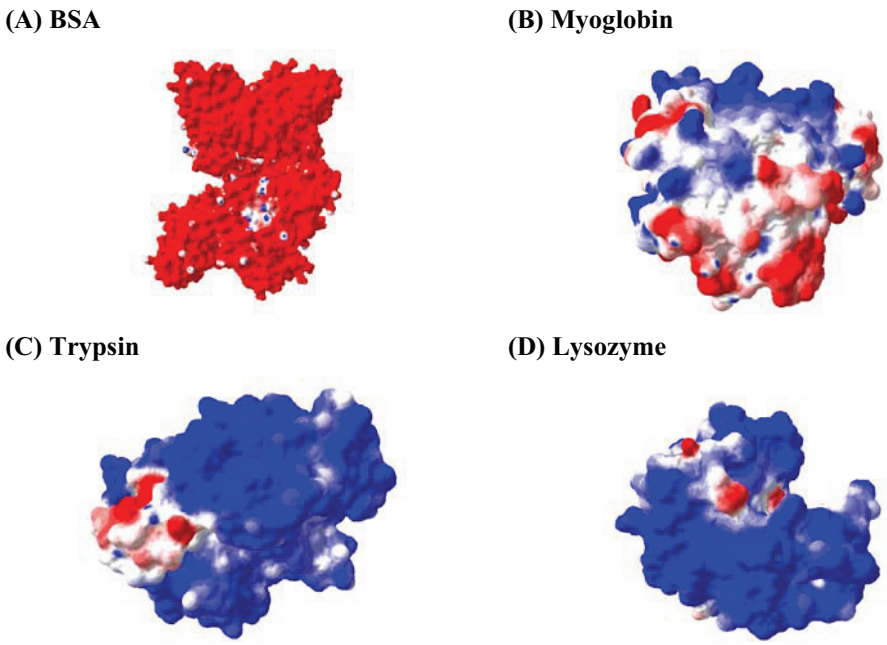


Figure 5-10: Surface charge of the investigated proteins as obtained by CLC Protein Workbench (http://www.clcbio.com). Blue areas in a protein surface represent positive charges; red areas illustrate negative charges at pH 7.

To take a step further, the proposed mechanism can also be used to reconcile the observed correlation between increased protein molecular weight and higher partition coefficients (chapter 5.3). An increase in protein size will naturally provide a higher contact area between the protein and the phase-forming compounds, thereby allowing a stronger interaction and better extraction efficiencies.

The assumption of electrostatic interaction between the proteins and the IL-cation is further supported by results obtained from gel electrophoresis. Figure 5-11 shows an SDS PAGE of the four model proteins in aqueous solution and in the upper phase of IL-based ATPS. Obviously, the presence of ionic liquid within the samples has an effect on the run of proteins within the gel. For all model proteins in aqueous solution a sharp band can be observed (line 2, 4, 7 and 9). However, samples from the upper phase of an IL-based ATPS show a deceleration of the protein run as well as an enlargement of the bands themselves (line 3, 5, 8, 10). While the band shift for the largest protein BSA is only marginal, lysozyme and myoglobin show a strong retention in their running behaviour as well as a significant broadening of the band itself. Taking into account the possible electrostatic interaction of the IL-cation with the charged protein surfaces, the observed band shifts could be explained by an increase of protein size due to the attachment of IL-cations to the protein. Depending on the number of cations as well as their ethylene glycol-chain, the protein size will increase considerably. The SDS PAGE shows a shift of the trypsin band from 23 kDa to 26-30 kDa, for lysozyme from 13 kDa to 18-30 kDa, for myoglobin from 17 kDa to 18-28 kDa and for BSA from around 52 kDa to 60 kDa. (In this context it has to be emphasized that the molecular weight of proteins in the gel is not in accordance with the proteins' molecular weight described above due to non-reducing conditions during electrophoresis.) The molecular weight of Ammoeng^{TM} 110 varies in dependence of the ethylene glycol-chain length between 0.5 1.2 kDa per cation, indicating that the increased protein retention and

blurriness of bands could be explained by the attachment of 2 up to 30 cations to the proteins. Nevertheless, it has to be pointed out that the enhanced retention and the band broadening might also be due to an interference of the ionic liquid with the SDS PAGE run.

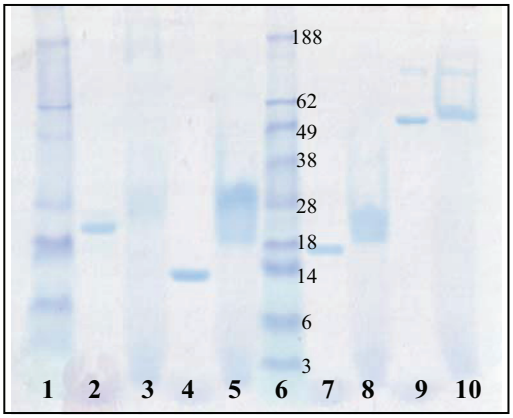


Figure 5-11: SDS PAGE of the four model proteins in aqueous solution and in the upper phase of IL-based ATPS. Samples on the gel are arranged as follows: (1) Protein standard in presence of 5 wt % IL, (2) trypsin in aqueous solution, (3) trypsin in upper phase of IL-based ATPS, (4) lysozyme in aqueous solution, (5) lysozyme in upper phase of IL-based ATPS, (6) protein standard [kDa], (7) myoglobin in aqueous solution, (8) myoglobin in upper phase of IL-based ATPS, (9) BSA in aqueous solution, (10) BSA in upper phase of IL-based ATPS.

The results discussed within this chapter clearly demonstrate that the system pH of IL-based ATPS exhibits a strong effect on protein partitioning. Although electrophoresis cannot be used to definitely prove a direct interaction between the proteins and the IL, electrostatic interaction is proposed to be the main driving force governing protein partitioning within IL-based ATPS. However, in order to obtain a more complete picture of protein-IL-interaction, more sophisticated methods like e. g. isothermal titration calorimetry need to be applied.

5.6 Statistical analysis of driving forces for protein partitioning

Due to the complexity of the partitioning phenomenon, it is difficult to predict protein partitioning behaviour and select separation conditions for the optimum extraction of a target protein. The results described above clearly demonstrate that for the case of IL-based ATPS no simple correlation between the investigated protein characteristics and the partitioning behaviour can be found, although electrostatic interaction and protein molecular weight exhibit the greatest influence. However, models which can predict separation *a priori* are very useful and would facilitate the experimental approach considerably. If protein partitioning could be reliably predicted, extraction in IL-based ATPS could be optimised by calculation only. However, the quantitative modelling of protein partitioning poses an extremely complex problem because of its dependence on a broad array of factors. Hence, regression analysis was applied as a tool for modelling and analysing the experimental data obtained from the protein partitioning in IL-based ATPS (see also chapter 9.3.8). Using the dependent variable 'partition coefficient log *K*' in the regression equation as a function of the independent (explanatory) variables, a best fit of data was obtained by using the least square method.

All in all, a set of data based on around 150 experiments was analysed, thereby taking into account the influence of system parameters (pH and temperature) as well as protein properties (molecular weight, surface charge, surface area, volume, sphericity and hydrophobicity). It was found that the partition coefficient log *K* could best be explained by Equation 5-5, with x_{pH}, x_T, x_H and x_Z and x_{MW} coding for the applied pH and temperature and the protein's hydrophobicity (surface → interior), surface charge and molecular weight, respectively. Interestingly, the parameters surface area, volume and sphericity are not included in the equation and therefore seem to be of minor importance within the partition process.

$$\log K \quad b_0 + b_1 \cdot x_{pH} \cdot x_{MW} + b_2 \cdot x_{pH}^2 + b_3 \cdot x_Z \cdot x_H + b_4 \cdot x_Z + b_5 \cdot x_T^2 + b_6 \cdot x_{pH} \cdot x_Z + b_7 \cdot x_Z^2 + b_8 \cdot x_T \cdot x_Z$$

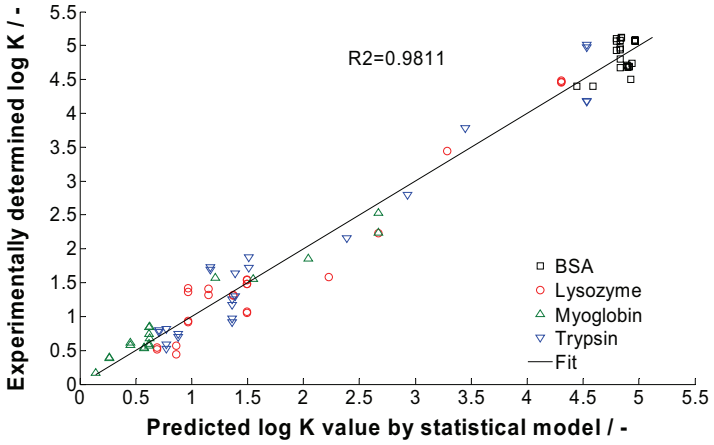
Equation 5-5: Prediction of the logarithm of partition coefficient for protein partitioning in IL-based ATPS.

The parameter estimates as well as the standard errors for Equation 5-5 are given in Table 5-6. All in all, the equation can be used to predict log *K* with an accuracy of R^2 0.9811 (Figure 5-12). It has to be emphasized that the order of explanatory variables within the predicting model equation reflects the importance of the influencing parameters for partitioning as is also indicated by an increase of the standard error with increasing number of terms. Hence, pH, charge and molecular weight of the proteins are of major importance for the prediction of the partition coefficient log *K*. A reduction of the number of explanatory variables within the statistical model from 8 to 5, will result in a drop out of the variables multiplied with b_6, b_7 and b_8, thereby decreasing the correlation coefficient to R^2 0.9713.

Table 5-6: Parameter estimates for the prediction of the partition coefficient (log *K*) in IL-based ATPS.

Term	Estimate	Standard error / %
Intercept b_0	-4.13	26.1
b_1	$1.74 \cdot 10^{-5}$	17.0
b_2	0.05	25.9
b_3	-0.62	38.8
b_4	-0.47	52.5
b_5	$-8.96 \cdot 10^{-5}$	85.4
b_6	0.04	89.5
b_7	0.002	75.1
b_8	-0.001	86.9

All in all, the results obtained by statistical analysis provide further evidence of the proposed electrostatic interaction between the model proteins and the phase-forming compounds in IL-based ATPS. Since the obtained model equation is strongly influenced by the parameters pH, charge and molecular weight, these factors have the highest impact on the partition process.



$$log\ K = -4.13+1.74\cdot10^{-5}\cdot x_{pH}\cdot x_{MW}+0.5\cdot x_{pH}^2-0.62\cdot x_Z\cdot x_H-0.47\cdot x_Z-8.96\cdot10^{-5}\cdot x_T^2+0.04\cdot x_{pH}\cdot x_Z+0.002\cdot x_Z^2-0.00\cdot x_T\cdot x_Z$$

Figure 5-12: Statistical analysis of the factors influencing protein partitioning. The predicted values of partition coefficient as proposed by Equation 5-5 are plotted against the experimentally determined results.

To further evaluate and verify the statistical model, the extraction of two additional proteins within IL-based ATPS was investigated, pepsin (pdb code 3pep) and hemoglobin (pdb code 1g09). Since the results of these experiments were not used for fitting the model Equation 5-5, they can be applied to verify the reliability of the predicted log *K* value.

Pepsin and hemoglobin both are proteins with high molecular weight of 35 kDa and 63 kDa, respectively. At a pH value of 6.9, pepsin is negatively charged (-38) while hemoglobin is positively charged (+8). The experimental conditions, the protein properties and the resulting partition coefficients of the partition experiments within the IL-based ATPS are summarised in Table 5-7. At first sight, the experimental and predicted log *K* values seem to be in disagreement. However, taking into account the minimum detectable protein concentration in the lower phase, the measured partition coefficient log *K* of around 4.4 represents the partitioning of around 100 % protein to the IL-enriched upper phase. Thus, a predicted log *K* value higher than 4.4 represents the complete extraction of protein towards the upper phase and can be explained by the linear nature of the model.

All in all, it could be demonstrated that the statistical model described above was able to predict the complete extraction of pepsin and hemoglobin to the upper phase of IL-based ATPS. It provides a useful tool for estimating partition coefficients of proteins *a priori* and facilitates the experimental approach considerably. Nevertheless, it has to be emphasized that the model only applies to the investigated system of 12 wt % IL and 20 wt % K_2HPO_4/KH_2PO_4 and does not account for changes in the system composition, since the main objective of statistical analysis was focused on relating the protein properties to the partition coefficient within IL-based ATPS.

Table 5-7: Conditions, experimental and calculated log *K* values for pepsin and hemoglobin in IL-based ATPS.

PDB	x_{pH}	x_{MW}	x_Z	x_H	x_T	Predicted log *K*	Experimental log *K*
3pep	6.9	34515.8	-38	-0.185	4	9.953	4.424
1g09	6.9	63910	8	-0.435	4	6.773	4.383

5.7 Distribution of amino acids

The partitioning experiments of model proteins and their statistical analysis revealed the contribution of different protein properties (at varying extents) to the partition coefficient. Since amino acid residues determine the protein surface properties, it may be possible to deepen the understanding of the driving forces for protein partitioning by investigating the distribution of single amino acids in IL-based ATPS. Amino acids only differ from each other by one characteristic group to which clear physical and chemical properties can be assigned. Using the 20 natural amino acids, it could be possible to investigate the effect of each amino acid residue on the extraction efficiency of proteins.

Amino acids are amphoteric molecules; depending on the pH, amino acids are anions, cations or neutral. Moreover, polar or nonpolar groups are incorporated in the side chain causing different hydrophobicities or hydrophilicities. In general, amino acids can roughly be divided into 4 groups based on the amino acid side chain: (1) nonpolar amino acids (hydrophobic): glycine, alanine, methionine, proline, valine, phenylalanine, leucin, isoleucin, trpytophan, (2) polar, uncharged amino acids (neutral): serine, cysteine, threonine, tyrosine, asparagine, glutamine, (3) acidic amino acids (net negative charge at pH 7): aspartic acid, glutamic acid and (4) basic amino acids (net positive charge at pH 7): lysine, arginine, histidine. A general overview on the characteristics of amino acids is given in Figure 5-13.

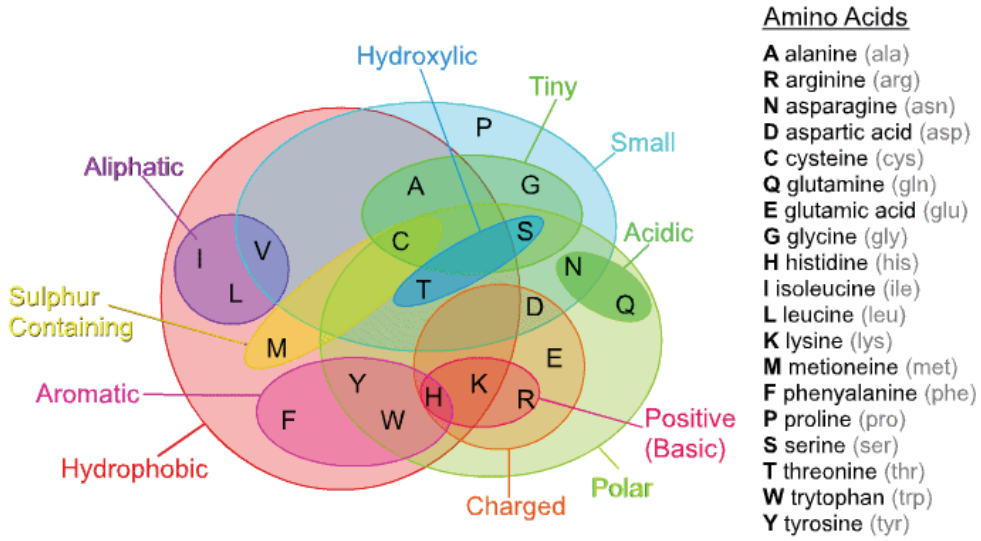


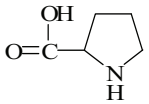
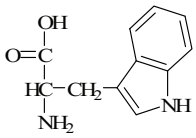
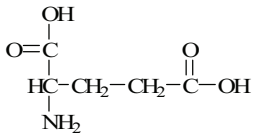
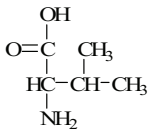
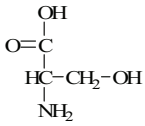
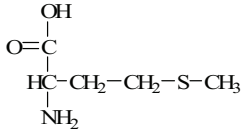
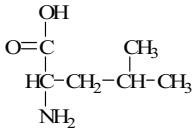
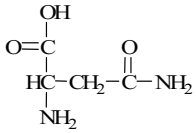
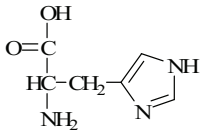
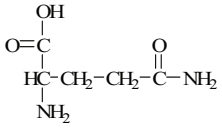
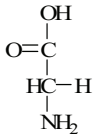
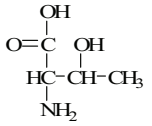
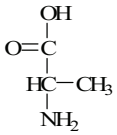
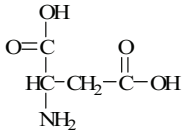
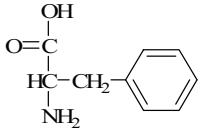
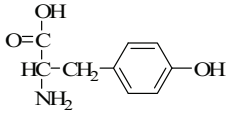
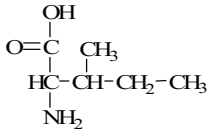
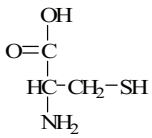
Figure 5-13: Schematic illustration of the differences in amino acid properties. Source: http://www.dreamingintechnicolor.com/InfoAndIdeas/AminoAcids.gif.

The partitioning behaviour of amino acids or low molecular weight peptides in conventional ATPS has been investigated and reported by several research groups during the last years (Cohen et al. 1995; Eiteman and Gainer 1992; Shang et al. 2004; Tintinger et al. 1997; van Berlo et al. 2000; Zaslavsky et al. 2000). Generally, it was found that electrostatic interaction and hydrophobicity are two main factors affecting the partitioning. Shang et al. (2004) have reported that within PEG/phosphate salt-systems, the salt can change the electrostatic charge of systems and cause amino acids with positive charge to prefer the salt-rich phase. Amino acids with a nonpolar side chain prefer the polymer-rich phase because of their stronger

hydrophobicity. With increasing pH and PEG molecular weight of the ATPS, an increase of the distribution ratios due to the change of electrostatic interaction and hydrophobicity was observed. However, most reports so far focus on the investigation of only selected amino acids and lack a detailed study on the effects of system composition and properties. Regarding the extraction of amino acids by ILs, Wang et al. (2005) investigated the partitioning of five different amino acids between water and $[C_4mim][PF_6]$, $[C_6mim][PF_6]$, $[C_6mim][BF_4]$ and $[C_8mim][BF_4]$. They found the partition coefficients of aromatic amino acids to be higher then those of aliphatic amino acids and attributed this observation to the presence of the hydrophobic pyrrole ring and additional CH_2-groups within the IL cation. The hydrophobicity of the amino acids thus was proposed to be a main driving force for amino acid partitioning. Additionally it was suggested, that electrostatic interactions between the cationic form of the amino acids and the anion of the IL are responsible for a higher degree of extraction in low pH ranges.

In order to obtain a sufficient overview of the properties of amino acids governing partitioning, all 20 proteinogenic amino acids were partitioned in the standard IL-based ATPS. Unfortunately, it was not possible to determine the partition coefficients of arginine, cysteine and lysine due to overlapping signals of the IL and the amino acids within LC/MS measurements. Nevertheless, interestingly, it could be demonstrated that the standard procedure for amino acid quantification using the Phenomenex EZ:faast kit is applicable for amino acid determination in the presence of the ionic liquid Ammoeng^{TM} 110 (see appendix Figure 11-1and Figure 11-2 for LC/MS diagrams). The results of amino acid partitioning show some interesting features regarding the partition coefficients (Table 5-8). While prolin, glutamin, serin, leucin and histidin preferably partition to the lower phase (negative value for log K), the other amino acids get enriched in the upper phase (positive log K value) and glycin partitions more or less equally between the phases. The highest partition coefficients were found for glutamic acid, threonin, aspartic acid and tyrosin ($\log K > 1$), indicating the extraction of more than 80 % of each amino acid to the upper IL-enriched phase.

Table 5-8: Partition coefficients of amino acids in IL-based ATPS consisting of 12 wt % Ammoeng^{TM} 110 and 20 wt % K_2HPO_4/KH_2PO_4 at pH 7 and 25°C. The partition coefficient log *K* is defined as the ratio of amino acid concentration in the upper phase to the concentration in the lower phase.

AS	log *K*	Structure	AS	log *K*	Structure
Pro	-1.003		Trp	0.572	
Glu	-0.535		Val	0.596	
Ser	-0.203		Met	0.640	
Leu	-0.063		Asn	0.734	
His	-0.037		Gln	0.748	
Gly	0.142		Thr	1.013	
Ala	0.318		Asp	1.227	
Phe	0.325		Tyr	1.512	
Ile	0.330		Cys	n. d.	

To provide a general overview on the amino acid properties within the IL-based ATPS at pH 7, Table 5-9 illustrates the physical and chemical characteristics in relation to the partition coefficient log *K* (see also Figure 5-13). All in all, no general correlation between the aromatic/aliphatic or hydrophilic/hydrophobic character of the amino acids and the partition coefficient could be observed contrary to reports by Wang et al. (2005). It was found though, that amino acids which are negatively charged at the investigated pH of 7 (glutamic and aspartic acid) get enriched in the upper IL-containing phase, while positively charged amino

acids such as histidin stay in the lower phase. This observation is in accordance with the assumption of electrostatic interaction between the IL cation and negatively charged residues as described for the model proteins above (see chapter 5.5). Still it has to be emphasized that further experiments at different pH values will be necessary to provide a deeper understanding on the driving forces and should be the subject of future studies. Determination of the partition coefficients of each amino acid at its isoelectric point, as well as above and below the pI, would provide an interesting approach to reveal direct influences of charge on the extraction efficiency. These investigations were not conducted within this thesis since the main emphasis was placed on relating the results of amino acid partitioning to the standard system used for the partitioning of model proteins.

Table 5-9: General overview on the physical and chemical characteristics of amino acids within the IL-based ATPS at pH 7 in relation to an increasing partition coefficient log *K*.

Amino acid	log *K*	Lower phase	Positive	Negative	Hydro-phob	Polar	Ali-phatic	Aro-matic	Small	Tiny
Pro	-1.003	✖							✖	
Glu	-0.535	✖				✖				
Ser	-0.202	✖				✖			✖	✖
Leu	-0.063	✖			✖		✖			
His	-0.037	✖	✖		✖	✖		✖		
Gly	0.142								✖	✖
Ala	0.318				✖				✖	✖
Phe	0.325				✖			✖		
Ile	0.330				✖		✖			
Trp	0.572				✖	✖		✖		
Val	0.596				✖		✖		✖	
Met	0.640				✖					
Asn	0.734					✖			✖	
Gln	0.748			✖		✖				
Thr	1.013				✖	✖			✖	
Asp	1.227			✖		✖			✖	
Tyr	1.512				✖	✖		✖		

Applying regression analysis in order to analyse the experimental data in a statistical approach, the dependent variable ‘partition coefficient log *K*’ in the regression equation was set as a function of the independent variables volume (x_v), Van der Waals volume (x_{Waals}), accessible surface area (x_{ASA}), molecular weight (x_{MW}), hydrophobicity (x_H) and isoelectric point (x_{pI}) of the amino acids. The properties of amino acids taken into account are summarised in Table 5-10.

Table 5-10: Properties of amino acids that were used for statistical analysis of amino acid partitioning in IL-based ATPS. Data were obtained from http://prowl.rockefeller.edu/aainfo/contents.htm and from the hydrophobicity (H_3, hydropathy) index (Kyte and Doolittle 1982).

Amino acid	Volume / Å^3	Van der Waals volume / Å^3	Accessible Surface Area / Å^2	MW / g mol^{-1}	Hydrophobicity H_3 / -	pI / -
Ala	92	67	67	89.1	1.8	6
Asn	135	96	113	132.118	-3.5	5.4
Asp	125	91	106	133.1	-3.5	2.8
Gln	161	114	144	147.13	-3.5	5.7
Glu	155	109	138	146.15	-3.5	3.2
His	167	118	151	155.16	-3.2	7.47
Ile	169	124	140	131.17	4.5	5.9
Leu	168	124	137	131.18	3.8	6
Met	171	124	160	149.21	1.9	5.7
Phe	203	135	175	165.19	2.8	5.5
Pro	129	90	105	115.13	-1.6	6.5
Ser	99	73	80	105.09	-0.8	7.9
Thr	122	93	102	119.12	-0.7	5.9
Trp	240	163	217	204.225	-0.9	6
Tyr	203	141	187	181.19	-1.3	7.8
Val	142	105	117	117.15	4.2	6

It was found that the logarithm of partition coefficient could best be described by Equation 5-6 which takes into account the volume of amino acids, their molecular weight and isoelectric point.

$$\log K \quad 4.9636 - 0.056556 \cdot x_V + 0.010349 \cdot x_{Waals} \cdot x_{pI} \quad 1.06 \cdot x_{pI} + 0.027034 \cdot x_{MW}$$

Equation 5-6: Calculation of the predicted log *K* value for amino acids in IL-based ATPS.

All in all, the equation can be used to predict log *K* with an accuracy of R^2 0.67822 (Figure 5-14) indicating that no simple correlation between these parameters and the partitioning of amino acids in IL-based ATPS exists. Nevertheless, again it was found that the molecular weight as well as the charge of amino acids (represented by the pI value) seems to be of great importance for the extraction process, while hydrophobicity was excluded from the equation due to an insufficient correlation with the partition coefficient.

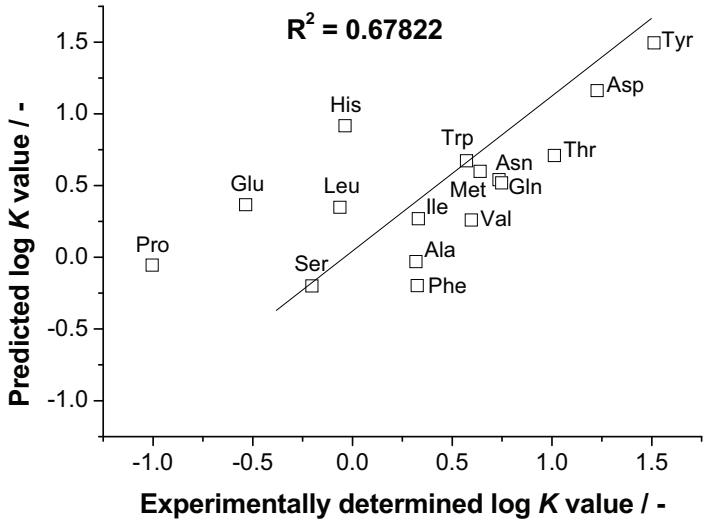


Figure 5-14: Correlation between the predicted and experimentally determined log *K* value for the partitioning of amino acids in IL-based ATPS consisting of 12 wt % Ammoeng™ 110 and 20 wt % K_2HPO_4/KH_2PO_4 at pH 7 and 25°C.

It can be stated that IL-based ATPS are not only suitable for the extraction of proteins but can also be used for the purification of amino acids. Due to the variation of partition coefficients between individual amino acids, IL-based ATPS could also be applied to selectively extract one amino acid from a mixture of amino acids. The conventional separation of amino acids includes ionic exchange, liquid membrane with extractants and reversed micelle methods (Adachi et al. 1991; Itoh et al. 1990). Most of these processes use organic solvents. Therefore, IL-based ATPS represent an interesting alternative downstream method for these small biomolecules as well. However, as it has already been pointed out above, further experiments will be required to optimise an IL-based ATPS with regard to the extraction problem.

A step further was taken by assigning the obtained partition coefficients of each amino acid to the calculation of distribution of the model proteins. Starting from the exposed surface area of amino acids in the proteins, the contribution of each amino acid in the proteins' surface on the partition coefficient was taken into account by multiplying the log *K* value of each amino acid with its surface area within the protein (see appendix, Table 11-1). The sum of these individual contributions was correlated to the partition coefficient of the model proteins in IL-based ATPS resulting in a very good correlation of R^2 0.99514 (Figure 5-15)[4]. Hence it can be concluded that although no sufficient correlation between the amino acid properties and their partitioning in IL-based ATPS could be found, the partition coefficients of amino acids and their contribution to a proteins' surface can be used to explain and relate the partitioning behaviour of proteins in IL-based ATPS.

[4] The calculation was done following the procedure as proposed for the calculation of hydrophobicity (Berggren et al. 2002).

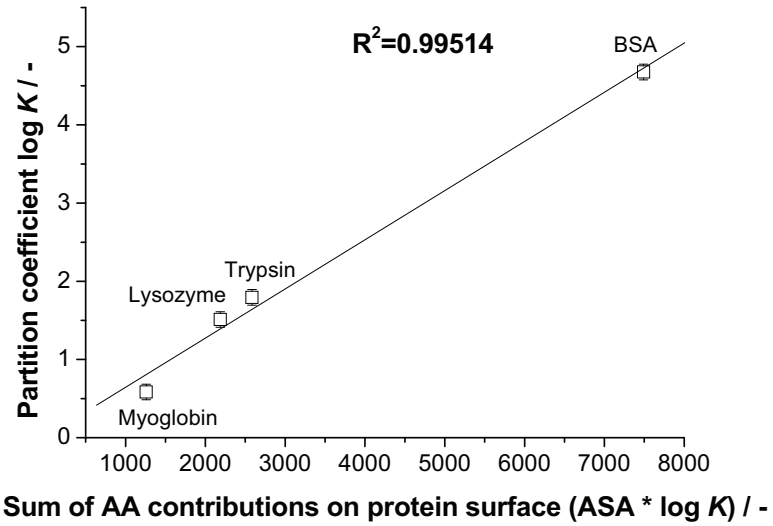


Figure 5-15: Correlation between contribution of each amino acid (AA) on a protein surface (sum of the product of amino acid surface area ASA and log *K* value of each amino acid in IL-based ATPS) and the partition coefficient of model proteins.

The approach of calculating the relative contribution of each amino acid on a proteins surface, as well as Equation 5-5 which was fitted for the partitioning of model proteins, can both be applied within future purification challenges. They provide researchers with the opportunity to decide by calculation only, whether the IL-based ATPS can be used to separate different amino acids or proteins and enzymes from each other. Supposing that the calculated partition coefficients differ significantly from each other, for instance if one protein shows a negative log *K* value (stays in the lower phase) and the other one a positive log *K* value (is extracted to the upper phase), IL-based ATPS provide a useful and advantageous separation technique. Generally, it can be proposed that an increase of negative charge associated with a highly accessible charged surface area will facilitate the extraction of a target compound towards the IL-enriched upper phase of IL-based ATPS.

5.8 Interims Summary

The successful industrial application of IL-based ATPS in downstream processing is clearly influenced by the ability to understand or even predict the partitioning of a target molecule between the phases. An IL-based ATPS using Ammoeng^{TM} 110 has been applied to investigate the main driving forces of protein partitioning within these systems. By studying the partitioning of different model proteins (myoglobin, lysozyme, trypsin and BSA), correlations between the protein and system properties could be found, thereby revealing the main driving forces of the partition process. The main results discussed within this chapter can be summarised by the following points:

- Surprisingly, only a very weak correlation was found between the protein **hydrophobicity** and the partition coefficient.
- A strong dependency could be observed between protein **charge** and **molecular weight** and the partition behaviour.
- Based on these findings, **electrostatic interaction** has been identified as the main driving force of protein partitioning in IL-based ATPS. The interaction itself was proposed to depend on two different mechanisms of interaction between the proteins and the phase-forming compounds of the IL-based ATPS: (1) the negatively charged residues of proteins interact with the positively charged ammonium cation and (2) the positively charged amino acids on a proteins' surface interact with the inorganic salts' anions H_2PO_4 and HPO_4^2, which in turn again allow the interaction with the positively charged IL-cation.
- With increasing **pH**, a higher number of HPO_4^2 anions will interact with the more negatively charged protein surface, thereby compensating the positive surface charge and allowing stronger electrostatic interaction with the IL-cation. Additionally, a higher **accessible contact area** between the protein surface and the phase-forming compounds contributes to a stronger interaction and increases the partition coefficient.
- **Statistical analysis** of experimental results further supported the proposed mechanism and the established statistical model based on the model proteins' physico-chemical properties provides a good start for predicting protein partitioning in IL-based ATPS.
- Experiments on the distribution of **amino acids** within IL-based ATPS confirmed again that charge and molecular weight play a decisive role in the partitioning process. Interestingly, it was possible to relate the contribution of the partition coefficient of each amino acid on the model proteins' surfaces to their partition behaviour within IL-based ATPS resulting in a high correlation coefficient of R^2 0.995.
- An approach based on the relative contribution of each amino acid on a protein surface, as well as a statistical model fitted for the partitioning of model proteins, can both be applied within **future purification challenges**. They provide researchers with the opportunity to decide by **calculation** only, whether the IL-based ATPS can be used to separate different amino acids or proteins and enzymes from each other.

All in all it can be concluded, that partitioning of proteins within IL-based ATPS represents a complex phenomenon that can only be explained by a combination of several protein properties and has to take into account a broad array of factors.

6 Results and discussion part III: Application of IL-based ATPS for downstream processes[5]

In times of molecular engineering and protein design, the development of new and biocompatible extraction methods for the separation and purification of enzymes and proteins is gaining increasing importance. Despite the versatile advantages of conventional ATPS, the aqueous nature of these systems entails some drawbacks with regard to the purification of industrially interesting enzymes and proteins. The occurrence of side reactions as well as the low solubility of numerous pharmaceutical substrates in aqueous media often prevents the combination of enzyme purification with enzyme-catalysed reactions. Many enzymes are known to be very sensitive and instable, thus losing activity during the purification process. Moreover, the phase-forming compounds within the enzyme containing phase might interfere with the enzyme catalysed reaction. Therefore, in many cases subsequent purification steps are needed in order to separate the phase-forming compounds from the enzyme containing solution (Rito-Palomares 2004).

The IL-based ATPS established within this work might hold the potential to overcome these drawbacks. In order to investigate the application of IL-based ATPS for the biocompatible extraction of catalytically active enzymes, the purification of two different alcohol dehydrogenases (ADH) from crude cell extracts of *E. coli* was chosen as an example for an industrially interesting downstream process.

6.1 Alcohol dehydrogenases (ADHs)

Dehydrogenases belong to the enzyme class of oxidoreductases (EC 1.x.x.x) and therein represent the largest group followed by oxidases, oxygenases and peroxidases. Generally, alcohol dehydrogenases (ADHs) catalyse the interconversion of alcohols, aldehydes and ketones and require either $NAD(P)^+$ or NAD(P)H as cofactor which is consumed in equimolar amounts compared to the substrate. Due to the fact that ADHs display a wide variety of substrate specificities and are involved in an astonishingly wide range of metabolic processes in all living organisms, they are of great scientific interest. As a consequence, their capability to catalyse chemo-, stereo-, and regioselective reactions has drawn and focused attention to their use as attractive biocatalysts for industrial applications within food, pharmaceutical and fine chemicals industries. Alcohol dehydrogenases are being increasingly applied for asymmetric synthetic transformations due to the growing demand for enantiopure pharmaceuticals (Bradshaw et al. 1992; Ferloni et al. 2004; Kruse et al. 1996; Wong and Whitesides 1994) and biosensors (Jones 1986; Kruse et al. 1996; Leone et al. 2001).

However, all in all scientific research has revealed that many ADHs are of limited use for technical applications due to problems such as low substrate specificity, narrow substrate spectrum, poor operational stability and low enantioselectivity (Table 6-1). Moreover, as long

[5] Parts of this chapter have been published in: Dreyer S, Kragl U (**2007**). Verfahren zur Extraktion von Biomolekülen (Process for the extraction of biomolecules) *Patent 102007001347.9, 04/01/2007* and
Dreyer S, Kragl U. (**2008**). Ionic liquids for aqueous two-phase extraction and stabilization of enzymes. *Biotechnology and Bioengineering* 99(6):1416-1424.

as the use of ADHs is restricted to aqueous reaction media, the scope of industrial bioconversions, especially for the production of fine chemicals, is limited by a variety of constrains:

- the operational instability of ADH enzymes and cofactors (Jones and Beck 1976; Ooshima et al. 1981; Orlich and Schomaecker 1999; Walt et al. 1984; Wong and Whitesides 1981),
- the insolubility of many of the substrates and products in water (Groger et al. 2004),
- product inhibition of the enzyme (Lee and Whitesides 1985),
- lack of stability of some substrates and products in aqueous solutions (Keinan et al. 1986b) and
- the difficulty of product and enzyme recovery from aqueous medium.

In principle, most of these problems might be overcome by switching from water to organic solvents as reaction medium. Nevertheless, the toxicity of the organic solvents towards the enzyme and environmental concerns enforce the application of other non-conventional media which should be non-toxic, non-hazardous and readily separated from the enzyme and the product. In this regard, the use of ionic liquids is of great interest.

Table 6-1: Drawbacks for the technical application of selected alcohol dehydrogenases.

Problem	ADH from	Reference
Low substrate specificity	Yeast (Y ADH), horst liver (HL ADH), *Thermoanaerobium brockii* (TB ADH), *Thermoanaerobacter ethanolicus* (TE ADH)	(Hummel 1997; Keinan et al. 1986a)
Narrow substrate spectrum	Yeast (Y ADH), horst liver (HL ADH), *Thermoanaerobium brockii* (TB ADH), *Thermoanaerobacter ethanolicus* (TE ADH)	(Hummel 1997; Keinan et al. 1986a)
Low enantioselectivity	Yeast (Y ADH), horse liver (HL ADH)	(Hummel 1997)
Poor operational stability	Yeast (Y ADH), horse liver (HL ADH)	(Hummel 1997)

In 1997 and 2004, the recombinant production of two new and stereoselective ADH enzymes, namely the *(R)*-specific alcohol dehydrogenase from *Lactobacillus brevis* (LB ADH) and the *(S)*-specific alcohol dehydrogenase from a thermophilic organism (T ADH) has been reported in literature (Daussmann and Hennemann 2004; Hummel 1997). These enzymes were found to exhibit high substrate specificity, a broad substrate spectrum and a high enantioselectivity and are therefore of great interest for industrial application. Hence, LB ADH and T ADH were chosen as model enzymes in order to investigate the potential application of IL-based ATPS for the biocompatible extraction of catalytically active enzymes.

6.2 Extraction of alcohol dehydrogenase from *Lactobacillus brevis*

6.2.1 Effect of the ionic liquid on LB ADH activity and stability

With regard to the purification of enzymes, the main obstacle in developing a suitable extraction method represents the maintenance of the stability and catalytic activity of the biocatalyst. In organic solvents, which are used in conventional extraction processes, proteins tend to denature irreversibly. Aqueous two-phase systems are particularly suitable for extraction of enzymes by providing the natural aqueous environment for proteins. Nevertheless, phase components such as PEG and salt are well-documented as having various effects on protein structures such as solubility and stability modulations and PEG-protein associations. It is therefore essential to evaluate the general impact the phase-forming compounds of ATPS may have upon biomolecular structure and function.

LB ADH as well as T ADH can be used for the reduction of the substrate acetophenone to 1-phenylethanol with high enantioselectivity. LB ADH produces the *(R)*-1-phenylethanol while T ADH can be applied for the production of *(S)*-1-phenylethanol (Figure 6-1, part 1). In order to regenerate the expensive cofactor and thereby make a synthesis profitable, a substrate-coupled cofactor regeneration can be used by adding the reduced cosubstrate 2-propanol to the reaction mixture (Figure 6-1, part 2).

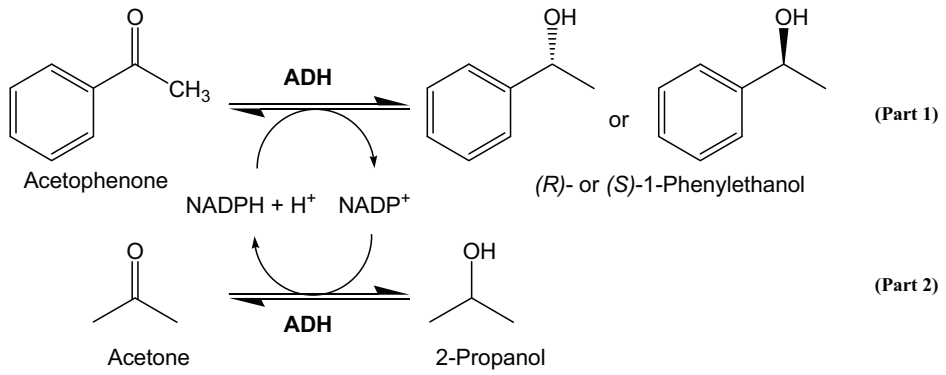


Figure 6-1: ADH catalysed reduction of acetophenone. Part 1 was used for the photometric determination of ADH activity by following the consumption of NADPH at 340 nm. Part 2 represents the extension for acetophenone reduction with substrate coupled cofactor regeneration.

The effect of the ionic liquid Ammoeng^{TM} 110 on enzyme activity of LB ADH was investigated by the addition of different amounts of IL to the standard activity assay. Using acetophenone as substrate, the consumption of NADPH during the enzymatic reaction can easily be followed by photometric measurements (Figure 6-1, part 1). Surprisingly, the experimental investigations revealed an activating effect of the ionic liquid Ammoeng^{TM} 110 on LB ADH. The addition of IL resulted in an increased enzyme activity of up to 126 % (in the presence of 30 wt % Ammoeng^{TM}110) as compared to the pure aqueous solution. As illustrated in Figure 6-2, there is no general trend of increasing activity with increasing amount IL. However, already the addition of 1 wt % Ammoeng^{TM} 110 results in an enhanced enzyme activity of 110 % and is therefore beneficial for the biocatalytic activity of LB ADH.

Keeping in mind the direct interaction of proteins and ionic liquid, the activating effect might be ascribed to direct influences on the biocatalyst due to changes in the active site structure, the transition state structure or the enzyme periphery. Similar effects have previously been reported for the influence of organic solvents on biocatalysts (Kvittingen et al. 1992; Ryu and Dordick 1992).

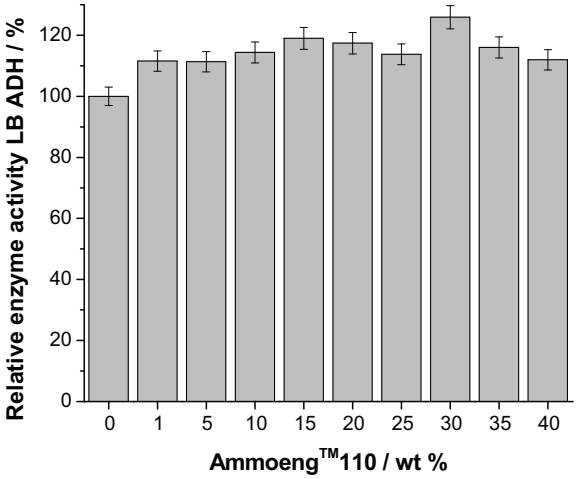


Figure 6-2: Enzyme activity of LB ADH at standard conditions (30°C, pH 7) in the presence of increasing amounts ionic liquid as compared to the enzyme in aqueous buffer solution.

With regard to the purification of active biocatalysts, another important factor to be considered is the enzyme stability in the presence of the phase-forming compounds. By storing the enzyme LB ADH with varying amounts IL, the enzymatic activity was followed over time. Since the optimum activity of LB ADH was found to be at 30°C, the enzyme was stored at this temperature as well as under refrigerated conditions and enzymatic assays were performed following the standard conditions as described previously (Riebel 1996). Interestingly, it was found that besides an activating effect, the IL also exhibits a stabilising effect on LB ADH since the half life time both at 4°C and 30°C was increased significantly by the addition of Ammoeng™110 (Figure 6-1). More precisely, by increasing the amount of IL present during storage from 0 wt % up to 30 wt % the half life of LB ADH at 30°C was enhanced gradually up to a factor of 9.8 while storage at 4°C resulted in an increasing half life time up to a factor of 7.9.

These results further support the observed thermal stabilisation of BSA by the ionic liquid Ammoeng™ 110 (chapter 5.2). Generally, a stabilising effect of ILs on enzymes has already been reported several times before (Zhao 2005). It was announced that for the case of enzyme catalysis employed in aqueous solutions of hydrophilic ILs, the stabilising effect of the ionic liquid follows the Hofmeister series. Therefore, chaotropic cations and kosmotropic anions should be most suitable for stabilising enzyme activity (Collins 2004; von Hippel and Schleich 1969; Wiggins 1997). However, the finding that Ammoeng™ 110 exhibits a strong stabilising effect is not consistent with these reports since the ammonium cation with oligoethylene glycol units is quite large and therefore likely to be only slightly chaotropic. With regard to the IL anion, Cl is known as a borderline ion (Baldwin 1996; Collins and

Washabaugh 1985; Jenkins and Marcus 1995). Thus the stabilising effect of Ammoeng^{TM} 110 cannot be explained by the Hofmeister series alone.

Table 6-2: Half life time of enzymes in the presence of different amounts ionic liquid Ammoeng^{TM} 110.

Concentration of ionic liquid in enzyme solution / wt %	**Half life time ($t_{1/2}$) of LB ADH / d**	
	4°C	**30°C**
0	20.9	0.6
1	43.9	1.2
5	94.9	2.6
10	94.9	3.0
15	100.8	4.1
20	135.9	5.5
30	165.0	5.9

The incorporation of oligoethylene glycol units within the cation and its structural similarity to polyethylene glycol should also be considered to be involved in the stabilising effect of Ammoeng^{TM} 110. Polyethylene glycol has been reported to stabilise proteins in solution (Boyd and Yamazaki 1993; Hyun et al. 1993; Ichikawa et al. 1988) and enhance the refolding of proteins from inclusion bodies while averting aggregation (Cleland et al. 1992; Cleland and Randolph 1992; Cleland and Wang 1990a; Cleland and Wang 1990b). Evidence suggests that PEG causes preferential hydration of globular proteins by steric exclusion of PEG from the local vicinity of the protein, although PEG may interact, possibly by binding, with accessible non-polar surface residues (Shulgin and Ruckenstein 2006). Keeping in mind the proposed electrostatic interaction between proteins and the ionic liquid cation, two mechanisms are suggested to contribute to the stabilising effect: The electrostatic interaction between IL and enzyme is more pronounced with the native than the denatured protein state which usually exhibits a significantly more hydrophobic exposed surface area. A direct interaction with the active site structure, the transition state structure or the enzyme periphery also explains the observed enzyme activating effect. In addition, the enzyme solution gets more densely packed with increasing amount of IL and thereby favours the compact native enzyme state over the denatured, inactive and unfolded state, explaining the observed enhancement of LB ADH stability.

6.2.2 Effect of compatible solutes

Due to the structural similarity between the ionic liquid Ammoeng^{TM} 110 and so-called compatible solutes, further investigations on the stabilising influence of the IL were performed by comparison with these biomolecules.

Compatible solutes are accumulated in microorganisms in response to increased extracellular salt concentrations, but also as a response to other environmental changes such as increased temperature. By accumulation of these organic compounds, microorganisms control their internal water activity, maintain the appropriate cell volume and turgor pressure, and protect intracellular macromolecules. The types of compatible solute used for osmotic balance include polyols and derivatives (e. g. glycerol, sorbit and mannit), sugars and derivatives (e. g. saccharose and trehalose), amino acids and derivatives (e. g. glutamic acid, proline and betaine) and tetrahydropyrimidin derivates (ectoine and hydroxyectoine) (Galinski 1995). In general, compatible solutes have low molecular mass, high solubility in water, and are nontoxic to organisms even when accumulated at a high concentration (Brown and Simpson 1972). Numerous studies have proven that they are not only compatible with the native state of proteins and membranes but also stabilise their structures against dehydration, heat shock and denaturing agents (Andersson et al. 2000; Goller and Galinski 1999; Lippert and Galinski 1992; Smirnoff and Cumbes 1989). Hence compatible solutes have also found widespread application for industrial use. Examples include the use of ectoines in medical skin care products or the stabilisation of enzymes and antibodies in pharmaceutical industry (Buenger and Driller 2004; Lentzen and Schwarz 2006).

For comparison with the enzyme activating effect of Ammoeng^{TM} 110, the activity of alcohol dehydrogenase from *Lactobacillus brevis* was tested in the presence of a set of compatible solutes (Figure 6-3). Results indicate that enzymatic activity of LB ADH does not seem to be significantly affected by the presence of compatible solutes. Only the addition of lysine and betaine show a slight increase of relative enzymatic activity of LB ADH to around 105 %. However, in comparison to the ionic liquid Ammoeng^{TM} 110 the activating effect is much smaller. Addition of the compatible solutes prolin, ectoine and hydroxyectoine even slightly decreases the enzymatic activity of LB ADH to 93 94 % of initial activity.

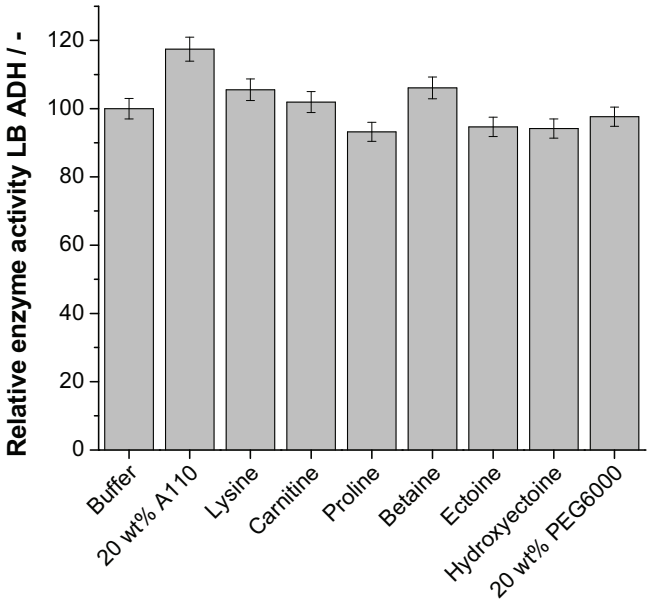


Figure 6-3: Relative enzymatic activity of LB ADH in the presence of different compatible solutes (500 mM) and PEG6000 under standard conditions of 30°C and pH 7.

Additionally, the stability of LB ADH was tested by storing the enzyme in the presence of the different compatible solutes as well as a solution of 20 wt % PEG6000 (Table 6-3). Obviously, all compatible solutes except hydroxyectoine - can be used in order to enhance the storage stability of LB ADH. However, it has to be pointed out that the storage temperature has a strong influence on the stabilising effect. While lysine is the best compatible solute for storing LB ADH at a temperature of 4°C, at 30°C the best stabilising effect could be obtained by addition of carnitine. Interestingly, these two compatible solutes are also the ones exhibiting the greatest structural similarity to Ammoeng™ 110. Moreover, the influence of the ILs Ammoeng™100 and Ammoeng™101 was tested and also revealed a strong stabilising effect on LB ADH which again confirms the molecules' structure and ionic character to be of main importance for the observed effects. On the other hand PEG6000 shows a strong stabilising effect at 4°C while decreasing the storage stability of LB ADH at 30°C from 0.6 to 0.4 days.

Table 6-3: Influence of the addition of compatible solutes, ionic liquids and PEG6000 on the stability of LB ADH if stored at 4°C and 30°C. Extinction coefficients are related to NADPH and slightly vary in dependence of the applied compatible solute or ionic liquid.

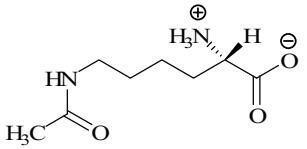
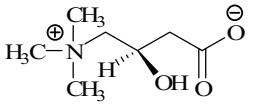
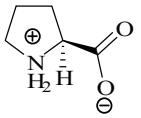
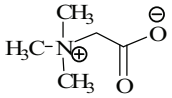
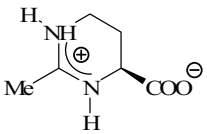
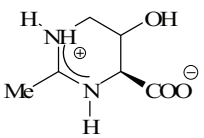
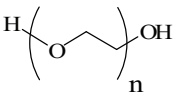
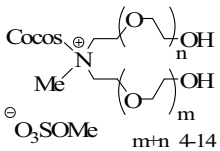
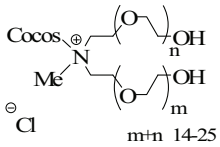
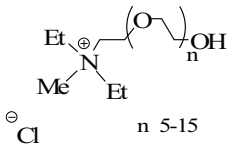
Compatible Solute / Ionic Liquid		**Extinction coefficient / mL µmol^{-1}**	**Half life time LB ADH /d**	
			4°C	**30°C**
Buffer	-	6.093	13.9	0.6
N_ε-Acetyl-L-Lysine		5.774	64.8	1.3
L-Carnitine		5.979	22.4	2.5
L-Proline		6.069	20.8	0.8
Betaine		5.744	27.9	0.8
Ectoine		5.977	25.2	1.4
Hydroxyectoine		6.008	11.7	0.8
20 wt % PEG6000		6.241	98.0	0.4

Table 6-3: Influence of the addition of compatible solutes, ionic liquids and PEG6000 on the stability of LB ADH if stored at 4°C and 30°C. Extinction coefficients are related to NADPH and slightly vary in dependence of the applied compatible solute or ionic liquid. Continued.

Compatible Solute/ Ionic Liquid		**Extinction coefficient /**	**Half life time LB ADH /d**	
		mL µmol^{-1}	**4°C**	**30°C**
20 wt % Ammoeng^{TM} 100	 m+n 4-14	6.012	36.5	2.3
20 wt % Ammoeng^{TM} 101	 m+n 14-25	6.001	33.2	2.4
20 wt % Ammoeng^{TM} 110	 n 5-15	6.033	135.9	5.5

All in all, the stability and activity enhancing effect of Ammoeng^{TM} 110 on LB ADH is clearly superior to the effect of compatible solutes, PEG6000, Ammoeng^{TM} 100 and Ammoeng^{TM} 101. Based on the fact that ionic liquids are salts and due to their structural similarity with compatible solutes, further indications about the mechanism of stabilisation could be given by previously established theories on the stabilising behaviour of compatible solutes (Arakawa and Timasheff 1983). A set of theories regarding protein-solute interactions have been proposed which can be roughly classified into two types: (1) those which postulate direct solute-macromolecule interactions and (2) those which hypothesize that macromolecular stability is mediated by solute-induced changes in water structure (see Hofmeister series). Basically, these theories involve the arguments which have already been discussed in the previous chapters for the action of the ionic liquid on proteins. The most common theory to explain the stabilising effect of compatible solutes proposes that these molecules are preferentially excluded from the surface of proteins (Liu and Bolen 1995; Timasheff 2002a; Timasheff 2002b). This in turn leads to preferential hydration of the protein. The increased osmotic pressure generated by the solutes will favour compact folded proteins, which expose less surface area than denatured proteins (Figure 6-4). Different interactions of organic solutes with folded and denatured proteins also contribute to their stabilisation effects. Cioni et al. have proposed that, compared to water, solutes have more unfavourable interactions with the peptide backbone and since unfolded protein has more available backbone, this biases the equilibrium to a folded protein (Cioni et al. 2005). Hence it is suggested that osmolytes which impart stability actually interact with the unfolded state of the protein, shifting the equilibrium to promote the folded configuration. The free energy of the denatured state is higher than that of the native state, making population of this state energetically unfavourable.

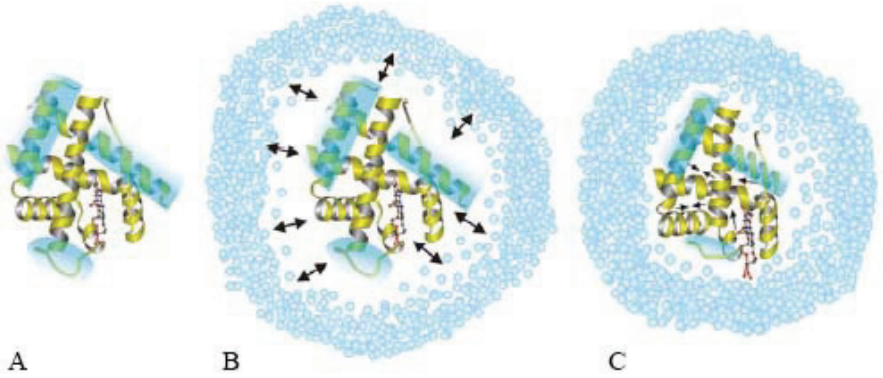


Figure 6-4: Illustration of protein stabilisation by compatible solutes through preferential exclusion. (A) Model of tertiary protein structure with hydration shell in native state; (B) Denaturing of protein under stress conditions. Compatible solutes are illustrated as spheres that are excluded from the protein surface which in turn leads to an unequal distribution in solution; (C) To reduce the energetically unfavourable distribution, the protein adopts a more compact conformation which is similar to the native state (Arakawa and Timasheff 1985; Bolen and Baskakov 2001). Picture from bitop AG, Witten, Germany, www.bitop.de.

However, the results obtained for the stabilising and activating effect of Ammoeng^{TM} 110 on LB ADH as well as the driving forces of protein partitioning in IL-based ATPS argue for a direct interaction of the IL cation with the protein. Hence, a preferential exclusion of the IL from the proteins' surface is unlikely. Nevertheless, strikingly the best stabilising effects on proteins were found for betaine, lysine, carnitine and the ionic liquid molecules, all of which possess a similar structure containing a flexible side chain of varying length and an ionic character. Taking into account that for instance PEG6000 could not be used to stabilise LB ADH at 30°C, the ionic character seems to be of major importance for the stabilisation mechanism. This observation again provides an indication of an interaction between the charged molecules and the enzyme's surface which results in a stabilisation of the native enzyme state. In addition, the effect of the flexible side chain has to be considered. Following partially the argumentation as proposed for the stabilising mechanism of compatible solutes, the enzyme within the solution might be surrounded by compatible solutes or IL molecules due to electrostatic interaction which in turn favour the more compact, densely packed and native enzyme state. The side chains of these molecules, however, could provide cavities which allow the retention of the essential hydration shell around the enzyme as well as the flexibility of the enzyme itself. It has to be emphasized though, that these explanations are just theoretical and need further confirmation e. g. through NMR measurements. By building a structurally detailed dynamic model of a protein from ^{15}N relaxation data, the effect of varying concentrations of compatible solutes or IL on the protein could provide further evidence on protein mobility or protein/solute interactions.

6.2.3 Effect of the inorganic salt on enzyme activity

In order to form an IL-based ATPS, an inorganic salt has to be added as the second phase-forming compound. Therefore, the influence of K_2HPO_4/KH_2PO_4 on the activity of LB ADH has to be taken into account. It has to be emphasized that the ratio of phosphate salts (1.82 : 1.00) provides a pH value of 7.0 even in the presence of ionic liquid and thereby ensures an optimum enzyme activity. Figure 6-5 illustrates the relative activity of LB ADH in the presence of increasing amounts inorganic salt. Up to a concentration of 10 wt % no effect

could be observed, while the activity slightly decreases at concentrations of 15-25 wt % and drops at 30 wt % to 55 % relative activity. Nevertheless, it can be concluded that concentrations as applied for the formation of IL-based ATPS will not significantly disturb the enzymatic activity of LB ADH if the enzyme is enriched in the IL-containing upper phase.

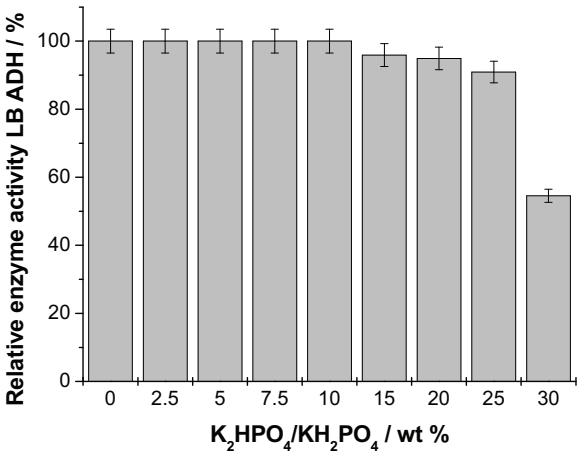


Figure 6-5: Influence of increasing concentrations of inorganic salt on the activity of LB ADH under standard conditions of 30°C and pH 7.

6.2.4 Purification of LB ADH by IL-based ATPS

Alcohol dehydrogenase from *Lactobacillus brevis* is recombinantly produced in *Escherichia coli* and is therefore to be extracted from the crude cell extract (partly purified by heat denaturation). It was demonstrated above that the composition of IL-based ATPS and the partitioning of proteins therein can be influenced by changing different parameters. However, in order to match the requirements of enzyme with regard to the maintenance of catalytic activity, the pH value of the IL-based ATPS was set to pH 7.0 (optimum pH for activity of ADH) enabled by the application of a mixture of K_2HPO_4 and KH_2PO_4 at a ratio of 1.00 : 1.82 as the second phase-forming compound. Moreover, the temperature during phase separation was set to 4°C in order to prevent thermal denaturation. Thus, the selection and optimisation of the IL-based ATPS with regard to an optimum purification of the enzyme is restricted to a variation of concentration of the phase-forming compounds Ammoeng™ 110 and K_2HPO_4/KH_2PO_4.

In a traditional approach for process optimisation in ATPS, one factor at a time would be varied while the other one is kept constant in order to subsequently relate the observed results to the changes of one parameter (Selber et al. 2000). The choice of parameters which are considered to be important and therefore should be characterised, represents an initial problem of optimisation. If two or more factors are chosen the number of experiments to be performed dramatically increases. Moreover, this strategy usually fails, because it tacitly assumes that a change in the value of one variable is independent of the other, which is not always the case (Pilipauskas 1999). Hence, the global optimum might be missed as demonstrated in Figure 6-6[6].

[6] For general information on the design of experiments see also: Ullmann. (**2004**). Ullmann's chemical engineering & plant design. *VCH Wiley*, Weinheim

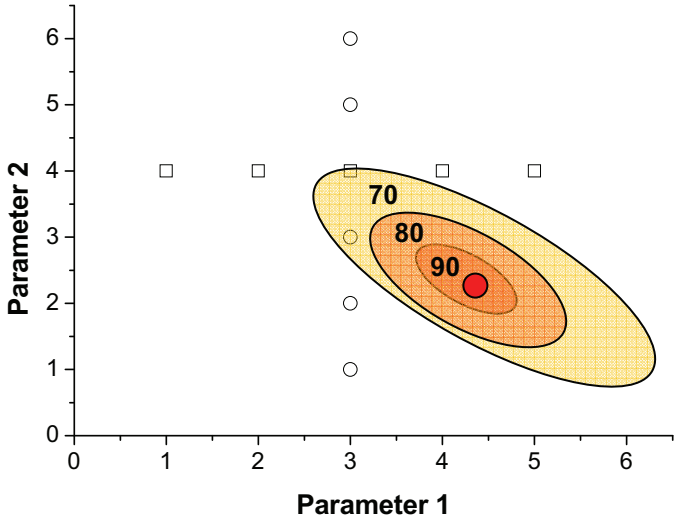


Figure 6-6: Example for the optimisation of ATPS by variation of one parameter at a time. Squares and circles are experimental points and mark subsequent experimental rows. If, e. g. first the squares are measured, the best point is taken for the next series of experiments marked by the circles. The optimum (illustrated by the coloured ovals with percentage of extraction efficiencies) is missed.

Due to the reasons stated above, the Box Wilson method of experimental design was used for optimising the purification of enzymes in IL-based ATPS. This method was first introduced by G. Box and K. Wilson in 1951 and belongs to a class of a more general methods called response surface methodology (RSM) (Box and Wilson 1951). RSM is a group of techniques used in the empirical study of relationships between one or more measured responses and a number of explanatory input variables. Using this technique, several parameters at a time are varied resulting in a reduction of the number of experiments while increasing the amount of information drawn from the obtained results at the same statistical validation (Kleppmann 2006). Box and Wilson suggested to use a first-degree polynomial model which can be estimated by a factorial experiment. This procedure is sufficient to determine which explanatory variables have an impact on the response variable of interest. When only significant explanatory variables are left, a more complicated design, such as a central composite design can be implemented to estimate a second-degree polynomial model which can then be used to optimise the response.

The design of experiments which should be performed to evaluate the coefficients of the model was done according to the Box Wilson composite rotatable design. Basically, the design includes ‘center points’ which are augmented with a group of ‘star points’ which allow estimation of curvature (Figure 6-7). The star points establish new extremes for the low and high settings for all factors. If the distance from the center of the design space to a factorial point is ±1 unit for each factor, the distance from the center to a star point is ±α with |α| 1.414.

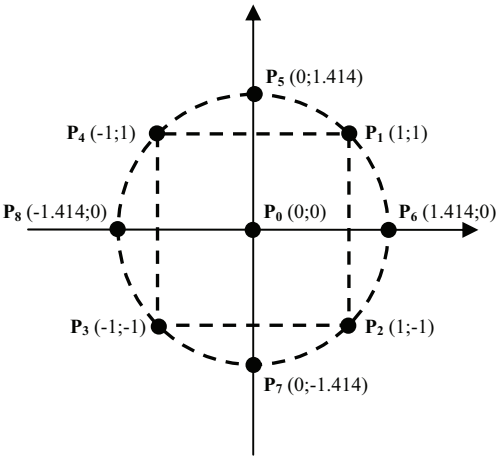


Figure 6-7: Schematic illustration of the design of experiments. A 2^2 factorial design with four star point (P_5, P_6, P_7, P_8) has been applied.

For optimisation of enzyme purification using IL-based ATPS, the concentration of ionic liquid (X_1) and inorganic salt (X_2) were set as independent values to be varied and specific enzyme activity was chosen as target parameter in a series of repeated partitioning experiments. The specific enzyme activity (U μg^{1} protein) represents an indicator for the purification of a catalytically active enzyme since the separation of undesirable protein results in an increase of specific activity. For the purpose of the experiment, the independent variables are each specified at five levels. Calculation of the variables X_i was done by coding them as x_i according to Equation 6-1 with x_i representing the coded value of the variable i, X_0 the value of the variable i at the center point of the investigated area, and ΔX_i the step size.

$$x_i \quad (X_1 \quad X_0)/\Delta X_i$$

Equation 6-1: Coding of the variables X_i for statistical calculations.

As an example, the real values of the independent variables in the first set of experiments are summarised in Table 6-4.

Table 6-4: Real values of the independent variables in the experimental plan (X_1: ionic liquid / wt %, X_2: inorganic salt / wt %).

Real values	**Coded values**				
	-1.414	-1.0	0.0	1.0	1.414
X_1	11.465	12.5	15	17.5	18.535
X_2	17.586	18	19	20	20.414

The analysis of experimental results by studying the pattern of responses was done by fitting a second-degree polynomial for describing the target value ‘specific enzyme activity’ in the IL-enriched upper phase. The model of regression is defined by Equation 6-2, where Y codes for the predicted target response ‘specific enzyme activity’, b_0 is the intercept term, b_i values are coefficients of the linear terms and b_{ij} values are coefficients of the quadratic terms.

$$Y \quad b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{12}x_1x_2 + b_{22}x_2^2$$

Equation 6-2: Model of regression for studying the pattern of responses.

Equation 6-2 can then be applied to obtain the relationship for calculating and predicting the specific enzyme activity in the upper IL-enriched phase. To exemplify this procedure, Equation 6-3 describes the fitted polynomial as obtained for the first set of experiments for optimisation of the extraction of LB ADH using IL-based ATPS.

$$Y \quad -99.684 + 2.1437x_1 + 9.1958x_2 \quad 0.022605x_1^2 \quad 0.082035x_1x_2 \quad 0.21185x_2^2$$

Equation 6-3: Second-degree polynomial for predicting specific activity of LB ADH in the upper phase of IL-based ATPS.

The parameter estimates and analysis are summarised in Table 11-2 (see appendix). The correlation coefficient obtained for the fitted model was R^2 0.771. A comparison of the experimental and predicted target value 'specific enzyme activity' in dependence on the applied concentrations of phase-forming compounds reveals a good agreement of results (Table 6-5).

Table 6-5: Comparison of the experimental and predicted results for specific enzyme activity of LB ADH in the upper phase of IL-based ATPS.

Ammoeng^{TM} 110 / wt %	**K_2HPO_4/KH_2PO_4 / wt %**	**Experimental specific activity / U µg^{-1} protein**	**Predicted specific activity / U µg^{-1} protein**
x_1	x_2	Y	Y
17.5	20	1.27	1.37
14	20	1.68	2.10
17.5	18	2.00	1.95
14	18	2.05	2.11
18.535	19	1.62	1.64
11.465	19	2.38	2.29
15	20.414	2.02	1.70
15	17.586	1.90	1.94
15	19	2.28	2.25
15	19	2.28	2.25
15	19	2.28	2.25
15	19	2.29	2.25
15	19	2.28	2.25
15	19	2.27	2.25

Graphical analysis of the results is demonstrated in Figure 6-8. The specific enzyme activity of LB ADH in the upper phase is plotted against the applied concentrations of ionic liquid and inorganic salt. Clearly, the experiments were already conducted in the maximum response area. Applying some Calculus, it can easily be shown that the function *Y* given in Equation 6-3 attains a local maximum at 10.6 wt % IL and 18.2 wt % inorganic salt.

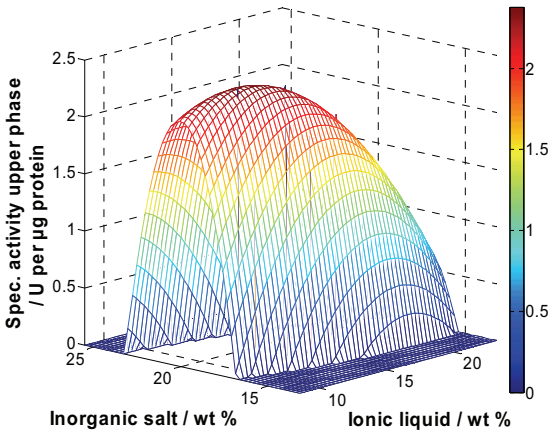


Figure 6-8: Illustration of the response surface as obtained for the partitioning experiments of LB ADH within IL-based ATPS.

Subsequently, the next level of experiments was designed using the calculated local maximum of specific activity at 10.6 wt % Ammoeng^{TM} 110 and 18.2 wt % inorganic salt as the center point of experiments. As step size, ΔX_{IL} was set to 0.7 wt % and ΔX_{Salt} was set to 1 wt %. The results of these experiments were again used to fit a second degree polynomial (Equation 6-4).

$$Y \quad -72.439 + 7.4396x_1 + 2.9317x_2 \quad 0.17330x_1^2 \quad 0.17744x_1x_2 \quad 0.011576x_2^2$$

Equation 6-4: Second round of experiments. Second degree polynomial for describing the specific enzyme activity in the upper phase.

The correlation coefficient R^2 between the experimental and predicted values for specific enzyme activity was 0.841 and a maximum enzyme activity of LB ADH could be found for a system containing 11.3 wt % IL and 19.2 wt % K_2HPO_4/KH_2PO_4. All in all, the purification factor for LB ADH was around 2.1 as a result of the separation of 46.5 % of total protein present in the cell extract. Regarding the enzyme activity in the IL-containing upper phase, specific activity could be increased up to 206.7 ± 5.3 % by a recovery of 95.0 ± 4.0 % of total activity (Table 6-6).

At this point it would be interesting to relate the observed partition behaviour of LB ADH to the models and predictions as described in chapter 5.6 and 5.7. Unfortunately, a comparison turns out to be unfeasible due to the reason that the enzyme LB ADH is present in a mixture of proteins and therefore facilitates the quantification of a single protein.

Table 6-6: Results for the purification of LB ADH by IL-based ATPS.

Ionic liquid / wt %	Salt / wt %	Partition coefficient *K* / -	Vol. activity[a] / %	Spec. activity[a] / %
0	0	-	100	100
11.3059	19.196	3.0 ± 0.1	440 ± 3.6	206.7 ± 5.3

[a] Results for the ionic liquid-enriched upper phase

To summarise, the results described above clearly prove that IL-based ATPS represent a biocompatible extraction method which can be used to selectively extract a targeted protein into the ionic liquid-containing upper phase while retaining or even stabilising its catalytic activity. The significant improvement of specific activity in the IL-containing upper phase strongly indicates that catalytically inactive proteins are separated to the lower phase. Additionally, the Box Wilson method of experimental design was found to be an applicable and useful approach for tracing down the optimum system conditions for enzyme partitioning by altering the concentration of phase-forming compounds.

6.3 Extraction of alcohol dehydrogenase from a thermophilic organism

Due to the successful application of the IL-based ATPS for the purification of LB ADH and the finding that the ionic liquid additionally stabilises the enzyme, further investigations were made on the extraction of a thermally unstable enzyme. Alcohol dehydrogenase from a thermophilic organism (T ADH) was chosen to realise this approach. It has been reported that during storage of T ADH in buffer the enzyme activity decreases significantly as indicated by a loss of volumetric activity from 167 U mL ^{1} to 85 U mL ^{1} within 30 min (Eckstein 2004). Similar to LB ADH, the enzyme can be applied for the enantioselective production of phenylethanol and is therefore of great industrial interest. However, the low stability and rapid deactivation of T ADH prevent the industrial application up to now.

6.3.1 Effect of the ionic liquid on T ADH activity and stability

First investigations were focused on the influence of Ammoeng^{TM}110 on the activity and stability of T ADH. As illustrated in Figure 6-9, the ionic liquid again showed an activity enhancing effect on the enzyme although the effect is not as pronounced as for LB ADH.

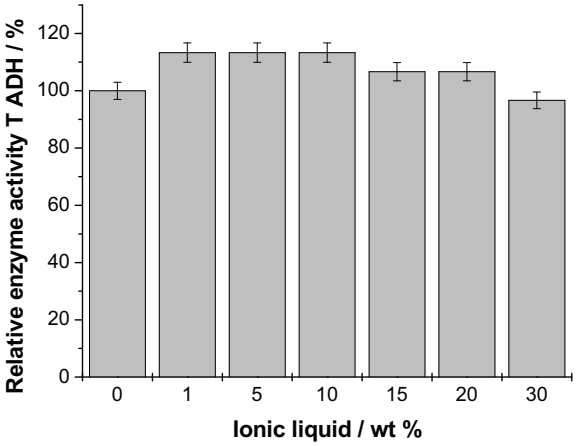


Figure 6-9: Enzyme activity of T ADH in the presence of different amounts Ammoeng^{TM}110 under standard conditions (30°C and pH 7).

With regard to the enzyme stability the effect of the ionic liquid revealed to be even more significant than for LB ADH. Starting from a very poor stability of the enzyme in pure aqueous solution ($t_{1/2}$ 0.5 h), the half life time of T ADH could dramatically be improved by the addition of the ionic liquid Ammoeng^{TM}110 to up to 36 days. Hence, the stabilising effect of the ionic liquid becomes even more apparent for thermolabile enzymes.

Table 6-7: Half life time of T ADH in the presence of different amounts ionic liquid Ammoeng^{TM} 110.

Concentration of IL in enzyme solution / wt %	**Half life time ($t_{1/2}$) of T ADH at 30°C / d**
0	0.015
1	0.39
5	1.35
10	8.0
15	9.6
20	24.1
30	36.1

6.3.2 Purification of T ADH by IL-based ATPS

Analogous to the procedure described for the optimisation of LB ADH extraction with IL-based ATPS, the purification of T ADH by using the Box Wilson method of experimental design was applied. Several rounds of experiments were conducted in order to optimise the partitioning of the target enzyme. The results of each set of experiments were used to fit a second-degree polynomial in order to predict the occurrence of a maximum response and to

create the best experimental conditions for the next set of experiments (see also appendix Equation 11-1, Table 11-3 and Figure 11-3). By using this approach, the best system composition for the purification of T ADH was found to be 10.6 wt % ionic liquid and 19.6 wt % inorganic salt, respectively. For the alcohol dehydrogenase T ADH 52.8 % of total protein were found to be extracted into the IL-containing upper phase. Moreover, specific activity in this phase was increased up to 403.4 ± 5.4 % and the recovery of total activity was 200.1 ± 4.0 % (Table 6-8). The observation of an increased recovery of total activity can be explained by the instability of T ADH which suffers from a strong activity loss during storage in phosphate buffer (Eckstein 2004). Hence, the purification of T ADH and its enrichment in the IL-containing upper phase takes advantage of the stability improving effect of Ammoeng^{TM} 110 that in turn explains the increase of recovered activity.

Table 6-8: Results for the purification of T ADH by an optimised IL-based ATPS.

Ionic liquid / wt %	**Salt / wt %**	**Partition coefficient *K* / -**	**Vol. activity[a] / %**	**Spec. activity[a] / %**
0	0	-	100	100
10.6059	19.61	3.0 ± 0.1	900 ± 3.8	403.4 ± 5.4

[a] Results for the ionic liquid-enriched upper phase

Within further experiments, the possibility of scaling-up the purification was investigated. By increasing the system volume by a factor of 3.5 (total system of 14 g), the optimum system composition was applied to purify T ADH on a larger scale and compare the obtained results with previous experiments. Not surprisingly, it was found that the extracted amount of protein as well as the specific activity of T ADH was the same as has been found during the smaller scale experiments. This observation is in good agreement with reports on PEG-based ATPS that allow a relatively straightforward scaling-up since the same partitioning can be obtained both in small laboratory scale and in large-scale extractions (Kula 1993). In conclusion, the optimum system composition of IL-based ATPS as obtained by experimental design should be suitable to bring forward the establishment of large-scale extractions of enzymes as well.

Additionally, it has to be emphasized that the stabilising effect of the IL present in the upper phase of ATPS can be utilised for enzyme storage. By simply separating the ADH-enriched and IL-containing upper phase of the optimised IL-based ATPS from the lower phase, it can be used as a storage medium for the enzyme. The approach renders possible an enhancement of the half life time of T ADH to 32 ± 4 days.

6.4 Further beneficial effects of the IL

As pointed out in chapter 6.1, the industrial application of ADHs is predominantly limited by the operational instability of enzymes, the insolubility of many of the substrates and products in water, substrate / product inhibition of the enzyme, the lack of stability of some substrates and products in aqueous solution and the difficulty of product and enzyme recovery from aqueous medium. In principle, most of these problems might be overcome by switching from pure aqueous to IL-containing reaction media. The first problem of restricted enzyme stability was already described above and could be overcome by the gentle extraction of T ADH using IL-based ATPS as well as the stabilising influence of Ammoeng^{TM} 110 on enzyme activity (chapter 6.3). Investigations related to the other obstacles are addressed in the following section.

6.4.1 Substrate solubility

The presence of the ionic liquid Ammoeng^{TM} 110 within the aqueous two-phase system exhibits additional advantageous effects especially with regard to the application of the IL- and enzyme-enriched upper phase for bioconversion. To start with, the ionic liquid Ammoeng^{TM} 110 can be used in order to enhance the solubility of hydrophobic substrates. Figure 6-10 exemplarily shows the increase of solubility of acetophenone (substrate of T ADH and LB ADH) in water and phosphate buffer enabled by the presence of ionic liquid. By adding 70 wt % IL to water, the concentration of solubilised acetophenone can be increased from 0.038 mol L^{-1} to 1.75 mol L^{-1}. This result is of special interest in the scope of industrial bioconversions due to the fact that many commercially interesting compounds are very poorly soluble in aqueous media.

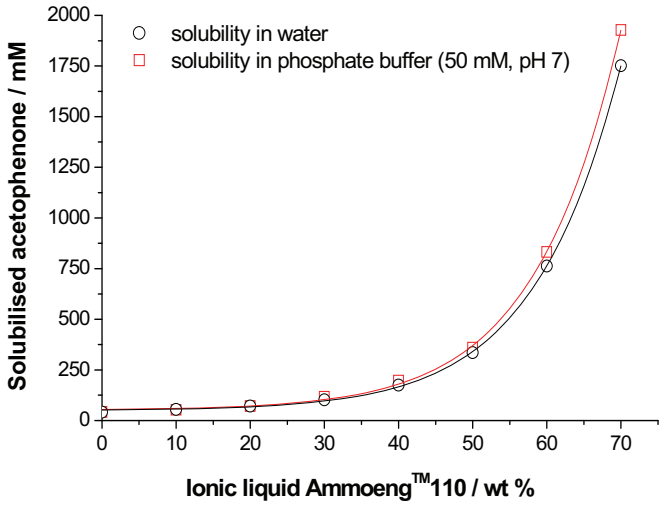


Figure 6-10: Solubility enhancing effect of the ionic liquid on acetophenone. The black line represents the concentration of acetophenone in water, the red line shows the solubility of acetophenone in phosphate buffer with increasing amount of ionic liquid present in the system at 25°C.

6.4.2 T ADH within biotransformation processes

With regard to the application of T ADH for biotransformation processes, further advantages can be drawn from the stabilising effect of the IL on the enzyme. Using the substrate-coupled cofactor regeneration within the bioconversion of acetophenone, the expensive cofactor can be regenerated and render the synthesis of *(S)*-1-phenylethanol more profitable (see also Figure 6-1, part 2). In theory the equilibrium conversion C_{calc} of acetophenone within the given system (using a cosubstrate/substrate ratio of 20) can be calculated from the substrates' oxidation-reduction potentials and is 90.0 % under the conditions used (Eckstein 2004). However, since the enzyme's half life time in phosphate buffer is very short (0.5 h at 30°C), the T ADH-catalysed reduction of acetophenone to *(S)*-1-phenylethanol is limited by the enzyme stability and only reaches experimental conversions up to 40 % (Figure 6-11, A) due to inactivation effects. However, the presence of 10 wt % ionic liquid within the reaction system provides a strong stabilising effect on T ADH and thus enables a significant increase of the conversion of acetophenone up to 91.0 ± 1.6 % (Figure 6-11, B). Moreover, at this point it has to be emphasized that the enantioselectivity of the T ADH-catalysed reaction remains high in the presence of all investigated concentrations of Ammoeng™110 (ee > 99 %).

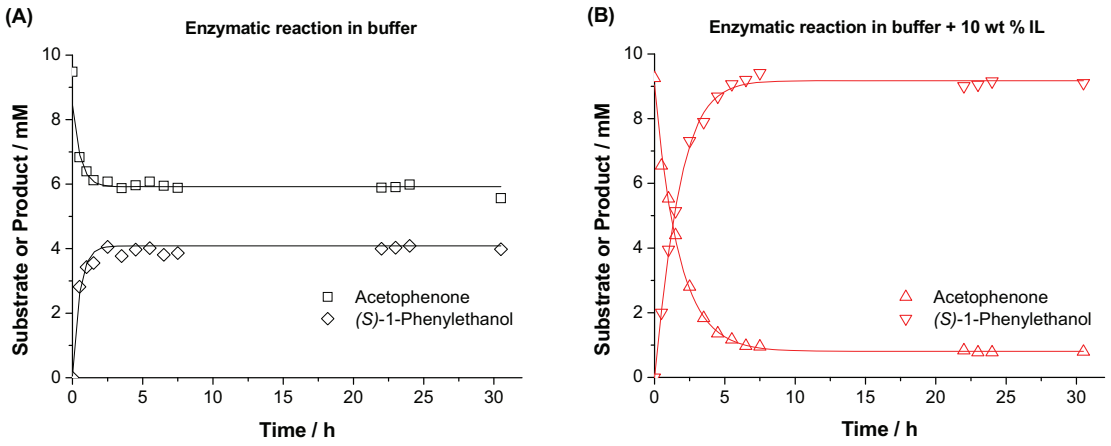


Figure 6-11: T ADH-catalysed reduction of 10 mM acetophenone with cofactor regeneration at pH 7 and 30°C, (A) reaction in phosphate buffer (50 mM, pH 7), (B) reaction in phosphate buffer containing 10 wt % Ammoeng™ 110.

To further point out the beneficial properties of the ionic liquid Ammoeng™ 110, Figure 6-12 demonstrates a combination of the substrate solubilising and enzyme stabilising effect of Ammoeng™ 110 on T ADH. The red line represents the conversion of 10 mM acetophenone in the presence of 10 wt % ionic liquid resulting in the formation of around 9.3 mM *(S)*-1-phenylethanol. The presence of IL within the system was then used to increase the solubility of the substrate acetophenone up to 60 mM (blue lines in Figure 6-12) and thereby enables the formation of 48 mM product by performing the enzyme-catalysed reaction. A comparison of the space-time yield (STY) or volumetric productivity for both reactions reveals that the addition of 10 wt % ionic liquid can be used to increase the maximum STY from 0.47 mol phenylethanol L^{-1} h^{-1} (10 mM acetophenone) up to 0.80 mol phenylethanol L^{-1} h^{-1} (60 mM acetophenone) after 1 h of reaction.

A closer look at the conversion of 60 mM substrate catalysed by T ADH reveals a restriction of conversion to 87 % though. In this context it must be emphasized that an increase of substrate concentration in the absence of ionic liquid results in a strong decrease of the catalytic activity of T ADH. By applying the enzyme in a buffer solution saturated with acetophenone (ca. 40 mM), the conversion was restricted to 4.8 % as a result of enzyme inactivation which can be attributed to substrate or cosubstrate inhibition effects. Therefore, it can be concluded that Ammoeng^{TM} 110 also provides a protecting effect against inactivation effects related to substrate inhibition.

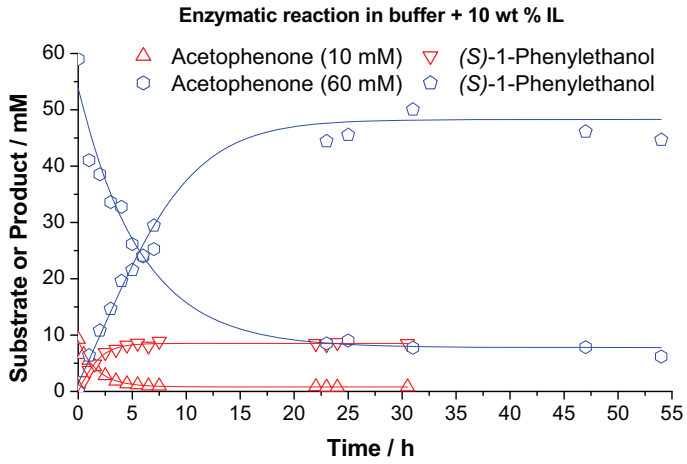


Figure 6-12: Combined effects of solubility enhancement and enzyme stabilisation of T ADH by the addition of 10 wt % ionic liquid. The cosubstrate 2-propanol was added in a 20-fold excess. Solid lines show the conversion of 10 mM acetophenone and broken lines the conversion of 60 mM acetophenone with cofactor regeneration at pH 7.0 and 30°C in the presence of 10 wt % IL.

A more detailed investigation on the influence of IL concentration on T ADH-catalysed conversion of acetophenone revealed that up to a concentration of 10 wt % IL the conversion gradually increases while maintaining the rate of conversion (Figure 6-13 A).

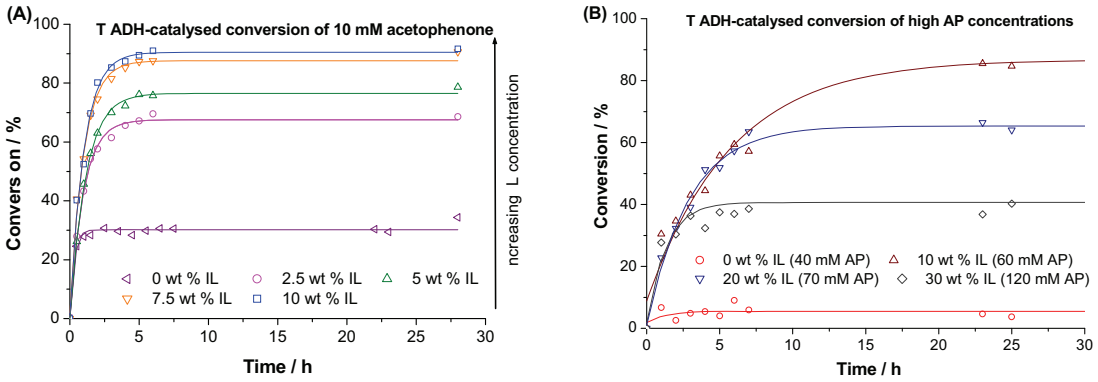


Figure 6-13: T ADH catalysed conversion of acetophenone at different concentrations of ionic liquid Ammoeng^{TM} 110. (A) Conversion of 10 mM acetophenone, (B) conversion of acetophenone (AP) saturated solutions.

This observation can be explained by the increasing stability of T ADH with increasing amount of IL. However, an increase in the concentration of acetophenone above 60 mM enabled by a higher concentration of IL results in a decrease of conversion rate and a reduced final product yield (Figure 6-13 B and Table 6-9). Since obviously an increased concentration of substrate and cosubstrate inhibits the enzyme, explanation can be found in the inactivating effect of these compounds which at high concentrations cannot be compensated by the stabilising effect of the IL. All in all it can be concluded that the ionic liquid Ammoeng^{TM}110 stabilises T ADH both against thermal denaturation and substrate inactivation.

Table 6-9: Conversion of increasing amounts acetophenone by T ADH.

Ammoeng^{TM} 110 / wt %	**Acetophenone / mM**	**Conversion after 54 h / %**	**Product amount after 54 h / mM**
0	10	40	4.0
0	40	4.8	1.9
10	10	91.0	9.3
10	60	89.5	53.7
20	71	66.5	47.2
30	117	45.4	53.1
40	197	21.9	43.1
50	359	3.2	11.5
60	832	1	8.3

Applying the upper T ADH-enriched phase of the optimised IL-based ATPS for the conversion of acetophenone, the observed advantages of enzyme stabilisation become evident and can be used to full capacity (Figure 6-14). The presence of around 27 wt % ionic liquid within the reaction system provides a strong stabilising effect on T ADH and enables a conversion of acetophenone up to 91.0 ± 1.9 %. Since the volumetric activity within the sample is increased by a factor of 9 due to the enrichment of T ADH in the IL-phase, the conversion rate is enhanced significantly. Increasing the substrate concentration to up to 60 mM still allows obtaining the maximum final conversion. However, further increases of substrate concentration result in a decrease of conversion rate and a reduced final product yield as has already been observed above. Additionally, the relatively high concentration of Ammoeng^{TM} 110 slightly decreases the conversion rate in comparison to a system containing 10 wt % IL (if the same volumetric enzyme activity is applied). This observation can be attributed to a higher viscosity of the medium and mass transfer limitations. Hence, when applying the upper phase of IL-based ATPS for the conversion of acetophenone catalysed by T ADH, a dilution of the sample has to be considered in order to ensure faster reaction rates. All in all, a compromise has to be found between the substrate concentration within the system, the rate of conversion and the final product yield.

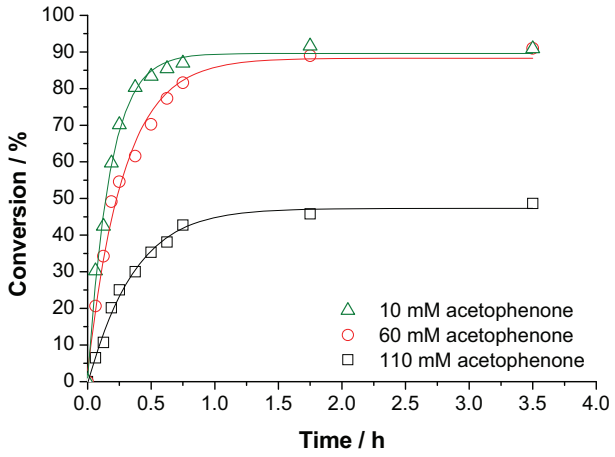


Figure 6-14: Application of the upper T ADH-containing phase of the optimised IL-based ATPS for the conversion of different concentrations acetophenone at 30°C and pH 7.

6.4.3 Recovery of product

Considering the process development with ATPS, a critical point to be addressed is the recovery of product from the phases. To separate proteins from the phase polymers in PEG-based ATPS, a number of methods can be used (Johansson 1994). If the protein and polymer differ strongly in their respective molecular weights (10 times or more), they can be separated by gel chromatography or ultrafiltration (Tjerneld et al. 1985; Veide et al. 1989). A more common way is to use ion-exchange chromatography (Johansson 1994). Other methods are those based on electrophoresis and two-phase techniques (Albertsson 1986; Johansson 1994; Tjerneld et al. 1987).

With regard to the IL-based ATPS, the enzyme-catalysed reaction can directly be performed by using the IL- and protein-enriched upper phase. Due to the stabilising effect of the ionic liquid on the enzyme, a separation of the biocatalyst from the phase components would be unreasonable. Separation of protein was therefore not further investigated but could theoretically be achieved e. g. by ultrafiltration and dialysis taking into account the huge difference in molecular weight between the proteins and the IL.

Nevertheless, an effective method for the extraction of product that is formed during the enzyme-catalysed reaction needs to be developed. In the case of T ADH-catalysed reduction of acetophenone, the product *(S)*-1-phenylethanol can easily be recovered by simple extraction with hexane yielding in an extraction efficiency of 41.5 ± 3.0 % within one extraction step. The extraction was also found not to be disturbed by the presence of ionic liquid (Figure 6-15). However, due to the reason that heptane is also a strong non polar which is less dangerous than hexane, future studies should consider the use of heptane as extracting agent.

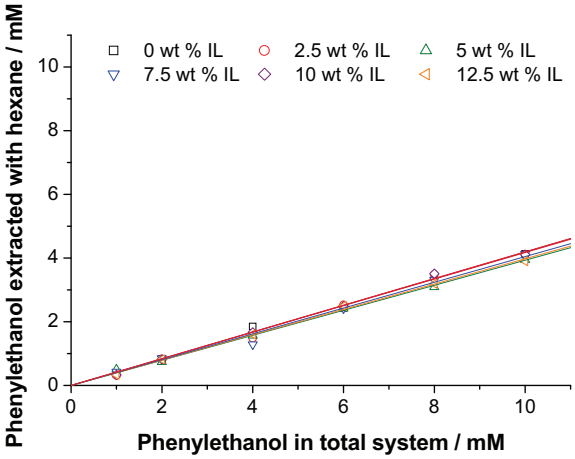


Figure 6-15: Extraction of *(S)*-1-phenylethanol by hexane in the presence of different amounts IL.

All in all it can be concluded that the application of IL-based ATPS for the purification of T ADH not only provides a suitable downstream method while retaining and stabilising enzyme activity, but indeed also renders the performance of T ADH-catalysed biotransformation processes possible. The main problems associated with the industrial application of T ADH can be overcome due to the solubility enhancing effect of the IL on hydrophobic substrates, the protective effect of the IL against inactivation and substrate inhibition effects and the easy recovery of product form the reaction medium.

6.5 Interims Summary

To underline the special advantages of IL-based ATPS, the purification of two different alcohol dehydrogenases, namely the *(R)*-specific alcohol dehydrogenase from *Lactobacillus brevis* (LB ADH) and the *(S)*-specific alcohol dehydrogenase from a thermophilic organism (T ADH), from crude cell extracts of *E. coli* was chosen as an example for an industrially interesting downstream process. At present, the industrial application of ADHs is predominantly limited by the operational instability of enzymes, the insolubility of many of the substrates and products in water and the difficulty of product and enzyme recovery from aqueous medium. In principle, most of these problems can be overcome by switching from pure aqueous to IL-containing reaction media as has been demonstrated within this chapter:

- The **purification** of LB ADH and T ADH was optimised with regard to the enrichment of the enzyme in the IL-containing phase. The **specific enzyme activity** in the upper phase could be increased to 206.7 ± 5.3 % for LB ADH and 403.4 ± 5.4 % for T ADH by variation of the concentration of phase-forming compounds. These results clearly prove that the IL-based ATPS represents a biocompatible extraction method which can be used to selectively extract a targeted protein into the IL-containing upper phase while retaining or even stabilising its catalytic activity.
- Additionally, the Box Wilson method of **experimental design** was found to be an applicable and useful approach for tracing down the optimum system conditions for enzyme partitioning by altering the concentration of phase-forming compounds.
- Interestingly, the presence of Ammoeng^{TM} 110 within the IL-based ATPS was found to be advantageous due to its **stability enhancing effect** on both ADH enzymes. In the presence of 30 wt % IL, the half life time of LB ADH was increased from 0.6 to 5.9 days and for T ADH from 0.015 to 36 days at 30°C. Additionally, a small increase in **activity** could be observed for both enzymes.
- Furthermore, Ammoeng^{TM} 110 provides a **solubility enhancing effect** on hydrophobic substrates. The amount of the ADH's substrate acetophenone within aqueous solution can be increased considerably by the addition of IL.
- Since substrate solubility and enzyme stability are both increased by the presence of IL, advantages become evident from the improved **conversion** (e. g. from 40 % to 91 % for T ADH) and **yield** within ADH-catalysed bioconversions. Additionally, it was found that **substrate inhibition** is reduced considerably for T ADH in reaction mixtures containing Ammoeng^{TM} 110.
- The **product** *(S)*-1-phenylethanol can easily be recovered from the reaction mixture by simple extraction with hexane.

All in all, IL-based ATPS take advantage of both the selective, gentle purification process of an aqueous two-phase extraction and the special properties of the applied ionic liquid. Hence, they offer the opportunity to combine the purification process of active biocatalysts with the performance of enzyme-catalysed reactions.

7 Summary

The development of new and innovative downstream methods for biomolecules is of great importance when seeking to meet the challenges of a growing market of industrial biotechnology. Therefore, the main objective of this thesis was to evaluate the potential of ionic liquid-based aqueous two-phase systems (IL-based ATPS) as an alternative or even superior purification technique. With respect to an industrial application, a special class of ionic liquids, called 'Ammoeng™' ILs, was chosen since these ILs are toxicologically listed and available in technical quantities at reasonable prices. The binodal curves of IL-based ATPS were determined and factors influencing the phase formation and composition (such as type of phase-forming compounds, temperature and pH) were investigated. In summary, it was found that the generation of an IL-based ATPS is mainly depending on the tendency of chaotropic salts to be salted-out by kosmotropic salts.

The successful industrial application of IL-based ATPS in downstream processing is clearly influenced by the ability to understand or even predict the partitioning of a target molecule between the phases. An IL-based ATPS, using Ammoeng™ 110 and K_2HPO_4/KH_2PO_4 as phase-forming compounds, has been used to investigate the main driving forces of protein partitioning within these systems. By studying the partitioning of different model proteins (myoglobin, lysozyme, trypsin and BSA), correlations between the protein- and system-properties could be found, thereby identifying electrostatic interaction as the main driving force of the partition process. Experiments on the distribution of amino acids within IL-based ATPS revealed similar trends as already observed for the model proteins. It has to be stated that partitioning of proteins within IL-based ATPS represents a complex phenomenon which can only be explained by a combination of several protein properties. Hence, a statistical model based on the proteins' physico-chemical properties has been established and provides a good start for predicting the protein partitioning in IL-based ATPS.

To underline the special advantages of IL-based ATPS, the purification of two different alcohol dehydrogenases (ADH) from crude cell extracts of *E. coli* was chosen as an example for an industrially interesting downstream process. Using experimental design, the purification of LB ADH and T ADH was optimised with regard to the enrichment of the enzyme in the IL-containing phase. The specific enzyme activity in the upper phase could be increased to 206.7 ± 5.3 % for LB ADH and 403.4 ± 5.4 % for T ADH by variation of the concentration of phase-forming compounds. Interestingly, the presence of Ammoeng™ 110 within the system was also found to be advantageous due to its stability enhancing effect on both ADHs. The half life time of LB ADH and T ADH was increased significantly in the presence IL. Furthermore, Ammoeng™ 110 provides a solubility enhancing effect on the hydrophobic substrate acetophenone. Since substrate solubility and enzyme stability are both increased, advantages become evident from the improved conversion (e.g. from 40 % to 91 % for T ADH) and yield within ADH-catalysed conversion. Moreover, substrate inhibition of T ADH was reduced considerably in reaction mixtures containing Ammoeng™ 110.

All in all it can be concluded that IL-based ATPS offer the opportunity to combine the purification process of active biocatalysts with the performance of enzyme-catalysed reactions thereby rendering these system an interesting alternative downstream processing method especially with regard to the application for industrial enzymes.

8 Outlook

Within the scope of this thesis, the applicability of ionic liquid-based aqueous two-phase extraction as an alternative or even superior downstream processing technique, especially for industrially interesting enzymes, has been proven feasible. In order to address the market potential of the IL-based ATPS developed within this thesis in particular, and future scientific research on IL-based ATPS in general, the outlook will be devided into two main parts dealing with these topics.

Market potential of IL-based ATPS

The great potential of IL-based ATPS clearly arises from the opportunity to combine the gentle purification process of active biocatalysts with the performance of enzyme-catalysed reactions by simply employing the IL-enriched, enzyme-containing upper phase within biotransformation processes. Hence, the prospective industrial application of IL-based ATPS is most likely anticipated to be within the field of biocatalysis. Today, development of downstream processes is offered by several small companies like bioURETIKON or Angel Biotechnology. The applied methods are mainly based on chromatography and/or ultrafiltration. The purification of enzymes by extraction with ILs has to compete with these techniques (Table 8-1). Especially the commonly used chromatographic procedures have several drawbacks which can be overcome by IL-based ATPS: The aim of the first step of protein separation is the removal of water (concentration of protein) and the change of media to provide a protease-free environment to stabilise the target protein. This step has to be as fast as possible to prevent degradation of the target protein catalysed by proteases. Assuming a thorough mixing, extraction using IL-based ATPS separation is much faster than any chromatographic procedure. Furthermore, it can be claimed that the development of chromatographic procedures did not keep up with the development of fermentation procedures during the last years. Especially the high product concentrations obtained in fermentations today (about 10 g L^{1}) and the high batch volumes (up to 20000 L) can turn out to be problematic. For ion exchange chromatography the fermentation solutions often have to be diluted by a factor of 3 to 6 to meet the requirements of pH value and conductivity of the solution. This may be reasonable at lab scale, but at industrial scale large amounts of buffer for dilution and large storage capacities have to be provided. Furthermore, the costs for chromatographic bed materials are dispensed. Expenses for the applied ILs can be minimised by recycling of the IL phase. In addition, ILs have become much cheaper during the last years thanks to improved production processes and large scale production.

Another chromatographic method, called affinity chromatography, is often used for the purification of monoclonal antibodies. The separation has to be performed at pH values < 3, which can lead to agglomeration and denaturation of the target protein. The separation by IL-ATPS is clearly superior because it works at moderate pH values between 6 and 8. A disadvantage which all chromatographic methods share is the large amount of solvents needed. For protein separation aqueous buffers, acids and bases are required not only for separation and elution, but also for regeneration of the column packing. Usually the amount of several bed volumes is required. As mentioned before, the volume of IL needed for extraction and IL-ATPS is much smaller, which results in lower chemical and equipment costs.

Furthermore, the low vapour pressure and flammability of ILs represent advantages with respect to environmental and safety aspects.

Table 8-1: Downstream processing of different product classes as performed today and by IL-based ATPS.

Product Class	**Examples**	**Downstream Processing Today**	**Advantages of IL-based ATPS Downstream Processing methods**	**Advantages for Customers and Society**
Proteins	Ovalbumin (proteomics, immunology)	Ultracentrifugation	Reduced solvent consumption	Cheaper and faster overall processes
	Soybean proteins (food nutrition)	Microfiltration	Better atom efficiency (due to IL recycling)	Advance towards more sustainable production processes
	Gluten (bakery)	Dialysis	Reduced energy consumption	Reduced CO_2 emission
	Collagen (cosmetic surgery)	Evaporation	Reduced equipment investments	Cheaper pharmaceuticals
	Monoclonal antibodies	Crystallisation	Faster processing	Reduced waste
Enzymes	Proteases (detergents)	Adsorption	Stabilisation of enzymes	
	Trypsin (leather industry)	Precipitation	Combination with enzyme-catalysed reaction	
	Papain (food industry)	ATPS	Improved yield and efficiency of biotransformations	
	Cholesterol esterase (pharmaceutical use)	Ion exchange / hydrophobic / interaction and affinity chromato-graphy	Less substrate / product inhibition	
	Glucose oxidase (pharmaceutical use)	Membrane filtration		
Amino acids	L-Glutamic acid (cosmetics, dietry supplement)			
	L-Lysine (food nutrition)			
	L-Threonine (pharmaceutical use)			
	L-Methionine (food nutrition)			

To mention an example, the recombinant production of alcohol dehydrogenases is usually performed in *E. coli* cells. The purification of enzyme from the cell extract is carried out by hydrophobic chromatography or interaction chromatography, followed by subsequent anion exchange and affinity chromatography. This procedure allows the purification of the target protein to homogeneity and the subsequent application of the enzyme for biotransformation processes. However, for many industrial bioconversions, a complete purification of the enzyme is not necessary as long as no side reactions or interfering contaminants are observed during the enzyme-catalysed reaction. In this context, IL-based ATPS offers the potential to integrate several unit operations into one process step - thereby saving time and costs. By applying the IL-ATPS the target enzyme can easily be extracted from a protein mixture and the upper phase can directly be used as reaction medium for the following bioconversion process (Table 8-2). In this context, the stabilising and solubility enhancing of the ionic liquid present in IL-based ATPS make the advantage of applying these systems even more apparent.

Table 8-2: Comparison of downstream processing of recombinantly produced enzymes by today methods and IL-based ATPS methods.

	Today's Downstream Processes	**IL-based ATPS Downstream Processes**
Procedure	Hydrophobic chromatography / interaction chromatography	Mixing of phase components and phase separation
	Anion exchange / affinity chromatography	Application of enzyme-containing upper phase for biotransformation
	Application of pure enzyme for biotransformation	
Equipment / Chemicals	Chromatographic bed materials (€ 500/kg)	Flasks / extractor
	Equilibration solution / eluent / washing solution / regenerating solution (€ 1/L)	Ionic Liquid (€ 30/kg), inorganic salt (€ 10/kg)
	High energy costs for equipment	Recycling of ionic liquid possible
Time	Hours – days	Minutes

The worldwide market for white biotechnology products was estimated to be nearly € 77 billion in 2005 (Caesar 2008). Since the demand for biotechnology products is on the rise, greater manufacturing capacities as well as more efficient downstream processes will be required in the near future. Due to the fact that the downstream processes account for 10-80 % of the manufacturing costs, innovative and effective new downstream technologies have a great potential by meeting the challenges of the growing market. The IL-based ATPS techniques can be applied in three major applications: downstream processing of proteins, enzymes and amino acids. As for the downstream processing of enzymes the market for IL-based ATPS is estimated to be about € 67 million. Enzymes for industrial use have a market of € 1.7 billion (Novozymes, 2006). Out of this, 40 % amount for downstream processes of which 10 % could presumably be replaced by IL-based ATPS.

Future research

However, in order to bring forward the industrial application of IL-based ATPS several issues need to be addressed first. It has to be pointed out that the transferability of IL-based ATPS for the purification of enzymes from other enzyme classes needs to be confirmed in further studies. First experiments on the distribution of Penicillin amidase (EC 3.5.1.11) were performed in the scope of this thesis and have already indicated the maintenance of activity within IL-based ATPS for enzymes other then alcohol dehydrogenases. In this context, it has to be emphasized that each individual protein to be extracted will necessitate an individual optimisation of the system itself with regard to the requirements for optimum catalytic activity. Using the Box Wilson method of experimental design as described in this thesis, the optimisation process can at least be significantly facilitated. Moreover, due to the gentle purification process and the stabilising effects as provided by IL-based ATPS, the application of these systems for the purification of instable proteins such as membrane-bound enzymes is of considerable interest as well. These play an important role as diagnostic tools or for drug testing experiments and therefore hold an enormous potential within pharmaceutical research.

With regard to the large-scale application of IL-based ATPS, naturally further experiments on the scalability of these systems will be required. Scaling-up on a small scale could successfully be shown within this thesis and has been reported to be relatively straightforward for ATPS in general. Nevertheless, investigations on continuous operation modes using IL-based ATPS would be of great interest for commercial purposes. For example, continuous counter-current column operation could provide operating convenience for contacting two-phase aqueous partitioning systems for enzyme extraction. Moreover, to render industrial processes more profitable and with regard to environmental concerns, an adequate method for recycling of the phase-forming compounds needs to be achieved. This could be done e. g. by ultrafiltration. In this context, a general account on the estimation of process costs in comparison to already established downstream methods has to be addressed. The cost of ionic liquid will probably provide the greatest hurdle to commercial progress of IL-based ATPS and has to be compared to the advantages which can be drawn from the system.

Besides the practical application of IL-based ATPS processes for the recovery of enzymes, the purification of pharmaceutical compounds such as monoclonal antibodies is of great interest within the field of biotechnology. The potential use of IL-based ATPS will, however, necessitate additional purification steps in order to meet the required purity standards. For a therapeutic use of proteins, the IL-based ATPS therefore needs to be combined with more selective procedures such as chromatography steps. Moreover, the purification of non-protein products like aroma compounds or metal ions could be of interest for industrial applications. Additionally, it is anticipated that a new potential trend of IL-based ATPS technique could be emerging from their extended use as analytical tools. By increasing the knowledge of protein partitioning in relation to structure, partitioning in IL-based ATPS could be applied in order to give further insight into conformational changes, oligomerisation of proteins and structure-function relationships. This approach could be realised e. g. by building a structurally detailed dynamic model of a protein from ^{15}N relaxation data and studying the effect of varying concentrations of IL on the protein in order to provide further evidence on protein mobility or protein/solute interactions.

On a scientific basis, the results obtained within this thesis lead to new insights into protein-IL interactions. Having high-flying expectations, a deeper understanding of these interactions, which could be provided by further experiments on e. g. amino acid partitioning at different pH values, will help on the way of solubilising proteins in neat, taylor-made ILs without losses in activity. This in turn will enable an enhanced technological utility of the biomolecules and offer an even greater market for biocatalysis.

The general observation of a stabilising effect of ionic liquids on proteins and enzymes can also be brought forward to the application within the stabilisation of whole cells. To state an example, ionic liquids can be used for the stabilisation and storage of mammalian cells such as erythrocytes[7]. Acting in a similar way as compatible solutes, the cells can be prevented from dehydration and losses of biological activity thereby increasing the storage stability within e. g. blood preservation.

[7] Experiments done on the stabilisation of erythrocytes by ionic liquids have been preformed in the scope of this dissertation. These results are not included within this thesis but have led to a patent application: Altrichter J, Kragl U, Dreyer S. (**2008**). Stabilisierung von Zellen durch ionische Flüssigkeiten (Stabilisation of cells by ionic liquids). *German patent application* 10 2008 039 734.2.

9 Materials and methods

9.1 Materials

Table 9-1: Amino acids, proteins and enzymes used within this thesis.

Substance	Supplier
Lysozyme from chicken egg white (code L6876), Lot 096K1237	Sigma Aldrich, Germany
Myoglobin from equine heart (code M1882), Lot 056K7008	Sigma Aldrich, Germany
Albumin from bovine serum (code A9418), Lot 057K12011	Sigma Aldrich, Germany
Trypsin from bovine pancreas (code 93610), Lot S39871-337	Sigma Aldrich, Germany
Pepsin from porcine gastric mucosa (code P7012), Lot 074K7718	Sigma Aldrich, Germany
Hemoglobin from bovine blood (code 51290), Lot 010K7618	Sigma Aldrich, Germany
ADH from *Lactobacillus brevis* (LB ADH); cell lysate	Jülich Fine Chemicals, Germany
ADH from thermophilic organism (T ADH); cell lysate	Jülich Fine Chemicals, Germany
L-amino acid kit, Lot 1297881 522506155	Sigma Aldrich, Germany

Table 9-2: Chemicals used within this thesis.

Substance	Supplier
Ammoeng^{TM} 100, Lot 537	Solvent Innovation, Germany
Ammoeng^{TM} 101, Lot 538	Solvent Innovation, Germany
Ammoeng^{TM} 102, Lot 99/632	Solvent Innovation, Germany
Ammoeng^{TM} 110, Lot 99/611, Lot 99/641	Solvent Innovation, Germany
Ammoeng^{TM} 111, Lot 99/637	Solvent Innovation, Germany
Ammoeng^{TM} 112, Lot 542	Solvent Innovation, Germany
Ammoeng^{TM} 120, Lot 99/546	Solvent Innovation, Germany
Ammoeng^{TM} 130, Lot 545	Solvent Innovation, Germany
Ammoeng^{TM} 520, Lot 543	Solvent Innovation, Germany
K_2HPO_4	Merck KGaA, Germany
KH_2PO_4	J. T. Baker, Netherlands
KOH	VWR, Germany
$(NH_4)_2SO_4$	Sigma Aldrich, Germany

Table 9-2: Chemicals used within this thesis. Continued.

Substance	Supplier
$MgCl_2 \cdot 6\ H_2O$	University Rostock
PEG6000	Merck KGaA, Germany
Protein Stabilizer Evaluation Kit, Lot 0716 (N_ε-Acetyl-L-Lysin, L-Carnitine, L-Proline, Betaine, Ectoine, Hydroxyectoine)	Biomol GmbH, Germany
Acetophenone; for synthesis	Merck KGaA, Germany
(*R*, *S*)-1-phenylethanol; for synthesis	Merck KGaA, Germany
NADPH tetrasodium salt; 96.4 %	Jülich Fine Chemicals, Germany
Bradford Reagent	Sigma Aldrich, Germany
NANOCOLOR ortho-Phosphate Standard test Chemical kit	Macherey-Nagel, Germany
NuPAGE® 10 % Bis-Tris Mini Gel	Invitrogen, Germany
NuPAGE® LDS Sample Buffer (4x)	Invitrogen, Germany
NuPAGE® Reducing Agent (10x)	Invitrogen, Germany
Novex SeeBlue® Pre-Stained Standard	Invitrogen, Germany
SimplyBlue Safe Stain	Invitrogen, Germany
EZ:faast™ kit for analysis of free amino acids	Phenomenex, Germany
10 mM ammonium formate in water	Riedel-de Haen, Germany
10 mM ammonium formate in methanol	Riedel-de Haen, Germany
2-propanol; > 99 %	J. T. Baker, Netherlands
n-hexane; 95 %	J. T. Baker, Netherlands
Acetone	Sigma Aldrich, Germany

Table 9-3: Equipment used within this thesis.

Apparatus	Supplier
Photometer Specord 200 UV-Vis	Analytik Jena, Germany
Capillary Ubbelohde viscosimeter (DIN); filling capacity 15 – 20mL, overall length 290mm, type 50113, capillary Ic, viscosity range 3 - 30 $mm^2\ s^{-1}$	Schott Instruments GmbH, Germany
HPLC pump K-1001	Knauer, Germany
HPLC column Chiralcel OD-H 5 µm	Daicel, Japan

Table 9-3: Equipment used within this thesis. Continued.

Apparatus	Supplier
HPLC column Nucleosil 100 C18	Macherey-Nagel, Germany
HPLC Variable Wavelength Monitor	Knauer, Germany
Software Eurochrom 2000	Knauer, Germany
LC MS Pump Plus-Finnigan Surveyor	Thermo Finnigan, Germany
LC MS Autosampler Plus-Finnigan Surveyor	Thermo Finnigan, Germany
UV-Detector PDA Plus - Finnigan Surveyor	Thermo Finnigan, Germany
MS-Detektor Finnigan LTQ	Thermo Finnigan, Germany
Software Xcalibur (Version 2.0 SR2)	Thermo Finnigan, Germany
Electrophoresis equipment	Sigma Aldrich, Germany

9.2 Methods for the characterisation of IL-based ATPS

In order to ensure reproducibility all experiments were carried out at least three times and the mean value of the obtained results was calculated.

9.2.1 Screening of ionic liquids

The general applicability of ionic liquids from the Ammoeng™ series for the formation of aqueous two phase systems was investigated by solubilising different amounts of IL in an aqueous solution (5 50 wt %) and adding increasing amounts of the inorganic salt mixture K_2HPO_4/KH_2PO_4. If the formation and separation of two aqueous phases was observed, the IL was classified as being capable to be used as phase-forming compound.

9.2.2 Determination of binodal curves

The binodal curves of ionic liquid-based aqueous two phase systems were determined by the cloud point method (Albertsson 1986). The weight of the added ionic liquid and inorganic salt in order to form a two phase system was determined using an analytical balance and is referred to as weight of IL or inorganic salt per weight of the whole system (wt %).

To determine the binodal curves different amounts of the ionic liquids (Ammoeng™ 100, Ammoeng™ 101 and Ammoeng™ 110) were weighed into a test tube and dissolved in water in order to obtain stock solutions with known amounts of ionic liquid. Subsequently, inorganic salt was added step by step to the aqueous IL-solution and vortexed until complete solubilisation. The point where the mixture became turbid or cloudy was noted and the amount of salt added was determined. Deionised water was then added until the solution

became clear and the mass of the mixture was noted. Afterwards inorganic salt was added till the mixture was turbid again and the above procedure was repeated over a whole range of concentrations. The same experiment was carried out starting from different stock solutions of dissolved inorganic salt in water and adding different amounts of ionic liquid until turbidity of the system occurred. Inorganic salts investigated were KOH, K_2HPO_4, KH_2PO_4 and a mixture of the inorganic salts K_2HPO_4 and KH_2PO_4 at a mass ratio of 1.82 : 1.00, respectively. In the following the salt mixture is always referred to as the inorganic salt K_2HPO_4/KH_2PO_4.

Furthermore the same experiments were conducted at different temperatures by storing the samples in a water bath in order to investigate the influence on phase separation and thus the location of the binodal curve.

9.2.3 Investigation of the system composition

In order to investigate the phase formation and composition of IL-based ATPS, the pH, the temperature and the concentration and type of phase-forming compounds was varied. The influence on phase volume was determined by measuring the phase height by a calliper gauge and calculating the phase volume by using Equation 9-1 that was fitted for the vials used within these experiments.

$$V = \left(\frac{\pi}{4}\right) d_{inside}^2 \times \left(h - h_{bottom}\right) \qquad [cm^3]$$

Equation 9-1: Calculation of the phase volume by measuring the phase height (d = diameter of vials = 1.2785 [cm]; h_{bottom} = thickness of the vial-bottom = 0.09475 [cm]; h = height of phase [cm].

Samples from the top and the bottom phase were carefully withdrawn and analysed for their content of phase-forming compounds. The amount of inorganic salt was investigated by using the NANOCOLOR ortho-Phosphate Standard Test Chemical kit from Macherey-Nagel (Düren, Germany). Determination of ionic liquid within the upper phase of IL-based ATPS was carried out via the Bradford assay. The interaction of the ionic liquid Ammoeng™ 110 with the dye Coomassie Brilliant Blue G-250 results in a maximum of absorption at 595 nm and shows a linear correlation to the quantity of ionic liquid up to a concentration of 15 wt % IL. The concentration of ionic liquid can be determined with an accuracy of ± 0.4 wt % by this method. A calibration curve was determined for different amounts of the ionic liquid Ammoeng™ 110 in aqueous solution and used for the calculation of IL content in the samples: 1000 µL of an adequately diluted sample was mixed with 1000 µL Bradford reagent and incubated at 20°C for 15 min. Afterwards the absorption at 595 nm was determined by a photometer (Specord 200, AnalytikJena, Germany).

9.2.4 Viscosity measurements

Viscosity measurements of the phases of IL-based ATPS were performed by using an Ubbelhode viscosimeter with a filling capacity of 15 20 mL, overall length of 290 mm and a viscosity range of 3 - 30 $mm^2\ s^{-1}$. The viscometer was charged by introducing a sample from the top or bottom phase of IL-based ATPS through tube 1 into the lower reservoir resulting in

a liquid level between A and B (Figure 9-1). Afterwards, the viscometer is placed into a holder and inserted into a constant temperature water bath. The sample is incubated in the water bath until the temperature of the samples is constant. Tube 3 is sealed by a plug and suction is applied by tube 2 until the liquid level reaches the middle of the top bulb in tube 2. Subsequently, suction is removed from tube 2 and the plug is removed from tube 3. To measure the efflux time, the liquid sample is allowed to flow freely down past mark D, measuring the time for the meniscus to pass from mark C to mark D. The kinematic viscosity ν of the sample is calculated by multiplying the efflux time by the viscometer constant (0.03014 mm^2 s^{-2}). Dynamic viscosity η can be calculated by multiplication of the kinematic viscosity ν with the fluid density ρ which was determined by weighing defined volumes of the samples on an analytical balance.

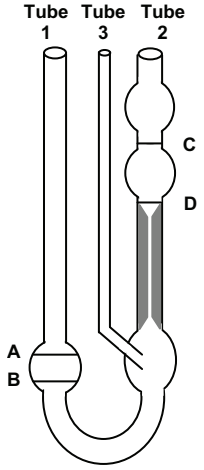


Figure 9-1: Schematic illustration of the Ubbelohde capillary viscometer.

9.3 Methods for investigating protein partitioning in IL-based ATPS

The standard system to investigate the driving forces of protein partitioning within ionic liquid-based aqueous two phase systems consisted of the ionic liquid Ammoeng™ 110 (12 wt %) and a mixture of the inorganic salts K_2HPO_4 and KH_2PO_4 (20 wt %) at a ratio of 1.82 : 1.00. To give a total system weight of 4 g, the ionic liquid Ammoeng™ 110 (0.48 g) was weighed into a glass vial and distilled water (2.62 g) was added. Afterwards, K_2HPO_4/KH_2PO_4 (0.8 g) was added and the vials were mixed until complete solubilisation of the phase-forming compounds. Subsequently, the protein solution (0.1 g) was added containing either myoglobin (1 mg mL^{-1}), lysozyme (2 mg mL^{-1}), trypsin (2 mg mL^{-1}) or BSA (2 mg mL^{-1}). The sample was mixed for 10 min on a horizontal shaker and phase separation was achieved by centrifugation or storage of the samples at a given temperature. Afterwards, the volume of each phase was measured by a calliper gauge and calculated by Equation 9-1, which was fitted for the vials used within these experiments. The distribution of proteins between the phases is described by the logarithm of the partition coefficient K, which is defined by Equation 9-2, where C_U and C_L represent the protein concentration in the upper and lower phase, respectively.

$$K = \frac{C_U}{C_L} \qquad [-]$$

Equation 9-2: Calculation of the partition coefficient.

9.3.1 Quantification of protein

The amount of protein within the two phase systems was determined by photometric measurements (Specord 200, AnalytikJena, Germany). A calibration curve was determined for each model protein by weighing different amounts of protein in water and determining the absorption at 280 nm for BSA, trypsin and lysozyme and 408 nm for myoglobin. The maximum of absorption at these wavelengths shows a linear correlation to the quantity of protein even in the presence of up to 30 wt % IL. Furthermore, for the determination of protein concentration in the presence of ionic liquid, one protein-free sample was prepared containing the same amount of ionic liquid and inorganic salt and used as a blank.

9.3.2 Effect of temperature

The model system to evaluate the effect of temperature on protein partitioning consisted of Ammoeng^{TM} 110 (12 wt %) and K_2HPO_4/KH_2PO_4 (20 wt %; ratio of salts 1.82 : 1.00) and was prepared as described above. Protein containing solution was prepared (1 mg mL ^{1} for myoglobin, 2 mg mL ^{1} for lysozyme, trypsin and BSA) and 0.1 g was added to the system. The sample was mixed for 10 min on a horizontal shaker. Phase separation was induced by storing the samples in a water bath for 1 2 h at a temperature of 4°C, 20°C, 40°C, 60°C and 70°C, respectively. Afterwards the volume of each phase was measured by a calliper gauge and samples from the top and the bottom phase were carefully withdrawn and analysed for their protein content.

9.3.3 Effect of pH

In order to investigate the influence of pH, an IL-based ATPS consisting of Ammoeng^{TM} 110 (12 wt %) and inorganic salt (20 wt %) in water was prepared. The pH of the systems was adjusted by variation of the salt ratio (Table 9-4). The phase-forming compounds were completely solubilised in water giving a total system weight of 3.9 g. Afterwards 0.1 g of the protein containing solution was added (1 mg mL ^{1} for myoglobin, 2 mg mL ^{1} for lysozyme, trypsin and BSA) and the sample was mixed thoroughly for 10 min. Phase separation was induced by storing the samples at 4°C for 1-2 h. Afterwards the volume of each phase was measured and samples from the top and the bottom phase were analysed for their protein content.

The total net charge of the model proteins at a given pH was calculated by using a web-based computer program (http://biophysics.cs.vt.edu/H++/index.php). The program takes as input a Protein Data Bank file (PDB, http://www.rcsb.org/pdb) and calculates the protein's total charge at given physical conditions of salinity (0.15), and internal and external dielectric (20 and 80, respectively) by using the Poisson-Boltzmann equation. PDB files used for calculations were 1ymb (myoglobin), 1dpx (lysozyme), 2g55 (trypsin) and 1ao6 (BSA).

Table 9-4: Ratio of salts used to adjust the system pH of IL-based ATPS composed of 12 wt % Ammoeng™ 110 and 20 wt % potassium salt.

pH of IL-based ATPS	Ratio of salts	
/ -	K_2HPO_4	KH_2PO_4
9	1.00	0
8.31	1.00	10.28
7.89	1.00	4.64
7.56	1.00	2.76
6.8	1.82	1.00
6.4	1.00	1.00

9.3.4 Calculation of surface area and hydrophobicity

The computer program GETAREA 1.1 (http://pauli.utmb.edu/cgi-bin/get_a_form.tcl) was employed to calculate the solvent accessible surface areas (ASA) of amino acid residues in the proteins as described by Fraczkiewicz and Braun (Fraczkiewicz and Braun 1998). The program takes as input a Protein Data Bank (PDB) file and a probe radius value, the default value 1.4 Å representing a water molecule. In order to calculate the surface hydrophobicity, different hydrophobicity scales were applied (Table 9-5). Assuming that each amino acid residue on the protein surface has a relative contribution to the surface properties, the exposed relative surface areas (r Σr_i) of each amino acid were calculated and multiplied with the assigned value in the utilised hydrophobicity scale by using Equation 9-3:

$$H = \sum_{i=1}^{20} r_i h_i$$

Equation 9-3: Calculation of the surface hydrophobicity of proteins.

The relative surface area is defined as r_i S_i/S, where S_i represents the surface area of the amino acid and S the total surface area of the protein. H is the calculated value of the surface hydrophobicity.

Table 9-5: Hydrophobicity values of the model proteins as calculated by the different hydrophobicity scales. [a] H_1 = Surface → Interior (Janin 1979), [b] H_2 = Octanol → Water (Radzicka and Wolfenden 1988), [c] H_3 = Hydropathy (Kyte and Doolittle 1982; Radzicka and Wolfenden 1988), [d] H_4 = Cyclohexane → Water (Radzicka and Wolfenden 1988), [e] H_5 = Peptide Slope System 1 (Berggren et al. 2002), [f] H_6 = Peptide Slope System 2 (Berggren et al. 2002), [g] H_7 = 1/m* (Hachem et al. 1996).

Amino acid	H_1[a]	H_2[b]	H_3[c]	H_4[d]	H_5[e]	H_6[f]
Ala	0.3	0.52	1.8	1.81	0.017	0.018
Arg	-1.4	-1.32	-4.5	-14.92	-0.031	-0.031
Asn	-0.5	-0.01	-3.5	-6.64	0.042	0.073
Asp	-0.6	-0.79	-3.5	-8.72	-0.003	0.006
Cys	0.9	0.52	2.5	1.28	0.017	0.018
Gln	-0.7	-0.07	-3.5	-5.54	0.042	0.073
Glu	-0.7	-0.79	-3.5	-6.81	-0.003	0.006
Gly	0.3	0	-0.4	0.94	0	0
His	-0.1	0.95	-3.2	-4.66	-0.021	-0.028
Ile	0.7	2.04	4.5	4.92	0.044	0.057
Leu	0.5	1.76	3.8	4.92	0.044	0.057
Lys	-1.8	0.08	-3.9	-5.55	-0.031	-0.031
Met	0.4	1.32	1.9	2.35	0.017	0.018
Phe	0.5	2.09	2.8	2.98	0.195	0.265
Pro	-0.3	0.52	-1.6	1.81	0.017	0.018
Ser	-0.1	0.04	-0.8	-3.4	0.017	0.018
Thr	-0.2	0.27	-0.7	-2.57	0.017	0.018
Trp	0.3	2.51	-0.9	-0.14	0.253	0.472
Tyr	-0.4	1.63	-1.3	2.33	0.216	0.29
Val	0.6	1.18	4.2	4.04	0.044	0.057

9.3.5 Hydrophobicity by protein precipitation

The method described by Hachem et al. (1996) was used for experimental determination of protein hydrophobicity. Protein (2 mg mL ^{1}) was dissolved in sodium phosphate buffer (pH 7, 0.05 M) and different amounts of ammonium sulphate were added to reach a salt concentration between 0.5 an 4 M (Hachem et al. 1996). After all salt was dissolved, the samples were left to equilibrate for 15 min at room temperature and then centrifuged at 14000 rpm for 30 min. Afterwards aliquots of the supernatant were taken and analysed for protein

concentration. For protein quantification a blank was prepared for each level of salt concentration and diluted in the same way as the solution containing the protein. Determination of the discontinuity point was done by plotting the logarithm of protein solubility against the concentration of salt.

9.3.6 Distribution of amino acids

The ionic liquid-based aqueous two phase system for the investigation of amino acid partitioning consisted of 12 wt % Ammoeng^{TM} 110 and 20 wt % of a mixture of the inorganic salts K_2HPO_4 and KH_2PO_4 (ratio 1.82 : 1.00). The phase-forming compounds were completely solubilised in distilled water giving a total system weight of 2.5 g. Afterwards, 1.5 g of a 24 mmol amino acid containing solution was added and the sample was mixed for 10 min on a horizontal shaker. Phase separation was induced by storing the samples for 1 2 h on ice. The volume of each phase was measured by a calliper gauge and calculated by using Equation 9-1 that was fitted for the vials used within these experiments. Samples from the bottom phase were carefully withdrawn and analysed for their amino acid content using the EZ:faast^{TM} kit for analysis of free amino acids (Phenomenex, Aschaffenburg, Germany) and LC/MS analysis. Amino acid analysis by the EZ:faast^{TM} kit comprises two basic steps: the extraction of amino acids from the sample by solid phase extraction and derivatisation of the samples with alkyl chloroformates. LC/MS measurements were performed using the LC/MS from Thermo Finnigan and the instrument settings as summarised in Table 9-6. The HPLC column included in the EZ:faast^{TM} kit and applied in the analysis was EZ:faast AAA-MS column 250 x 2.0 mm. The samples were analysed by using Xcalibur^{TM} software and database-library information that has previously been established in our group (Bathke 2008).

Table 9-6: Instrument settings for analysis of amino acids using LC/MS.

LC	Mobile Phase	A: 10mM Ammonium formate in methanol B: 10mM Ammonium formate in water
	Gradient	**Time /min** / **A / %** / **B / %** / **Flow rate / µL min^{-1}** 0 / 68 / 32 / 200 16 / 83 / 17 / 200 16.01 / 68 / 32 / 200 50 / 68 / 32 / 200
	Flow rate	200 µL min^{-1}
	Autosampler temperature	20°C
	Column temperature	35°C
	Injection volume	2 µL
	Washing volume	4000 µL
MS	Ion source	ESI
	Mode	Positive Ion
	Modus	Full scan
	Scan range	100-600 m/z
	Duration of analysis	50 min
	Segments	1

9.3.7 Denaturing electrophoresis

Gel electrophoresis was carried out according to the standard procedure described by Invitrogen by using a NuPAGE® 10 % Bis-Tris Mini Gel, NuPAGE® LDS Sample Buffer (4x), NuPAGE® Reducing Agent (10x) and the SeeBlue® Pre-Stained Standard from Invitrogen (Karlsruhe, Germany). The protein samples were prepared as summarised in Table 9-7 and incubated for 10 min at 70°C to provide a complete reduction of disulfides by the reducing agent. Afterwards, the samples were loaded onto a NuPAGE® 10 % Bis-Tris Mini Gel and the gel run for approximately 2 h at 75 V. Then, for washing purposes, the gel was transferred into 100 mL distilled water and heated in a microwave at 960 W for 45 s. This procedure is repeated three times and then 20 mL SimplyBlue Staining reagent containing Coomassie Brilliant Blue (Invitrogen) is added to the washing solution. Finally, the gel is transferred into 100 mL A. dest. and 20 mL NaCl solution (20 wt %) are added in order to ensure storability of the gel over several weeks.

Table 9-7: Sample composition for gel electrophoresis.

Reagent	Sample
Sample	x µL
NuPAGE® LDS Sample Buffer (4x)	3.75 µL
NuPAGE® Reducing Agent (10x)	1 µL
Aqua bidest	10.25 – x µL
Total volume	15 µL

9.3.8 Statistical analysis

Regression analysis was applied as a tool for modelling and analysing the experimental data obtained from the protein partitioning in IL-based ATPS. Using the dependent variable 'partition coefficient log *K*' in the regression equation as a function of the independent (explanatory) variables, a best fit of data was obtained and evaluated by using the least square method. The selection of the model to be applied is discussed in more detail in the following.

The use of many possible model variables, maybe also including collinear- and quadratic terms, could produce a quite big regression problem. This could result in problems with regard to a comprehensible analysis of the results. There is also often a tendency of increasing linear dependence when using more variables, which could cause different problems among others, concerning the stability, quality and interpretation of the parameters. Sometimes it is therefore desirable to decrease the number of variables. When lacking *a priori* knowledge, different statistically based selection methods must be used. The selection procedure used in this thesis was a method called 'forward backward selection' which represents a combination of two selection procedures (Sahlin 2005). (1) The 'forward selection' starts with an empty sub model and in every step one new variable is included in the model. The inclusion can be based on different selection criteria but is usually R^2. (2) The 'backward elimination' starts with an initial super model. The full model, including all predictor candidates, can be used as

the super model; i.e. the starting point of the selection algorithm. At every time step one predictor is excluded from the model.

However, neither the forward- nor the backward selections account for any copredictor effects. There is for instance a possibility that one variable is only significant in the presence of some other predictor or that one predictor becomes non-significant in the presence of two others and so on. In order to account for this, the forward selection is extended by introducing the possibility of excluding predictors at every time. By doing so, joint effects can come into consideration in the selection procedure. The algorithm includes one predictor in every step. After inclusion of one predictor there is the possibility to exclude one of the previously included predictors. The algorithm follows the general procedure as described by Sahlin (2005):

1. Having p predictors already included the test statistic for all the regressions containing the p predictors and one of the not yet included is calculated, respectively.
2. The variable giving the best test statistic is included in the $p + 1$ sub model.
3. Now having a $p + 1$-predictor model, the test statistic for all p regressions excluding one of the included predictors is calculated, respectively.
4. If one of the leave-one-out regressions produces a test statistic better than the one produced for the p-predictor model from the previous step, this p-model will be used for the next iteration step.
5. If none of the leave-one-out regressions produces a better test statistic, the $p + 1$-model will be used as starting point for the next iteration step.
6. The procedure is repeated until the desired model size is reached.

Implementation of this algorithm was done in a MATLAB script (see also appendix 11.5).

9.4 Methods for investigating enzyme purification by IL-based ATPS

9.4.1 Quantification of protein in protein mixtures (cell lysate)

Within experiments using complex protein mixtures such as cell lysates, the quantity of protein was determined via the Bradford protein assay. This assay is based on the adsorption of the dye Coomassie Brilliant Blue G-250 to the amino acids of proteins which results in a shift of the absorption maximum from 465 nm to 595 nm. 1000 µL of sample and 1000 µL of Bradford reagent were mixed in a cuvette and incubated at 20°C for 15 min. Afterwards the absorption at 595 nm was determined by a photometer (Specord 200, AnalytikJena) and protein quantity was calculated via a calibration curve with the standard protein BSA (Sigma-Aldrich) in the range of 0 10 µg mL^{1} protein. For the determination of protein concentration in the presence of ionic liquid, one protein-free sample was prepared containing the same amount of ionic liquid and used as a blank.

9.4.2 Partitioning of enzymes in IL-based ATPS

The partitioning experiments of enzymes in IL-based ATPS were performed at 4°C by weighing and mixing the phase-forming ionic liquid and inorganic salt in phosphate buffer (50 mM, pH 7.0) and then adding crude cell extract of *E. coli* to a final concentration of 0.1 wt %. The systems had a total weight of 4 g and were well mixed by a vortex mixer and stored on ice or centrifuged until phase separation was completed. The volume of each phase was measured by a calliper gauge and calculated by using Equation 9-1. Samples from the top and the bottom phase were carefully withdrawn and analysed for their protein content and enzyme activity. Protein concentration was determined via the Bradford assay and an identical sample of the corresponding phase from a system containing no protein was used as a blank. For determination of protein content in the lower phase, protein concentration of the upper phase was subtracted from total protein that was added to the system. The partition coefficient *K* was calculated by Equation 9-2 and enzyme activity was determined via the photometric activity assay described below.

9.4.3 Photometric ADH activity assay

The reduction of acetophenone by alcohol dehydrogenases was chosen to exemplify the extraction of catalytically active biomolecules by ionic liquid-based ATPS. Enzyme activity was determined via a photometric assay following the consumption of NADPH at 340 nm with a spectrophotometer (Specord 200, AnalytikJena) (Figure 6-1, part 1). Activity [U] was defined as 1 µmol min ^{1} acetophenone converted in 50 mM phosphate buffer (1 mM $MgCl_2$) at pH 7.0 and 30°C. The standard reaction mixture contained 10 mM acetophenone in 50 mM phosphate buffer (1 mM $MgCl_2$, pH 7.0) and 0.2 mM NADPH (Riebel 1996). 20 µL enzyme solution (0.1-1 U mL ^{1}) or 20 µL sample from the upper phase of the ATPS were added to give a total reaction volume of 1 mL. The consumption of NADPH was followed at 340 nm for 3 min. Volumetric enzyme activity was calculated by Equation 9-4. Specific enzyme activity is defined as units per µg protein and was calculated from the volumetric activity, the phase volume and protein content of the samples.

$$volumetric\ activity = \frac{slope \cdot 60 \cdot V_{tot}}{\varepsilon \cdot V_{sample} \cdot d} \cdot f \qquad [\mu mol\ min^{1}\ mL^{1}] \quad [U\ mL^{1}]$$

Equation 9-4: Calculation of volumetric enzyme activity. V_{tot} = total volume; V_{sample} = sample volume; ε = extinction coefficient; d = diameter of the cuvette; f = factor of dilution.

To investigate the influence of ionic liquid on enzyme activity, a certain amount of ionic liquid was added to the standard reaction mixture. Enzyme stability was determined by storing the crude cell lysate in phosphate buffer containing different amounts of ionic liquid. Samples of 20 µL were taken from this solution at different times to determine enzyme activity analogous to the procedure above.

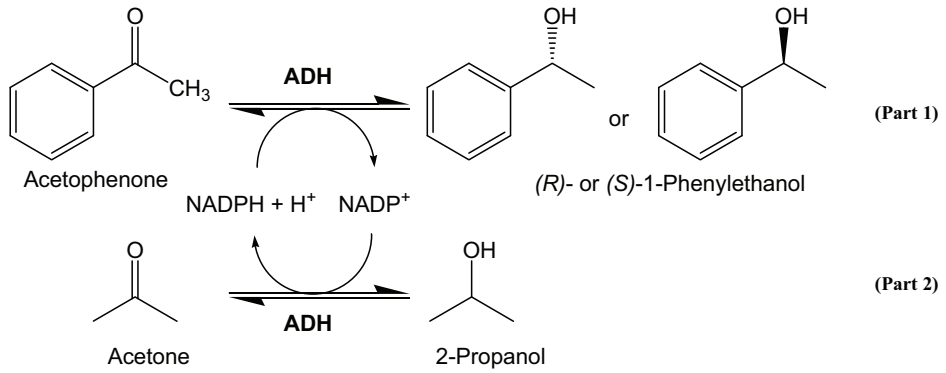


Figure 9-2: ADH catalysed reduction of acetophenone. Part 1 was used for the photometric determination of ADH activity by following the consumption of NADPH at 340 nm. Part 2 represents the extension for acetophenone reduction with substrate coupled cofactor regeneration.

9.4.4 ADH activity assay with cofactor regeneration

The activity of ADH was measured in a standard assay mixture consisting of a solution of acetophenone (10 mM), NADPH (0.2 mM) and 2-propanol (200 mM) in 50 mM phosphate buffer (pH 7.0, 1 mM $MgCl_2$) in a total volume of 8 mL (Figure 6-1, part 2). For investigating the influence of the ionic liquid a specific amount of Ammoeng^{TM} 110 was added to the system. The conversion of higher amounts of substrate was carried out by increasing the cofactor and cosubstrate concentration accordingly. To start the reaction, enzyme solution was added and the reaction was carried out under shaking (200 rpm) at 30°C. The enzyme-catalysed conversion of acetophenone into phenylethanol was followed by taking samples of 100 µL at selected times. These samples were extracted by the addition of 100 µL hexane and 1 min of vortexing. 50 µL of the upper organic phase were added to 950 µL of hexane and the samples were analysed via HPLC. Separation was carried out on a Chiralcel OD-H 5 µm column (Daicel, Japan) with 97 % Hexane, 3 % 2-propanole and 0.1 % acetic acid. The flow rate was 1 mL min^{1} at a temperature of 40°C. Substrate and product were measured spectrophotometrically at 220 nm (Variable Wavelength Monitor, Knauer, Germany). A HPLC diagram by way of example is given in Figure 11-4 (see appendix).

9.4.5 Influence of compatible solutes

A set of compatible solutes was investigated with respect to their influence on ADH activity and stability: N_{ε}-acetyl-L-lysine, L-carnitine, L-proline, betaine, ectoine and Hydroxyectoine. 500 mM of each compatible solute were added to the standard reaction mixture of the photometric ADH activity assay. Enzyme stability was determined by storing the crude cell lysate in phosphate buffer containing 500 mM compatible solute. Samples of 20 µL were taken from this solution at different times to determine enzyme activity analogous to the procedure above.

9.4.6 Experimental design for the optimisation of enzyme purification

In order to optimise the partitioning of enzymes within IL-based ATPS, the Box Wilson experimental design was used to setup the experiments (Box and Wilson 1951; Kleppmann

2006). The concentration of ionic liquid (X_1) and inorganic salt (X_2) were chosen as independent variables in a series of repeated experiments and the specific enzyme activity Y [U μg^{-1} protein] in the ionic liquid containing upper phase was set as the target parameter to be optimized. For statistical calculations, x_i was used as coded value of the variable i and is defined by Equation 9-5, where X_i is the variable, X_0 is the value of the variable at the center point of the investigated area, and ΔX_i is the step size.

$$x_1 = (X_1 - X_0) / \Delta X_i$$

Equation 9-5: Definition of x_i as the coded value of the variable i.

Equation 9-5 summarises exemplarily the real values of the independent variables in the experimental plan.

Table 9-8: Real values of the independent variables in the experimental plan (X_1: ionic liquid [wt %], X_2: inorganic salt [wt %]).

Real values	**Coded values**				
	-1.414	-1.0	0.0	1.0	1.414
X_1	11.465	12.5	15	17.5	18.535
X_2	17.586	18	19	20	20.414

For analysing the pattern of responses MATLAB script was used in order to fit a second-degree polynomial (see also appendix 11.4). The model of regression has the form:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_{11} x_1^2 + b_{12} x_1 x_2 + b_{22} x_2^2$$

Equation 9-6: Model of regression for the analysis of responses. A = predicted response; b_0 = intercept term; b_i = coefficients of the linear terms; b_{ij} = coefficients of the quadratic terms.

The occurrence of a local maximum value for function Y given in Equation 9-6 was determined and the calculated values for x_1 and x_2 were used as center points for the next step of Box Wilson-experiments until a global maximum was found.

9.4.7 Solubility of acetophenone

Different amounts of ionic liquid were dissolved in water or phosphate buffer (50 mM, pH 7.0). Afterwards acetophenone was added to the aqueous ionic liquid solution until two distinct phases were formed and samples were shaken over night at 220 rpm. The acetophenone-saturated lower phase was analysed by HPLC on a Nucleosil 100 C18-column (Macherey-Nagel, Düren, Germany). The eluent was MeOH / H_2O 1 / 1 and the flow rate was 0.8 mL min^{-1} at a temperature of 25°C. Detection of acetophenone was carried out at 242 nm.

10 Literature

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11 Appendix

11.1 LC/MS analysis of amino acids

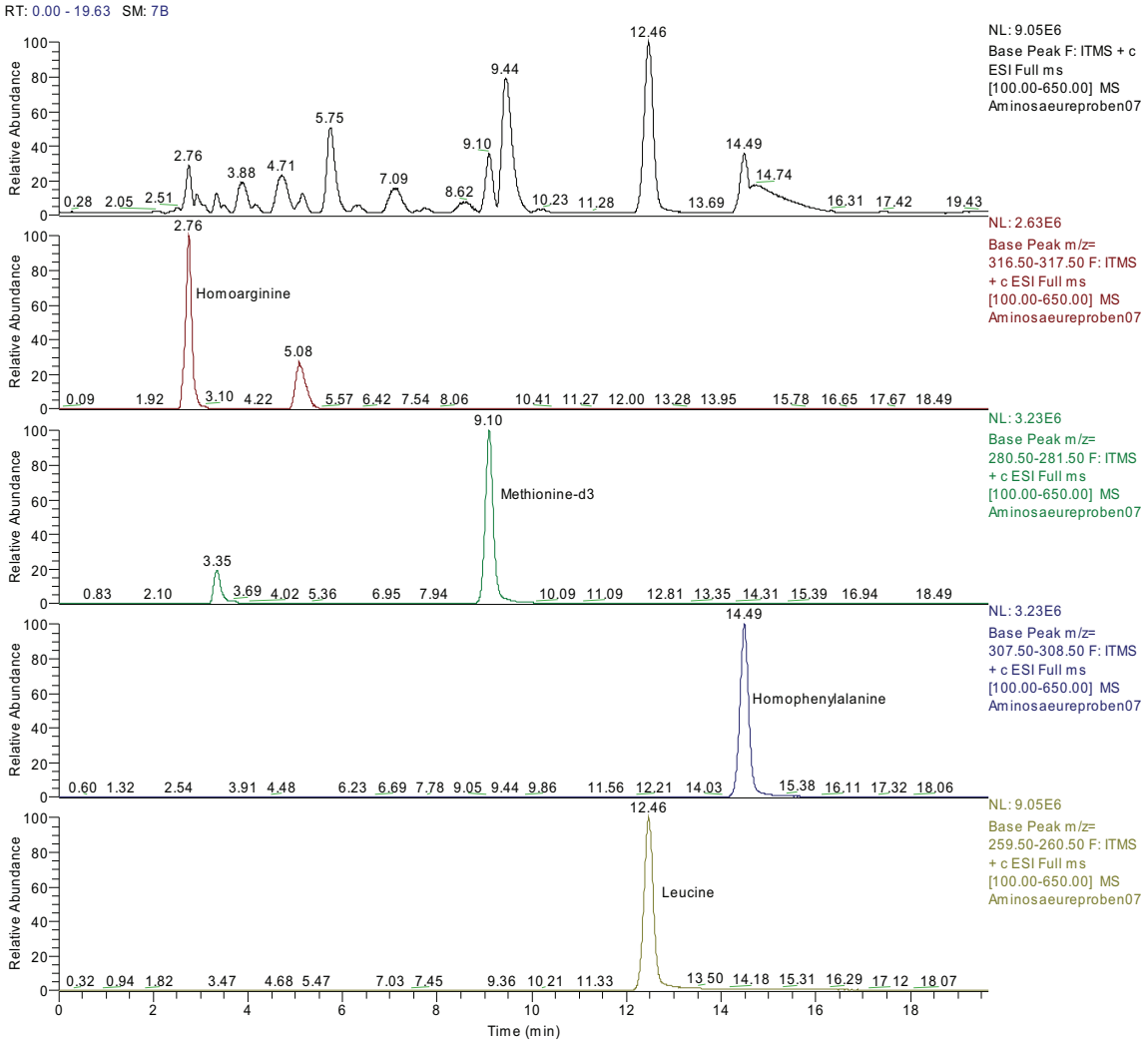

Figure 11-1: LC/MS analysis of amino acids within IL-based ATPS at the example of leucine. The top chromatogram represent the full scan of a sample from the lower phase of IL-based ATPS containing three internal standards and leucine. The lower diagrams represent chromatograms of the internal standards homoarginine, methionine-d3 and homophenylalanine as well as the investigated amino acid leucine.

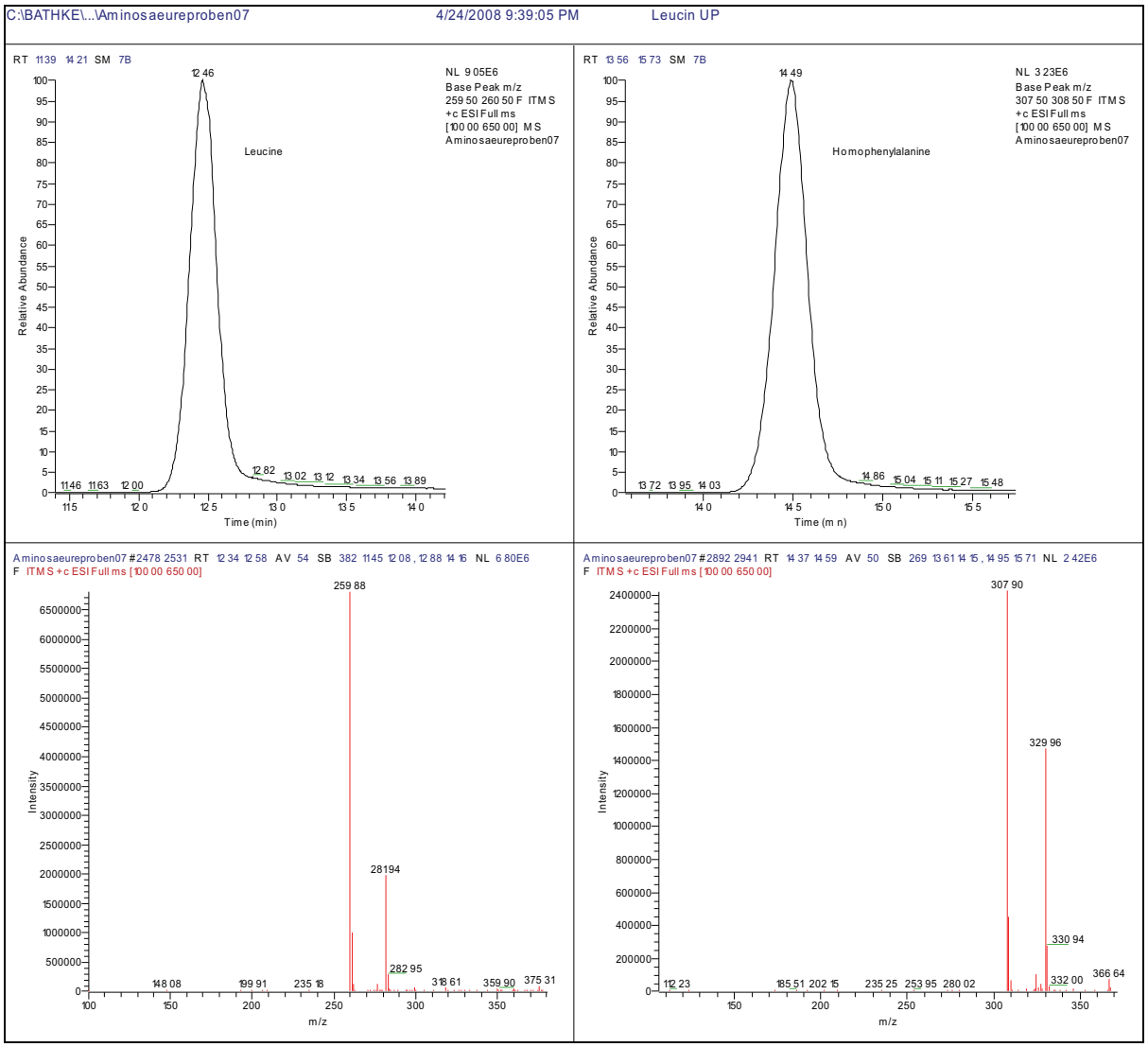

Figure 11-2: Chromatogram and MS spectrum at the example of leucine and homophenylalanine as obtained by LC/MS analysis.

11.2 Surface area of amino acids

Table 11-1: Data of surface area of amino acids within proteins and log *K* values of amino acids as used for the correlation with the partition coefficient in IL-based ATPS for the model proteins.

Amino acid	**log *K***	**Surface area of amino acids within proteins / Å^2**			
	in IL-based ATPS	**Myoglobin**	**Trypsin**	**Lysozyme**	**BSA**
Ala	0.318	649.63	365.8	271.69	4567.38
Arg	n. d.	141.02	149.08	1515.48	2554.18
Asn	0.734	210.34	829.87	940.75	1690.89
Asp	1.227	665.78	156.53	488.15	4880.67
Cys	n. d.	0	139.02	78.89	961
Gln	0.748	410.68	629.14	250.82	2398.6
Glu	-0.535	1078.28	264.73	105.95	11425.61
Gly	0.142	643.15	722.58	580.66	338.88
His	-0.037	670.39	123.64	34.54	1505.73
Ile	0.330	193.56	206.74	105.16	257.27
Leu	-0.063	309.88	273.23	210.99	2182.31
Lys	n. d.	1896.47	1141.21	496.2	10990.71
Met	0.640	5.78	3.04	0.16	446.63
Phe	0.325	130.11	115.14	92.49	893.79
Pro	-1.003	310.82	306.33	206.66	2495.9
Ser	-0.203	189.46	1851.48	320.58	1593.09
Thr	1.013	388.15	608.67	370.3	3100.9
Trp	0.572	15.96	103.95	233.14	103.03
Tyr	1.512	38.57	615.96	118.76	749.07
Val	0.596	139.6	376.63	219.33	1853.92

11.3 Experimental design using the Box Wilson method

Table 11-2: Parameter estimates for the second-degree polynomial as fitted to experimental results for LB ADH.

Variable		Estimate	Standard error
b_0	Intercept	-99.684	107.619
b_1	Linear Term	2.144	3.711
b_2	Linear Term	9.196	9.975
b_{11}	Quadratic Term	-0.023	0.042
b_{12}	Quadratic Term	-0.082	0.183
b_{22}	Quadratic Term	-0.212	0.252

Table 11-3: Comparison of the experimental and predicted results for specific enzyme activity of T ADH in the upper phase of IL-based ATPS.

Ammoeng^{TM} 110 / wt % x_1	**K_2HPO_4/KH_2PO_4** / wt % x_2	**Experimental specific activity / U µg^{-1} protein** Y	**Predicted specific activity / U µg^{-1} protein** Y
11.306	19.196	3.04	3.08
9.906	19.196	2.21	2.26
11.306	17.196	1.81	1.82
9.906	17.196	1.49	1.51
11.596	18.196	2.32	2.30
9.616	18.196	1.53	1.49
10.606	19.61	3.21	3.15
10.606	16.782	1.74	1.73
10.606	18.196	2.88	2.79
10.606	18.196	2.74	2.79
10.606	18.196	2.88	2.79
10.606	18.196	2.84	2.79
10.606	18.196	2.66	2.79
10.606	18.196	2.76	2.79

$$Y \quad -136.92 + 16.551\, x_1 + 4.9684\, x_2 \quad 0.91523\, x_1^2 + 0.17970\, x_1 x_2 \quad 0.17506\, x_2^2$$

Equation 11-1: Second-degree polynomial for describing the specific enzyme activity of T ADH in the upper phase of IL-based ATPS.

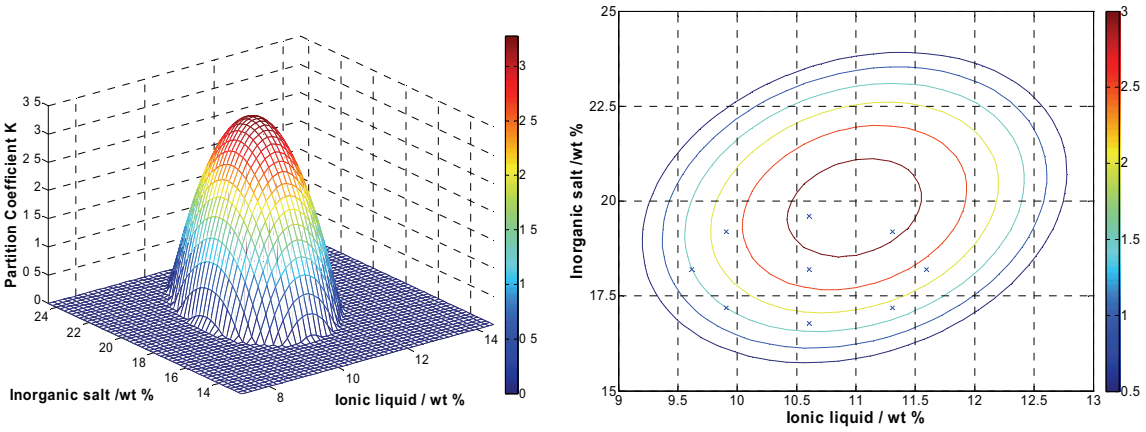

Figure 11-3: Illustration of the response surface as obtained for the partitioning experiments of T ADH within IL-based ATPS. (A) Meshplot, (B) Contour plot.

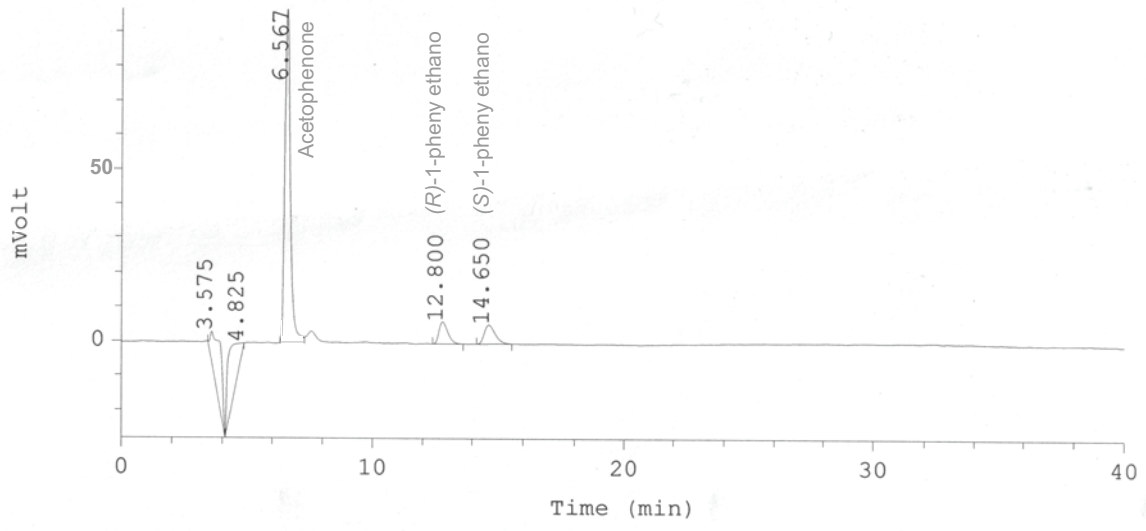

Figure 11-4: Examplary HPLC diagram of a solution containing 10 mM acetophenone and 10 mM of a racemic mixture of 1-phenylethanol.

11.4 MATLAB implementation of experimental design using a Box Wilson approach

For pattern analysing, efficient experimental design and graphical interpretation, the following MATLAB script was used. The script runs on MATLAB version 7.3.0 (R2006b) with the Statistics Toolbox.

```
%
% Construction matrix
%
C = [ones(size(x(:,1))) x(:,1) x(:,2) x(:,1).*x(:,2) x(:,1).^2 x(:,2).^2];
coefficients = {
    'Intercept'
    x1_name
    x2_name
    [x1_name '*' x2_name]
    [x1_name '^2']
    [x2_name '^2']
};
[n,p] = size(C);

% b0 b1 b2 b12 b11 b22
b = C\y;
y_hat = C*b;

r2 =1   ( (y y_hat)'*(y y_hat) )  / sum((y   mean(y)).^2)
sig2 = 1/(n p)*sum((y   y_hat).^2);

F_quant = finv(0.95, p, n p);
y_cv = zeros(size(y));

for iii = 1:n
    %
    % Cross validation
    %
    index = [1:(iii 1) (iii+1):n];
    b_cv = C(index,:)\y(index);
    y_cv(iii) = C(iii,:)*b_cv;

end

b_conf = zeros(size(b));
for iii = 1:p
    %
    % Conf interval 95%
    %
    invCC = inv(C'*C);
    b_conf(iii) = sqrt( invCC(iii,iii) * p * sig2 * F_quant );

end

r2_cv =1   ( (y y_cv)'*(y y_cv) )  / sum((y   mean(y)).^2)

%
% Rejection of non significant variables
%

if(reject_non_significant)
    sub_model_index = [1; find(abs(b(2:end)) > b_conf(2:end))+1];
```

```
else
    sub_model_index = [1:length(b)];
end

C_sub = C(:,sub_model_index);

[n_sub, p_sub] = size(C_sub);

b_sub = C_sub\y;

y_sub_hat = C_sub*b_sub;

r2_sub =1   ( (y y_sub_hat)'*(y y_sub_hat) )  / sum((y   mean(y)).^2)
sig2_sub = 1/(n p_sub)*sum((y   y_sub_hat).^2);

F_quant_sub = finv(0.80, p, n p_sub);

b_sub_conf = zeros(size(b_sub));
for iii = 1:p_sub
    %
    % Conf interval 95%
    %
    invCC_sub = inv(C_sub'*C_sub);
    b_sub_conf(iii) = sqrt( invCC_sub(iii,iii) * p_sub * sig2_sub *
F_quant_sub );

end

b_sub_tmp = b_sub;
b_sub_conf_tmp = b_sub_conf;

b_sub = zeros(size(b));
b_sub_conf = zeros(size(b));

b_sub(sub_model_index) = b_sub_tmp;
b_sub_conf(sub_model_index) = b_sub_conf_tmp;

H = [2*b_sub(5) b_sub(4) ; b_sub(4) 2*b_sub(6)];
a = [ b_sub(2);  b_sub(3)];


[stepx,D] = eig(H);
lambda = eig(H);

next_exp = zeros(size(box_wilson));

if(sum(lambda > 0)>0)
    disp('No local maximum');
    max_exsists = 0;
else
    max_exsists = 1;
    x_max = H\a;
    c_max = [1 x_max(1) x_max(2) x_max(1)*x_max(2) x_max(1)*x_max(1)
x_max(2)*x_max(2)];
    y_max = c_max*b_sub;


    stepfactor = 5;
    steplengthx = (3*stepfactor*sqrt(sig2)./( lambda)).^(1/3);

    for iii = [1:size(stepx,1)]
        stepx(:,iii) = steplengthx(iii)*stepx(:,iii)
    end
```

```
    for iii = [1:size(box_wilson,1)]
        next_exp(iii,:) = x_max' + box_wilson(iii,1)*stepx(:,1)' +
box_wilson(iii,2)*stepx(:,2)';
    end

    %plot model
    x1 = [0.75*min(x(:,1)) : (1.25*max(x(:,1)) 0.75*min(x(:,1)))/50  :
1.25*max(x(:,1))];
    x2 = [0.75*min(x(:,2)) : (1.25*max(x(:,2)) 0.75*min(x(:,2)))/50  :
1.25*max(x(:,2))];
    [X1,X2] = meshgrid(x1,x2);

    Y = b(1)*ones(size(X1)) + b(2)*X1 + b(3)*X2 +b(4)*X1.*X2 + b(5)*X1.*X1
+ b(6)*X2.*X2;

    f1 = figure(1);
    clf
    mesh(X1,X2,max(min(Y,max_plot_y), min_plot_y))
    axis([min(x1) max(x1) min(x2) max(x2) min_plot_y max_plot_y])
    xlabel(x1_name);
    ylabel(x2_name);
    zlabel(y_name);
    hold on

    for iii = [1:length(y)]
        epsx1 = (max(x(:,1))   min(x(:,1)))/100;
        xxx = x(iii,1);
        XX1 = [xxx epsx1 xxx xxx+epsx1 ;xxx epsx1 xxx xxx+epsx1 ;xxx epsx1
xxx xxx+epsx1];
        epsx2 = (max(x(:,2))   min(x(:,2)))/100;
        xxx = x(iii,2);
        XX2 = [xxx epsx2 xxx xxx+epsx2 ;xxx epsx2 xxx xxx+epsx2;xxx epsx2
xxx xxx+epsx2]';
        YY = min_plot_y * ones(3,3);
        YY(2,2) = y(iii);
        mesh(XX1,XX2,YY);
    end

    f2 = figure(2);
    clf
    contour(X1,X2,max(min(Y,max_plot_y), min_plot_y))
    axis([min(x1) max(x1) min(x2) max(x2)])
    xlabel(x1_name);
    ylabel(x2_name);
    grid on
    hold on
    plot(x(:,1),x(:,2),'x');

    saveas(f1, meshplot_name, 'emf');
    saveas(f2, contourplot_name, 'emf');

end % if exists maximum

ofile = fopen(txt_file_name, 'w');

fprintf(ofile, 'Box Wilson Results\n');
fprintf(ofile, '                  \n');
fprintf(ofile, '\n');
fprintf(ofile, 'Date: %s\n', date);
fprintf(ofile, '\n');

fprintf(ofile, 'Variables:\n');
```

```
fprintf(ofile, '%s\n%s\n',x1_name, x2_name);
fprintf(ofile, '\n');

fprintf(ofile, 'Modelled entity:\n');
fprintf(ofile, '%s\n',y_name);
fprintf(ofile, '\n');

fprintf(ofile, 'Fitted parameters with 95%% individual confidence
intervals\n');
for iii = 1:p
    fprintf(ofile,'%20s %25.4e +/  %12e \n',
coefficients,b_sub(iii),b_sub_conf(iii));
end
fprintf(ofile,'\n');
fprintf(ofile,'R^2 = %f\n', r2_sub);
fprintf(ofile,'\n');

if(max_exsists)

    fprintf(ofile, 'Model gives maximum %s = %e at:\n', y_name, y_max);
    fprintf(ofile, '%s = %e\n',x1_name,x_max(1));
    fprintf(ofile, '%s = %e\n',x2_name,x_max(2));
    fprintf(ofile, '\n');

    fprintf(ofile, 'Box wilson for next experiment:\n');
    fprintf(ofile, 'x_max = (%e,%e)\t(%s,%s)\n', x_max(1),x_max(2),x1_name,
x2_name);
    fprintf(ofile, 'step1 = (%e,%e)\t(%s,%s)\n',
stepx(1,1),stepx(2,1),x1_name, x2_name);
    fprintf(ofile, 'step2 = (%e,%e)\t(%s,%s)\n',
stepx(1,2),stepx(2,2),x1_name, x2_name);
    fprintf(ofile, '\n');

    fprintf(ofile, 'Table for next box wilson setup\n');

    fprintf(ofile, 'Experiment %20s %20s\n\n', x1_name,x2_name);
    for iii=1:size(box_wilson,1)
        fprintf(ofile, '\t%3d    %20.5e %20.5e\n',
iii,next_exp(iii,1),next_exp(iii,2));
    end

else
    fprintf(ofile, 'Model does not give any local maximum\n');
end


fprintf(ofile, '\n\n');
fprintf(ofile, 'Copyright S. Sahlin and S. Dreyer %s 2006\n',laborant);
fclose(ofile);
```

11.5 MATLAB Implementation of Forward Backward Selection Algorithm

The implementation of the forward backward selection algorithm described in chapter 9.3.8 was implemented with the following MATLAB script. The script runs on MATLAB version 7.3.0 (R2006b) with the Statistics Toolbox.

```
clear all;

%
%
% Tool for analysis of linear regression models
%
%


%
% Input y,C, column names names
%

%
% Outputs
%
%
%   Regression coefficents (together with F statitics),
%   Residual plots
%
%

measurement_data_susanne;


y = data(:,1);
y_name = table_header(1);


C_base_idx = [2 3 4 5 6 7 8];
C_base = [ones(size(y)) data(:,C_base_idx )];
C_names = {'1', table_header{C_base_idx }};

C = C_base;
for iii = 2:size(C_base,2)
    for jjj = iii:size(C_base,2)
        C = [C, C_base(:,iii).*C_base(:,jjj)];
        C_names =[C_names{iii} '*' C_names{jjj}]};

    end
end

model_index = 1;
not_in_model = [2:size(C,2)];

groups = data(:,2:5);
group_symbols = {'r*', 'bx', 'ko', 'm+'};
group_names = table_header(2:5);

y_maps = [y, log10(y)];
y_map_names = {'1', 'log10'};
y_map_index = 1;
```

```
n_parameters = 5;

    y = y_maps(:,y_map_index);

    best_iii = zeros(1,30);

    iii = 0;
    while iii < n_parameters +1
        iii = iii +1;
        q_value = zeros(size(not_in_model));

        for jjj = 1:length(not_in_model)
            q_value(jjj) = cross_validationsum_sq( C(:,[model_index
not_in_model(jjj)]), y);

        end

        best_iii(iii) = min(q_value);

        put_in_model = min(find(q_value == min(q_value)));
        disp([ '+ ' C_names{not_in_model(put_in_model)}]);

        model_index = [model_index not_in_model(put_in_model)];
        not_in_model = not_in_model([1:(put_in_model 1)
(put_in_model+1):end]);


        if(iii >=2)
            throw_out = true;

            while(throw_out)
                throw_out = false;
                q_value = max(q_value)*ones(1, length(model_index) 1 );

                for jjj = 2:length(model_index)
                    q_value(jjj) = cross_validationsum_sq(
C(:,[model_index([1:(jjj 1) (jjj+1):end]) ]), y);
                end

                if(min(q_value) < best_iii(iii   1))
                    iii = iii   1;
                    best_iii(iii) = min(q_value);

                    throw_out_idx =  min(find(q_value == min(q_value)));

                    disp([ '   ' C_names{model_index(throw_out_idx)}]);


                    not_in_model = [not_in_model
model_index(throw_out_idx)];
                    model_index = model_index([1:(throw_out_idx 1)
(throw_out_idx+1):end]);

                    if(iii>=2)
                        throw_out = true;
                    end
                end
            end
        end
```

```
    end

    n = length(y);
    p = n_parameters;
    C_m = C(:,model_index(1:n_parameters));
    b_hat = C_m\y;

    y_hat = C_m*b_hat;

    r2 =1   ( (y y_hat)'*(y y_hat) )  / sum((y   mean(y)).^2)
    sig2 = 1/(n p)*sum((y   y_hat).^2);

    F_quant = finv(0.95, p, n p);

    b_conf = zeros(size(b_hat));
    for iii = 1:p
        %
        % Conf interval 95%
        %
        invCC = inv(C_m'*C_m);
        b_conf(iii) = sqrt( invCC(iii,iii) * p * sig2 * F_quant );

    end

    r2_cv = 1   ( cross_validationsum_sq( C_m, y)  )  / sum((y
mean(y)).^2)
    disp([y_map_names{y_map_index} '( ' y_name{1} ' ) = ']);
    for iii = 1:n_parameters
        disp(['( ' num2str(b_hat(iii)   ) ' +/  ' num2str(b_conf(iii)) ' )
* ( ' C_names{model_index(iii)} ' )' ] )
    end

    figure(1);
    clf
    grid on
    hold on

    plot_groups=0;
    if plot_groups == 1
        for iii = 1:length(group_names)
            plot(C_m(groups(:,iii)==1,:)*b_hat, y(groups(:,iii)==1),
group_symbols{iii})
        end
    else
        plot(C_m(:,:)*b_hat, y(:), 'x')
    end

    plot( [min([C_m*b_hat; y])  max([C_m*b_hat; y])], [min([C_m*b_hat; y])
max([C_m*b_hat; y])] ,'g')
```

Curriculum vitae

PERSONAL DETAILS

Name	Susanne Elisabeth Dreyer
Date of birth	16.08.1979
Place of birth	Troisdorf, Germany

EDUCATION

Aug 1989 – May 1998	*Gymnasium Altenforst Troisdorf, Germany* High School education; university-entrance diploma (Abitur)
Oct 1998 – Oct 2000	*Friedrich-Wilhelms-Universität Bonn, Germany* Biology student; intermediate diploma (Vordiplom)
Oct 2000 – Nov 2004	*University of Aachen (RWTH), Germany* Biology student (Diplom); diploma thesis at the department of biotechnology (Prof. Dr. Hartmeier) on the topic 'Basic experiments for the production, immobilisation and application of carboxylesterase A of *Bacillus coagulans*'
Sept 2001 – Mar 2002	*Imperial College London, Great Britain* Biology student as participant of the Socrates-Erasmus programme of study abroad exchanges (scholarship)
Mar 2004 – May 2004	*Università di Milano, Italy* Guest researcher within the department of industrial microbiology (Prof. Dr. Molinari) to perform parts of the diploma thesis within the VIGONI project (financially supported by DAAD and the Italian CRUI)
Since Jul 2005	*University of Rostock, Germany* PhD student at the Department of Chemical Engineering (Prof. Dr. Kragl), partly financed by a scholarship of Degussa Stiftung (Evonik Industries) and the SPP1191-Ionic Liquids

EXPERIENCES

Apr 2002 – May 2002	*University of Aachen (RWTH), Germany* Research internship 'Characterisation of enzymes and immobilisation matrices for biochemical modification of lipids', Department of Biotechnology, RWTH Aachen
Jun 2002 – July 2002	*Bioreact GmbH Bonn, Germany* Internship at Biotechnology company Bioreact GmbH, working on solid state/substrate fermentations
Oct 2002 – Jan 2003	*University of Aachen (RWTH), Germany* Scientific student assistant working on the project 'Fermentation optimisation of recombinant *E. coli* strains for the production of Formiat-Dehydrogenase', Department of Biotechnology RWTH Aachen
May 2004 – Jun 2004	*University of Aachen (RWTH), Germany* Scientific supervision of two exchange students
Apr 2005 – Jun 2005	*Bioreact GmbH Troisdorf, Germany* Internship at Biotechnology company Bioreact GmbH, working in the production, chemical analysis and quality management
Sept 2006 – Dec 2006	*University of Rostock, Germany* Scientific supervision of the Bachelor thesis 'Characterization of aqueous two phase systems based on ionic liquids' at Department of Chemical Engineering University Rostock

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe und keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe. Die den Werken wörtlich entnommenen Stellen sind als solche kenntlich gemacht.

Rostock, 11.10.2008