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Reverse micellar extraction of amino acids and complex enzyme mixtures

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ABSTRACT

In this work, the reverse micellar extraction with the anionic surfactant dioctyl sodium sulfosuccinate (AOT) in isooctane for the extraction of biomolecules is investigated. The forward (FE) and backward extraction (BE) of amino acids and mixtures of these as well as the application for complex enzyme mixtures like Celluclast 1.5L and Novozyme 188 are presented. The selective separation of the mixtures of amino acids (Mix1: Asparagine, aspartic acid and arginine, Mix2: Glutamine, glutaminic acid and histidine) are presented. The efficiency in mixtures of amino acids is lower in comparison to pure amino acids, where no interaction occurs at corresponding process conditions. The experimental results are compared to predictions based on the thermodynamic model COSMO-RS. This is the first time that the partition behavior of various amino acids at different ionization states is predicted in this way.

The selected enzyme mixtures Celluclast 1.5L and Novozyme 188 are relevant for the conversion of biomass to bioethanol in biorefineries. Therefore an extraction and recycling of these enzymes during the process can be economically beneficial. The focus lies on the investigation of different influencing parameters like salt concentration (ionic strength) and pH of the aqueous phase in order to retrieve a high selectivity and high activity of the enzymes. At a pH lower than the pI of the proteins the highest extraction is observed and the activity of the enzymes has been maintained to at least 80% with respect to the maximum activity. The applicability of the reverse micellar extraction for these systems has therefore successfully been demonstrated. However, improvements in terms of back extraction are needed.

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1. Introduction

Surfactant-based separation processes such as the reverse micellar extraction are of high potential for various applications like downstream processing and enzyme recovery. Reverse micelles are formed in organic solutions and depending on the water content of the system, may have a small or large water core for hosting molecules. These aggregates can be used to solubilize different molecules according to their hydrophobicity, size and charge.

Since downstream processing is one of the integral parts of bioprocesses, an extraction method is needed, that can efficiently purify the desired target molecules with reasonable technological effort. In order to obtain very high purities of over 99% techniques like chromatography are favorable, but in terms of large product streams and less demands of purity, continuous extractions are advantageous [1,2]. Especially for the extraction of hydrophilic compounds from aqueous media the reverse micellar extraction is promising and easy to scale up (realization in extraction column), compared to other methods [2,3].

This technique has been of great interest since many years, but still fundamental investigations are not complete. The most cited papers concerning the extraction of amino acids come from the group Leodidis and Hatton [4–8] and one of the earliest publications of the selective extraction of enzyme mixtures originated from Goklen and Hatton [9]. But amino acids have not been studied extensively in mixtures so far and nearly no modeling approaches on the partition behavior at different ionization states are present.

In case of proteins most studies deal with ideal purified proteins like lysozyme [9], ribonuclease A [9,10], α-labtalbumin [10], α-chymotrypsin [11,12], or cytochrome C [9]. As described in literature [13,14], especially the recovery of enzymes from fermentation or hydrolysis broths is challenging, since many interfering substances are present, e.g. other proteins, nutrients, byproducts, salts and acids. Additionally, for the recycling of enzymes from product streams for their reuse, a method with small operational costs is needed.

The studies presented here aim to close the gaps in terms of investigation of amino acids in the reverse micellar system with AOT/isooctane. Especially, predictions of the partition behavior are essential for finding optimal process conditions. Moreover these predictions build the basis for future modeling of whole

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proteins, if their amino acid surface distribution is known in dependence of the pH.

The feasibility of the extraction of the widely used enzyme mixtures Celluclast 1.5L and Novozyme 188 is investigated. Therefore the results presented in this work can pave the way for more industrial applications of the reverse micellar extraction of biomolecules.

2. Material and methods

2.1. Material

Amino acids: L-phenylalanine (Phe), L-tyrosine (Tyr), L-tryptophan (Trp), L-asparagine (Asn), L-glutamine (Gln) from Sigma–Aldrich GmbH, L-arginine (Arg), L-histidine (His) and L-aspartic acid (Asp) from Sigma Chemical Co., L-glutaminic acid (Glu) from Merck KGaA (all purities ⩾98%).

Proteins: BSA (bovine serum albumin, CAS 9048-46-8, Sigma–Aldrich GmbH, ⩾98%), Celluclast 1.5L (Novozymes A/S), Novozyme 188 (Novozymes A/S). Chemicals for activity test (NREL): D(+)-glucose-monohydrate (CAS 14431-43-7, E.Merck KG, ⩾99,5%), sodium hydroxide (CAS 1310-73-2, Carl Roth GmbH, ⩾99%), 3,5-dinitrosalicylic acid (CAS 609-99-4, ABCR GmbH & Co., KG, 97%), potassium sodium tartrate tetrahydrate (CAS 6381-59-5, Carl Roth GmbH, ⩾98%), hydrochloric acid (CAS 7647-01-0, Carl Roth GmbH, 37%), filterpaper (type Whatman grade 1). Chemicals for activity test (PNPG): p-nitrophenol-α-D-glucopyranoside (CAS 2492-87-7, Sigma–Aldrich GmbH, ⩾98%), p-nitrophenol (CAS 100-02-7, Sigma–Aldrich GmbH), sodium carbonate (CAS 497-19-8, Sigma–Aldrich GmbH, ⩾99.0%). Buffer solutions (1) citrate buffer: citric acid monohydrate (CAS 5949-29-1, Carl Roth GmbH), sodium hydroxide (CAS 1310-73-2, Carl Roth GmbH), (2) TRIS–HCl buffer: Trizma base (CAS 77-86-1, Sigma–Aldrich GmbH), trizma hydrochloride (CAS 1185-53-1, Sigma–Sigma–Aldrich GmbH) all of analytical grade.

Other chemicals: Potassium chloride (CAS 7447-40-7, Carl Roth GmbH,), sodium chloride (CAS 7647-14-5, Carl Roth GmbH), magnesiumchlorid-hexahydrate (CAS 7791-18-6, Carl Roth GmbH), calciumchlorid-dihydrate (CAS 10035-04-8, Merck KGaA), isooctane (CAS 540-84-1, Carl Roth GmbH), and AOT (dioctyl sodium sulfosuccinate, CAS 577-11-7, SERVA GmbH) all of analytical grade; Buffer for amino acids: natriumactetat-trihydrat (CAS 6131-90-4, Merck KGaA) and acetic acid (CAS 64-19-7, Sigma–Aldrich GmbH; Karl-Fischer: hydranal titrant 5 and hydranal-solvent (Sigma–Aldrich GmbH); Bradford reagent (Sigma–Aldrich GmbH).

2.2. Phase-transfer method: forward (FE) and backward (BE) extraction

Generally, different techniques of the solubilization of target compounds are possible: Injection method, dry addition or phase-transfer method [12]. Since our aim is to investigate methods for large-scale applications, only the phase-transfer method is suitable. The phase-transfer method consists of two steps, named in literature as the forward (FE) extraction and the back extraction (BE) [2]. The method is presented in Scheme 1. The FE is used to extract the target molecules from an aqueous phase into the organic micellar phase via contacting both phases until equilibrium conditions are reached. The BE is needed for the extraction of the target molecules into a fresh aqueous phase.

The fundamental steps of the extraction procedure are similar between the extraction of amino acids or proteins. Therefore, the extraction steps are explained briefly in Sections 2.2.1 and 2.2.2 and differences are highlighted.

2.2.1. Amino acid extraction via phase-transfer

The extraction was performed in 10 mL volumetric glasses (type: Hecht Assistant NS 12, 5/21). All experiments were performed at least in triplicate and the error was analyzed according to the student-t-distribution with a confidence interval of 68.26%. For the FE, the volumes of both phases were 4 mL. The organic phase consisted of 4 mL with $c_{AOT} = 0.2$ mol/L. The samples were sealed with parafilm to avoid fluid loss during mixing and centrifugation. The extraction was performed in the overhead shaker (type: Heidolph Reax 2) for 30 min by ca. 80 rpm. In order to achieve a fast phase separation the samples have been centrifuged for 15 min at 4000 rpm. After the extraction both phases were carefully separated and analyzed.

For the BE 2 mL of the organic phase from the FE were mixed with 2 mL of fresh distilled water or buffer solution (0.05 mol/L TRIS–HCl). The mass transport was much slower [15] in the BE and the mixing time was set to 60 min. The conditions for the centrifugation maintained unchanged.

2.2.2. Enzyme extraction via phase-transfer

The extraction of enzymes (Celluclast 1.5L and Novozyme 188) was performed in a similar manner as the amino acids extraction by the phase-transfer method. The salt concentration of all samples was $c_{KCl} = 0.1$ mol/L, a commonly used concentration for the extraction of proteins [13]. The stock enzyme solution was prepared whilst the enzymes and the citrate buffer were mixed 1:20 (1 mL of enzyme solution and 19 mL of citrate buffer solution). The stock solutions were stored at +5 °C at pH = 4.8. Both enzymes were also analyzed as a mixture. For this purpose the ratio of Novozyme 188 and Celluclast 1.5L was set to 1:5 (1 mL of Novozyme 188 and 4 mL of Celluclast 1.5L), which is the common ratio used in biomass conversion [13,16–18]. The stock solutions for the mixtures were prepared with 1.25:20 in order to obtain the same concentration of Celluclast 1.5L, whereby this means, that the concentration of Novozyme is increased.

The extraction was performed in 10 mL volumetric glasses (type: Hecht Assistant NS 12, 5/21) and the volumes of both phases were 4 mL, as established for the amino acid extraction. The liquid phase contained 3.4 mL of 0.05 mol/L citrate buffer, 0.2 mL of 2 mol/L salt solution (KCl) and 0.4 mL of enzyme stock solution. The organic phase consisted of 4 mL with $c_{AOT} = 0.1$ mol/L. For the threefold determination the liquid phase was prepared in 50 mL falcon tubes (type: Sarstedt HD-PE). The extraction was performed in an overhead shaker (type: Heidolph Reax 2) for 15 min by ca. 80 rpm, so that the extraction process conditions were the same as for the amino acids.

The BE was also performed in 10 mL volumetric glasses (type: Hecht Assistant NS 12, 5/21). After the FE 3 mL of organic phase were added to 3 mL of fresh aqueous phase. The aqueous phase contained 0.05 mol/L of TRIS–HCl buffer and 1 mol/L of KCl. The high ionic strength was necessary to support the release of the proteins as reported in literature [13]. The pH of TRIS–HCl buffer was amounted to 9.2. The backward extraction was performed in the overhead shaker for 45 min at ca. 80 rpm. Afterwards both phases were separated for analysis.

2.3. Analysis methods

The concentrations of aromatic amino acids have been measured by UV–VIS spectroscopy (type: Thermo SCIENTIFIC Evolution 300/UV–VIS). Phe has been measured at 257 nm, Tyr 275 nm at and Trp at 279 nm. All other amino acids have been measured by Reversed-Phase-Chromatography with fluorimetric detection after a pre-column derivatization with o-Phthal in the central lab at Hamburg University of Technology. Only the concentration of the amino acids in the aqueous solution is directly measurable, so that

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