Enzyme Recovery using Reversed Micelles

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op gezag van de rector magnificus,
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STELLINGEN

1. Bij de door Aprahamian *et al.* gegeven bepalingsmethode van de hoeveelheid grensvlak in een vloeistof-vloeistof dispersie, gebaseerd op surfaktant depletie, is ten onrechte geen rekening gehouden met de verhoging van de grensvlakspanning bij toenemende hoeveelheid grensvlak.

   Aprahamian, E., F.F. Cantwell, en H. Freiser (1985) *Langmuir*, **1**, 79.

2. De door Patil *et al.* gegeven kreatieve fit van de door hun gemeten stofoverdrachtscoëfficiënten van eiwitten in waterige twee fase systemen geeft aan dat deze auteurs meer waarde hechten aan het kunnen verklaren van onderzoeksresultaten dan aan statistische betrouwbaarheid.

   Patil, T.A., S.B. Sawant, J.B. Joshi en S.K. Sikdar (1988) *Chem. Eng. J.*, **39**, B1.

3. Bij de beschrijving van de stofoverdracht van eiwitextrakties in holle vezel membranen door Dahuron en Cussler komt onvoldoende tot uiting dat hierbij afwijkingen tussen experimentele en theoretische waarden tot een faktor 20 normaal zijn. Niet in het model meegenomen verschijnselen hebben blijkbaar een zeer grote invloed op dit type extraktie.

   Dahuron, L. en E.L. Cussler (1988) *AIChE J.*, **34**, 130.

4. De verklaring dat afschuifkrachten verantwoordelijk zijn voor het feit dat hydrogenase in omgekeerde micellen sneller wordt geïnaktiveerd indien de vloeistof vaker wordt doorborreld met waterstof gas gaat voorbij aan een ander effekt van dit gas, nl. het veranderen van de redox toestand van het enzym, hetgeen een grote invloed heeft op de stabiliteit van hydrogenases.

   Castro, M.J.M. en J.M.S. Cabral (1989) *Enzyme Microb. Technol.*, **11**, 668.

5. Het bestuderen van de water opname kinetiek in omgekeerde micellen d.m.v. turbiditeitsmetingen aan een niet goed gedefinieerd systeem, zonder rekening te houden met het effekt van de druppelgrootte op de lichtverstrooiing draagt niet bij tot een beter begrip van het mechanisme van deze wateropname.

   Battistel, E. en P.L. Luisi (1989) *J. Colloid Interface Sci.*, **128**, 7.

6. Het verlies van surfactant zal de oorzaak zijn van het zeer geringe transport van eiwitten door vloeibare membranen van een omgekeerde micel fase, zoals gemeten door Armstrong en Li.

   Armstrong, D.W. en W. Li (1988) *Anal. Chem.*, **60**, 86.

7. Het doen van gedetailleerde voorspellingen over de efficiëntie van een enzymatische reaktie in omgekeerde micellen in een continu bedreven holle vezel reaktor op m^3 schaal op basis van enkele resultaten van batch experimenten in reageerbuizen getuigt van een ernstige onderschatting van de mogelijke opschaal effekten van een dergelijk proces.

   Gaathon, A., Z. Gross en M. Rozhanski (1989) *Enzyme Microb. Technol.*, **11**, 604.

8. Door de lange weg die nodig is voor het verkrijgen van bescherming en goedkeuring van routes voor de biotechnologische produktie en opwerking van voedingsmiddelen en medicijnen wordt bijna altijd gebruik gemaakt van verouderde en niet goed geoptimaliseerde produktie processen.

9. Het feit dat promovendi in hun wetenschappelijke stellingen meestal aangeven dat er in publikaties van anderen bepaalde zaken over het hoofd zijn gezien neemt niet weg dat ze regelmatig ideeën tegenkomen waaraan ze zelf nog niet gedacht hebben.

10. Een flexibel winkelopeningsbeleid zal de kwaliteit van de maaltijden van tweeverdieners verhogen.

M. Dekker
Enzyme Recovery using Reversed Micelles
Wageningen, 2 februari 1990

Voor Erica
Voor mijn ouders

VOORWOORD

Het in dit proefschrift beschreven onderzoek is tot stand gekomen dankzij de inzet van een groot aantal personen die ik hier graag wil bedanken.

In de eerste plaats wil ik hier noemen Klaas van 't Riet, zijn enthousiaste en stimulerende manier van begeleiden hebben een grote invloed gehad op mijn onderzoek. Naast zijn wetenschappelijke en creatieve inbreng ben ik hem ook dankbaar voor de manier waarop hij zijn deel van de sektie proceskunde runt. In korte tijd heeft hij enkele belangrijke fundamenten voor succesvol onderzoek aangebracht: een leuke groep goed gemotiveerde onderzoekers, die voorzien is van een uitstekende infrastruktuur.

Voor mijn onderzoek is de kennis uit meerdere vakgebieden van belang geweest. De begeleiding vanuit de vakgebieden Biochemie en Fysische- en Kolloid chemie is onmisbaar geweest. Bij de vakgroep Biochemie is in het begin Colja Laane bij mijn onderzoek betrokken geweest, later is dit overgenomen door Riet Hilhorst. Bert Bijsterbosch zorgde voor de ondersteuning vanuit de vakgroep Fysische- en Kolloidchemie. Naast hun begeleidende inbreng zijn genoemde personen ook van grote waarde geweest bij het corrigeren van de diverse publikaties die uit mijn onderzoek zijn voortgevloeid.

Binnen het kader van dit onderzoek hebben een aantal analisten een grote hoeveelheid werk verzet: bij Biochemie zijn dit in opeenvolgende perioden geweest: Jan Baltussen, Ron Wolbert en Peter Fijneman, bij Proceskunde was dit Peter Koenen.

Een groot aantal studenten heeft een bijdrage geleverd aan het omgekeerde micellen onderzoek in de vorm van een afstudeervak bij Proceskunde, Biochemie en/of Fysische- en Kolloidchemie. Ondanks het grote aantal wil ik ze hier toch allen bedanken voor hun inzet en ideeën: Stefan Weijers, Jacobiene Sanders, José Wijnans, Nico ter Burg, Eric Jan Reinierse, Libbe Tjalma, Frans den Ouden, Ria Stoop,

René Stunnenberg, Frans Roozen, Robbert Slingerland, Gijsbert Voskuilen, Henk Nachtegaal, Jens van der Pol, Hans van der Zandt, Jan Wattenberg, Dirk Heering, Albert Hamming, Marc Maste en Toine Bovee.

Het projekt is gefinancierd door de Stichting voor de Technische Wetenschappen (STW). Met het oog op de industriële toepassingsmogelijkheden van het onderzochte proces zijn halfjaarlijkse bijeenkomsten gehouden met vertegenwoordigers van een aantal geïnteresseerde bedrijven. Voor de gegeven adviezen en suggesties dank ik de leden van deze gebruikerscommissie: dr. ir. N.W.F. Kossen, dr. G.H. Schouten en drs. R.S. Hamstra (Gist-brocades); dr. ir. A.M. Trommelen en dr. N.K.H. Slater (Unilever); Dipl. Ing. R. Büchele (ENKA Membrana); dr. ir. K.H. Brunner (Westfalia Separator); ir. W.F. Jansen (Servo Delden); drs. N. Boots (STW).

De medewerkers van de werkplaats, fotolokatie, tekenkamer en magazijnen van de Centrale Dienst Biotechnion wil ik bedanken voor hun verrichte diensten.

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CONTENTS

OUTLINE OF THE THESIS

In biotechnology there is a need for new protein recovery processes, which combine a high selectivity for the desired product with a substantial concentration increase and easy scale-up. In this context, liquid-liquid extraction of an aqueous solution with an organic solvent containing reversed micelles presents itself as a promising process for the selective recovery of proteins form a fermentation broth. In this thesis research results on this recovery process are presented.

In Chapter 1 an introduction is given on reversed micelles and the factors which most predominantly affect the distribution behaviour of proteins between an aqueous phase and a reversed micellar phase. The selectivity of the extraction process is discussed in this part of the thesis.

Chapter 2 deals with the development of a continuous forward and back extraction of an enzyme using two mixer/settler units with the reversed micellar phase circulating between the units.

In Chapter 3 the modeling and optimization of this process is described. The optimization has been done with respect to maximum recovery of enzymatic activity and mimimum losses of surfactant from the reversed micellar phase.

The characterization of the mass transfer behaviour of an enzyme between an aqueous phase and a reversed micellar phase with respect to diffusional and interfacial processes is reported in Chapter 4.

In Chapter 5 the use of a temperature change to desolubilize the enzyme from the reversed micellar phase by affecting the phase behaviour is presented. This procedure has been used for the scale-up of the extraction process with centrifugal extractors.

A general discussion of the investigations is given in Chapter 6 of this thesis.

CHAPTER 1

THE DISTRIBUTION OF PROTEINS BETWEEN AN AQUEOUS AND A REVERSED MICELLAR PHASE

1.1. GENERAL INTRODUCTION

Reversed micelles are aggregates of surfactant molecules containing an inner core of water molecules, dispersed in a continuous organic solvent medium. These systems are optically transparent and thermodynamically stable. The considerable biotechnological potential of these systems is derived principally from the ability of the water droplets to dissolve enzymes, without loss of activity, in much the same way as does bulk water. The use of these systems in the biotransformation of apolar compounds, present in the bulk oil phase, has been described in a number of publications (Luisi, 1985; Martinek and Semenov, 1981; Hilhorst *et al.*, 1983; Laane *et al.*, 1987; Fletcher and Robinson, 1985).

Recently, it has been found that enzymes can be transported from one bulk aqueous phase to another *via* an intermediate reversed micellar phase, the transported protein molecules being transiently accommodated within the reversed micelles in this case. This phenomenon is a consequence of the reversibility of the phase transfer process. In addition to transport from a homogeneous solution phase,

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This chapter has been published as part of the review:
Dekker, M., R. Hilhorst, and C. Laane (1989) "Isolating Enzymes by Reversed Micelles", *Analyt. Biochem.*, **178**, 217.

proteins may also be transported from a solid phase, and even from the cytoplasmic compartment of bacteria. The latter phenomenon is based on the prior disruption of the bacterial cell wall by surfactant partitioning out of the reversed micellar phase.

Here, recent advances in separating enzymes by the use of reversed micelles are highlighted. The use of these media to convert apolar compounds enzymatically have been reviewed elsewhere (Luisi and Laane, 1986).

1.2. RECOVERY OF ENZYMES FROM AQUEOUS SOLUTION

The isolation of specific extracellular enzymes from a fermentation broth by conventional processes consists of the stepwise removal of undesired compounds from the broth. Because these steps are usually not specific for the desired enzymes, several steps are necessary to obtain an enzyme preparation of the required purity. Consequently, new isolation techniques, more selective for the required enzyme and easier to scale up are desirable. Liquid-liquid extraction procedures involving the use of reversed micellar systems have some very promising features in this respect.

The principle of this method is depicted schematically in Fig. 1. Firstly the enzyme is extracted from the aqueous phase to the reversed micellar phase under conditions such that the extent of phase transfer of the protein is maximal. Secondly the enzyme is recovered from the organic phase, by extraction of the reversed micellar phase with a second aqueous phase, under such conditions that the transfer of the enzyme from the reversed micellar phase to the aqueous phase is maximal.

By appropriate manipulation of physical parameters such as solution pH and ionic strength, which determine the distribution behaviour of the enzyme, it is possible to obtain manifold purification, and also concentration, of the required enzyme, in a straightforward process.

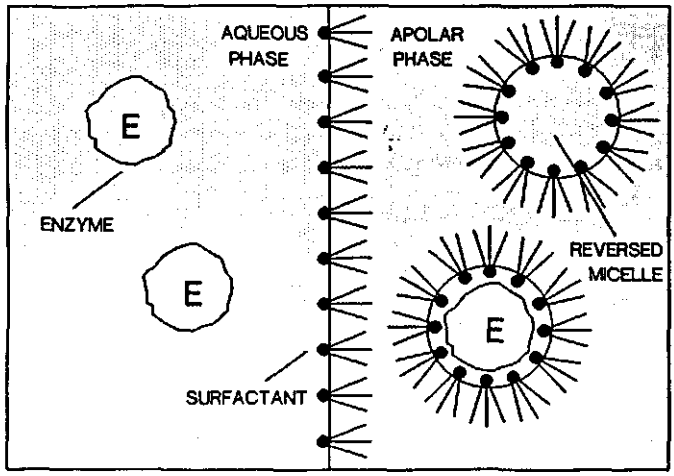

Fig. 1. Schematic representation of the liquid-liquid extraction of enzymes between an aqueous phase and a reversed micellar phase.

The first reports in literature on the transfer of proteins from an aqueous phase to a reversed micellar phase and *vice versa* were of Luisi *et al.* (1977, 1979), who illustrated this principle using a range of proteins. These investigations have been extended in more recent studies by groups at M.I.T. (Göklen and Hatton, 1985, 1986, 1987; Woll *et al.*, 1987, 1989) and in Wageningen (Van 't Riet and Dekker, 1984; Laane and Dekker, 1986; Dekker *et al.*, 1986, 1987[abc], 1988, 1989[ab]), for the development of a recovery process for proteins from aqueous solutions.

In the remaining part of this chapter the factors affecting the distribution of proteins between an aqueous phase and a conjugate reversed micellar phase will be discussed.

1.2.1. THE DISTRIBUTION OF PROTEINS

There is a large body of evidence which indicate that electrostatic interactions play a very important role in determining the distribution coefficient of a protein between a reversed micellar phase and a conjugate aqueous phase. This influence is

demonstrated clearly by the effect of aqueous phase pH, ionic strength and surfactant type (anionic, cationic or nonionic) on this distribution behaviour.

The individual effects exerted by each of these parameters will now be discussed separately.

1.2.1.1. Aqueous phase pH

The aqueous phase pH determines the ionization state of the surface charged groups on the protein molecule. Attractive electrostatic interactions between the protein molecule and the surfactant head groups, which form the internal surface of the reversed micelle, will occur if the overall charge of the protein is opposite to the charge of the surfactant head groups. This implies that, for cationic surfactants solubilization of the protein in reversed micelles is favoured at pH values above the isoelectric point (pI) of the protein, whilst the opposite is true for anionic surfactants.

Luisi *et al.* (1979) showed that the aqueous phase pH influences the transfer of α-chymotrypsin and pepsin to a reversed micellar phase of the cationic surfactant trioctylmethylammonium chloride (TOMAC) in cyclohexane. These results, however, could not be interpreted on basis of the pI values of these proteins.

The phase transfer of the enzyme α-amylase into reversed micelles of TOMAC/octanol in isooctane was found to occur only over a narrow pH range, well above the pI of the enzyme (Van 't Riet and Dekker, 1984). These results indicate that not only the sign of the protein charge is an important factor in protein solubilization, but also the surface charge density.

Using a reversed micellar system consisting of the anionic surfactant sodium di-2-ethylhexyl sulphosuccinate (commercial name Aerosol OT or AOT) in isooctane, Göklen and Hatton (1987) found almost complete solubilization of the three low molecular weight proteins (M_r = 12-14 kDal) ribonuclease A, cytochrome c and lysozyme. This solubilization took place in a range of 4 to 6 pH units just below the pI's of the three proteins (Fig. 2).

As the molecular weight of the protein increases, phase transfer can only be accomplished by increasing the value of (pH - pI). Thus in the case of α-chymotrypsin (M_r = 25 kDal) phase transfer was favoured in a pH range approximately 2 to 4 pH units below the pI of the protein; Bovine Serum Albumin (M_r = 68 kDal) was not transferred at all in the same system.

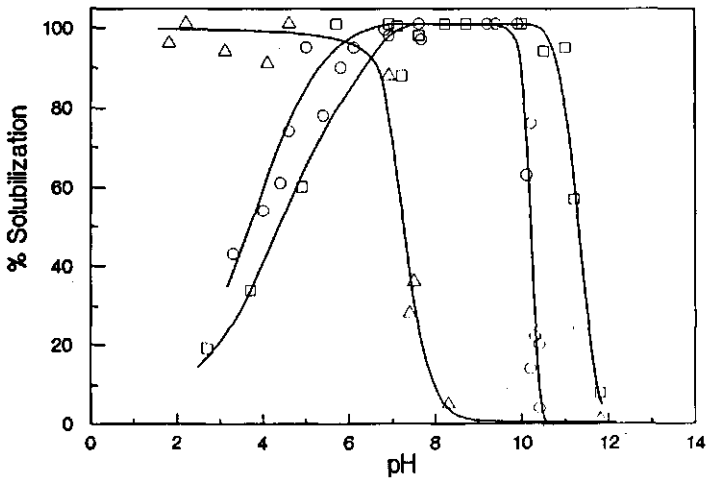

Fig. 2. The effect of pH on the solubilization of lysozyme, cytochrome c and ribonuclease A in AOT-isooctane solutions (From Göklen and Hatton, 1987).

The effect of pH on solubilization for proteins of different molecular weight might be explained by taking into account the fact that for larger proteins the size of the reversed micelle containing a protein molecule has to be significantly larger than the size of the empty reversed micelle. This energetically unfavorable transition of the micellar size has to be compensated for by more extensive electrostatic interactions in order to make the overall solubilization process feasible. This can be achieved by increasing the charge density of the protein by manipulation of the pH. For small proteins whose size is smaller than the size of the water pool inside a reversed micelle, solubilization occurs as soon as the net proteins charge is opposite to that of the reversed micellar interface.

This behaviour is demonstrated clearly by the linear correlation attained between the M_r of a protein and the difference between the pH of solubilization and the pI of the protein (Fig. 3, Wolbert *et al.*, 1989). The correlation holds for reversed micelles of both anionic and cationic surfactants.

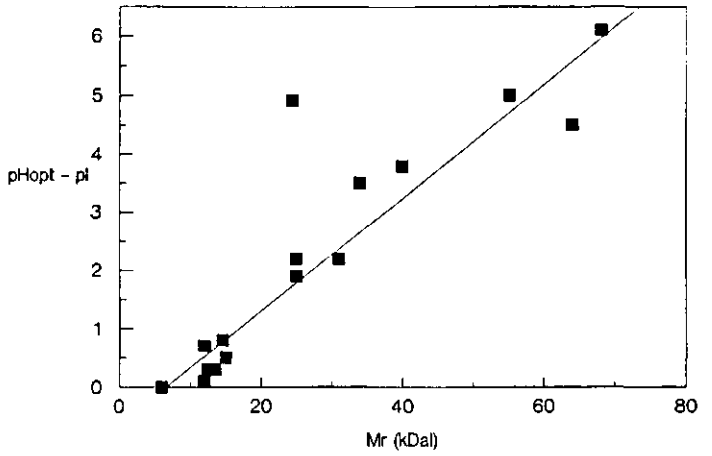

Fig. 3. Relation between the molecular weight of a protein and the difference between the proteins pI and the pH of optimal extraction into a reversed micellar phase of TOMAC in isooctane (From Wolbert *et al.*, 1989).

1.2.1.2. Ionic strength

The ionic strength of the aqueous phase determines the degree of shielding of the electrostatic potential imposed by a charged surface. This phenomenon causes at least two important effects in reversed micellar extraction. Firstly it decreases the electrostatic interaction between the charged protein molecule and the charged interface in the reversed micelle. Secondly it reduces the electrostatic repulsion between the surfactant head groups, resulting in a decrease in the size of the reversed micelles at higher ionic strength.

Göklen and Hatton (1986, 1987) showed the effect of ionic strength on the phase transfer of ribonuclease A, cytochrome c and lysozyme in a reversed micellar phase of AOT in isooctane. The extent of phase transfer to the reversed micellar phase decreases for all three proteins with increasing potassium chloride concentration, but the concentration required to initiate this decrease was found to be different for each

protein (Fig. 4). No rules can be deduced from these data with respect to the ionization state of the proteins used (expressed as the difference between the aqueous phase pH (6.7 - 7.1 in this study) and the pI of the proteins).

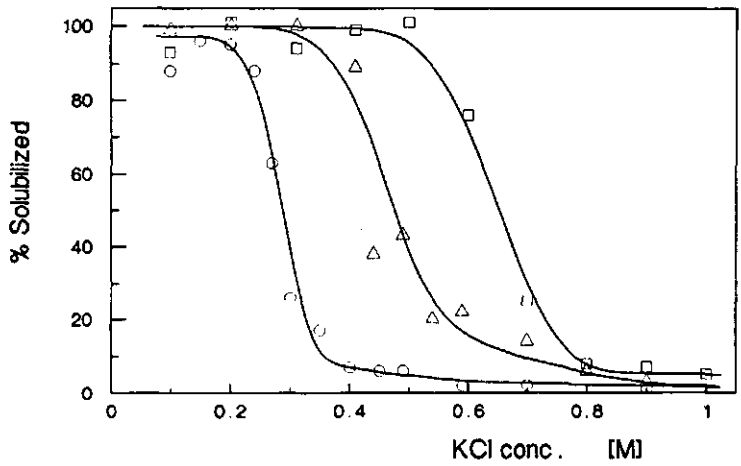

Fig. 4. The effect of the potassium chloride concentration on the solubilization of lysozyme, cytochrome c and ribonuclease in AOT-isooctane solutions (From Göklen and Hatton, 1987).

Meier *et al.* (1984) observed an increase in the amount of trypsin and peroxidase transferred to a reversed micellar phase of AOT in isooctane, using sodium salts to increase the ionic strength, but a decrease when using calcium salts. These effects were observed at both pH 7 and 10.

The fact that an increase in the ionic strength results in a shift in the pH profile of the distribution behaviour was demonstrated by Dekker *et al.* (1987c). The transfer of the enzyme α-amylase to a reversed micellar phase of TOMAC/octanol in isooctane was studied for different concentrations of sodium chloride as a function of the pH (Fig. 5).

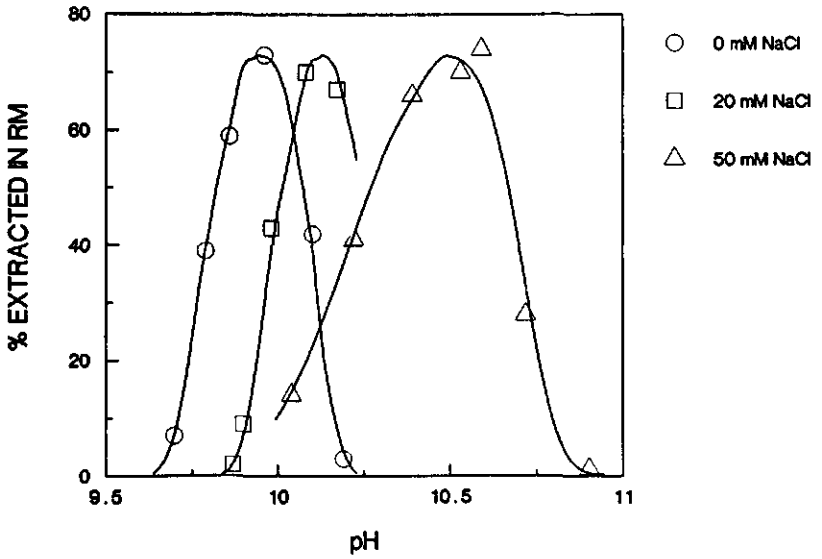

Fig. 5. Effect of pH on the transfer of α-amylase to a reversed micellar phase of TOMAC in isooctane at different ionic strengths (From Dekker *et al.*, 1987[c]).

As the salt concentration increased, a higher pH (resulting in a higher charge density on the protein) was required for maximal phase transfer. Thus once again, electrostatic interactions are implicated as being of primary importance in the phase transfer mechanism. For the results shown, it can be seen that increasing the ionic strength at pH 10.1, for example, first causes an increase in solubilization of α-amylase in the reversed micellar phase followed by a decrease in solubilization beyond an optimal ionic strength. The observation that not only the ionic strength, but also the type of ion influences the distribution behaviour of proteins implies specific interaction of ions with the protein and/or the surfactant headgroups.

The differences in the distribution behaviour of proteins on the pH and ionic strength of the aqueous phase has been used to advantage in the separation of a protein mixture (Gökden and Hatton, 1987). An aqueous solution of ribonuclease A, cytochrome c and lysozyme was extracted with a reversed micellar phase of AOT in isooctane. By using the results from Fig. 2 and 4 the three proteins could be separated by a series of three extractions.

Woll and Hatton (1987) showed that the aqueous phase pH and ionic strength

could be used to selectively extract an alkaline protease from a fermentation broth. By performing two series of extractions 42 % of the enzyme was recovered with a 2.2-fold increase in specific activity.

1.2.1.3. Surfactant type

As shown in the previous two sections, the distribution of proteins is dependent primarily on the difference in charge between the protein and the charged surfactant head groups. Therefore, in the absence of other effects, an opposite pH dependence of the distribution behaviour can be expected for reversed micelles stabilized by cationic and anionic surfactants.

In addition to the surfactant charge, other surfactant-dependent parameters may be cited. Among these are the size of the reversed micelles that are formed, the energy required to enlarge the reversed micelles, and the charge density on the inner surface of the reversed micelle.

Dekker *et al.* (1987[a]) showed the effect of the addition of a nonionic surfactant (Rewopal HV5: nonylphenolpentaethoxylate) to a reversed micellar phase of the cationic surfactant TOMAC in isooctane. The partitioning of α-amylase between the reversed micellar and the conjugate aqueous phase was found to be a strong function of the ratio of nonionic to ionic surfactant. An increase in the proportion of nonionic surfactant resulted in increased transfer of the enzyme to the reversed micellar phase. Additionally, phase transfer took place over a wider range of pH (Fig. 6).

This change in distribution behaviour might be explained by two effects; Firstly the charge density of the micellar interface will be influenced by the presence of the nonionic surfactant. Secondly the size and flexibility of the reversed micelles will be changed by the addition of the one-tail nonionic surfactant.

Until now we have only discussed the use of electrostatic interactions between the protein and the surfactant head groups to promote transfer to a reversed micellar phase. Another possibility is the use of biospecific interactions between an enzyme and a ligand (substrate analogue, product or an inhibitor). In principle, the ligand can be confined to the reversed micellar phase by conjugation to a suitable hydrophobic tail; in the reversed micelles, such a (polar) ligand would form a sitespecific surfactant headgroup. The effect of such an affinity surfactant, octyl-ß-D-glucopyranoside, has

been shown for the solubilization of concanavalin A in AOT reversed micelles (Woll *et al.*, 1987). The protein was transferred to the reversed micellar phase at pH values at which no transfer was observed without the affinity surfactant. This transfer was inhibited by the addition of free ligand in the aqueous phase, indicating that an affinity interaction was required for the solubilization at these pH values.

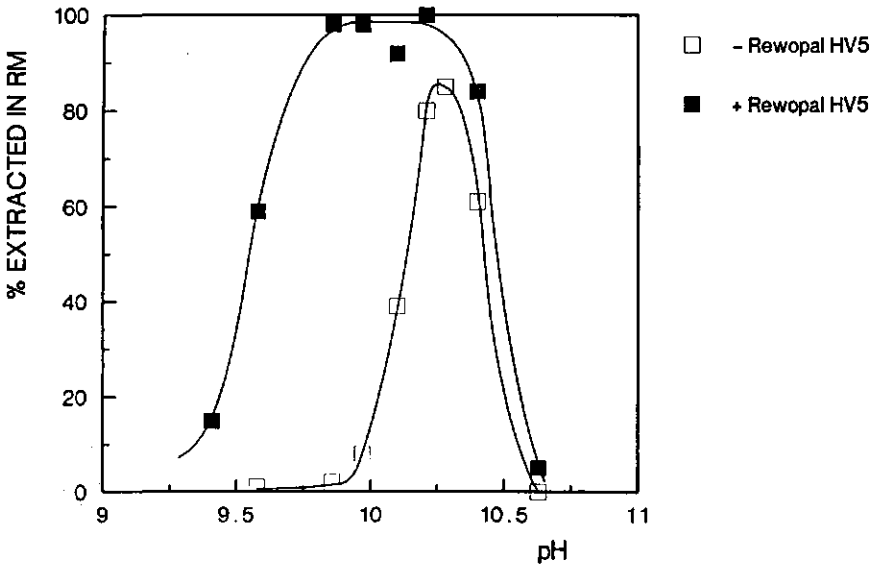

Fig. 6. Solubilization of α-amylase in a reversed micellar phase of TOMAC in isooctane in relation to the pH in the aqueous phase. Effect of the addition of nonionic surfactant to the reversed micellar phase (From Dekker *et al.*, 1987[a]).

In this system the solubilization is most likely due to a combination of electrostatic, affinity and possibly hydrophobic interactions. It would be interesting to explore this phenomenon further by substituting a nonionic surfactant for the ionic surfactants used previously. In this case electrostatic interactions between the protein and surfactant head groups are eliminated and affinity interactions, perhaps in combination with hydrophobic interactions, become decisive in determining the extent of phase transfer. This should result in a very selective extraction process.

1.2.1.4. Surfactant concentration

The concentration of surfactant in a reversed micellar phase that is in equilibrium with an aqueous phase has little effect on the size and structure of the reversed micelles. The extent of protein uptake from a conjugate aqueous phase therefore increases in proportion to the surfactant concentration in the reversed micellar phase (Woll *et al.*, 1989; Fletcher and Parrott, 1988). Solubilization of ribonuclease A and concanavalin A as a function of the AOT concentration could be described by a model based on a thermodynamic equilibrium between the concentration of protein - reversed micelle complexes and the concentration of free reversed micelles and free protein in the aqueous phase (Woll *et al.*, 1989). The distribution of α-chymotrypsin and pepsin in AOT reversed micelles has been described (Fletcher and Parrott, 1988) by expressing the protein partitioning based on the protein concentration in the water pools of the reversed micelles divided by the bulk aqueous phase concentration. This approach resulted in a partition coefficient which was independent of the surfactant concentration.

Since the total volume of the water pools varies linearly with the surfactant concentration ($w_{0,max}$, the molar ratio of water to surfactant in the micellar phase is approximately independent of the concentration of surfactant), the two descriptions are essentially the same.

In this context, the partitioning behaviour of the enzyme lysozyme proved to be exceptional; below a certain surfactant concentration the enzyme precipitated at the interface between the two phases. Yet increasing the surfactant concentration above this point resulted in the transfer of all of the protein to the reversed micellar phase. It was estimated that this concentration was just sufficient to provide a monolayer coverage for the total enzyme in the system.

1.2.1.5. Thermodynamic modeling

Several models of the distribution behaviour of proteins between a bulk aqueous phase and a reversed micellar phase have been developed (Fraaije, 1987; Maestro and Luisi, 1986; Woll and Hatton, 1987; Fraaije *et al.*, 1990). They are all based upon electrostatic interactions between the charges on the protein molecule and the

charges on the inner surface of the reversed micelle due to the ionic head groups of the surfactants. In addition to free energy change resulting directly from this interaction, an additional contribution arising from the redistribution of surfactant and protein-counterions, of other, free, ions, of water and of surfactant, on phase transfer must be considered.

Calculations have been made of the total free energy change upon the uptake of a hypothetical small protein in reversed micelles (Maestro and Luisi, 1986); no comparisons were made with experimentally determined distribution data.

In a more phenomenological approach, the distribution behaviour of cytochrome c between an aqueous phase and a reversed micellar phase of the cationic surfactant TOMAC in isooctane was analyzed with respect to the aqueous phase pH and ionic strength (Fraaije, 1987). From the experimental data calculations were made on the copartitioning of protons and other small ions accompanying protein uptake in reversed micelles. Around the optimal pH for protein transfer to the reversed micellar phase both protons and surfactant counterions (chloride) were found to be redistributed.

A third approach (Woll and Hatton, 1987) described the partitioning of ribonuclease A and concanavalin A as a function of aqueous phase pH and surfactant concentration. Four coefficients were used which depend on the reversed micellar system and the protein used.

All of the above models are useful in analyzing experimental data of protein partitioning and in indicating the most important system parameters affecting the value of the distribution coefficient. However the models cannot presently give accurate predictions of the distribution coefficient of a protein; many parameters are unknown or hard to quantify. These include hydrophobic interactions of the protein with the apolar phase, specific interactions of ions with the protein and surfactant, the free energy changes associated with the change in size of the reversed micelles on protein uptake and the distribution of charged groups on the protein molecules.

This last parameter has been neglected in all three approaches, they all assume an equal distribution of surface charge on a globular protein molecule. Wolbert *et al.* (1989) showed a strong effect of the symmetry of the distribution of charged groups on the surface of proteins on their distribution behaviour. Proteins with a more asymmetric charge distribution were found to partition more easily into the reversed micellar phase.

1.2.2. PROCESS DEVELOPMENT

For the large scale recovery of extracellular enzymes, an extraction with an organic solvent containing reversed micelles has interesting advantages over existing processes. A reversed micellar extraction combines the potential for concentration and purification of the enzyme in a single process. The liquid-liquid extraction technique in general is well known and apparatus and scale-up rules have been established for numerous applications, including the use in recovery processes for antibiotics and organic acids from fermentation broths.

The success of the type of extraction processes described here is critically dependent on the ability to direct the distribution coefficient of the desired enzyme between the aqueous phase and the reversed micellar phase, as discussed in section 1.2.1.

The remaining chapters of this thesis will be addressed to the development of this liquid-liquid extraction process for enzymes.

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CHAPTER 2

ENZYME RECOVERY BY LIQUID-LIQUID EXTRACTION USING REVERSED MICELLES

ABSTRACT

The recovery of α-amylase by liquid/liquid extraction, using a reversed micellar phase to transport the enzyme from one aqueous phase to another has been investigated. Reversed micelles are aggregates of surfactant molecules in an apolar solvent, surrounding an inner core of water. Reversed micelles of the cationic surfactant trioctylmethylammonium chloride were used to solubilize α-amylase in isooctane. A continuous forward and back extraction of the enzyme has been performed in two mixer/settler units, with the reversed micellar phase circulating between the two units. During the forward extraction the conditions (pH,ionic strength) favoured the transfer of α-amylase from the aqueous phase towards the reversed micellar phase. The reversed micellar phase containing the α-amylase was subsequently extracted with a second aqueous phase, which favoured the transfer of the enzyme towards the aqueous phase. In this way, the concentration of active α-amylase in the second aqueous phase was eight times that of the original solution. An enzyme

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activity loss of 30 % occurred during the extraction procedure. The forward and back extraction could be described in terms of the data on the distribution coefficients and the mass transfer rate constants.

2.1. INTRODUCTION

The recovery of an extracellular enzyme usually consists of the removal of undesirable compounds from the fermentation broth (e.g. microbial cells, debris, other proteins, salts and water). The conventionally employed processes are filtration, ultra-filtration, precipitation and drying. For more purified enzymes, chromatography processes are usually required. It would be very desirable to have access to a recovery step that is selective for the desired enzyme, that gives a considerable increase in the concentration of the enzyme and that can be scaled up easily.

A liquid-liquid extraction might serve these purposes. To be able to extract an enzyme from an aqueous phase into another liquid phase, one needs an immiscible phase, that can solubilize enzymes. A possible way to accomplish this is to create an aqueous two phase system, by the addition of certain polymers (Hustedt *et al.*, 1985). With this system several enzymes have been successfully recovered but one disadvantage is the costs of the polymer(s), which makes the recovery of these compounds necessary in a commercial application. Especially in the case of extracellular enzymes this will be a particular problem due to the large volumes of fermentation broth that are to be handled.

Another water immiscible phase that can solubilize enzymes is an apolar solvent containing reversed micelles. Reversed micelles are aggregates of surfactant molecules surrounding an inner core of water. Many enzymes have been succesfully solubilized by reversed micelles without loosing their catalytic activities (Hilhorst *et al.*, 1983, 1984[a], 1984[b]; Martinek *et al.*, 1977; Luisi *et al.*,1977, 1979, 1984; Wolf and Luisi, 1979; Bonner *et al.*, 1980; Meier and Luisi, 1980; Laane and Luisi, 1986). These reversed micellar systems containing enzymes have been used to study enzymatic reactions involving apolar substrates and to study enzymatic reactions at subzero temperatures (Douzou *et al.*, 1979). They might also serve as a model system for membrane adsorbed enzymes. Furthermore it has been demonstrated that proteins

can be transferred from an aqueous phase to a reversed micellar phase or *vice versa* (Luisi *et al.*, 1979; Meier *et al.*, 1984; Van 't Riet and Dekker, 1984; Göklen and Hatton, 1985) as shown schematically in Figure 1.

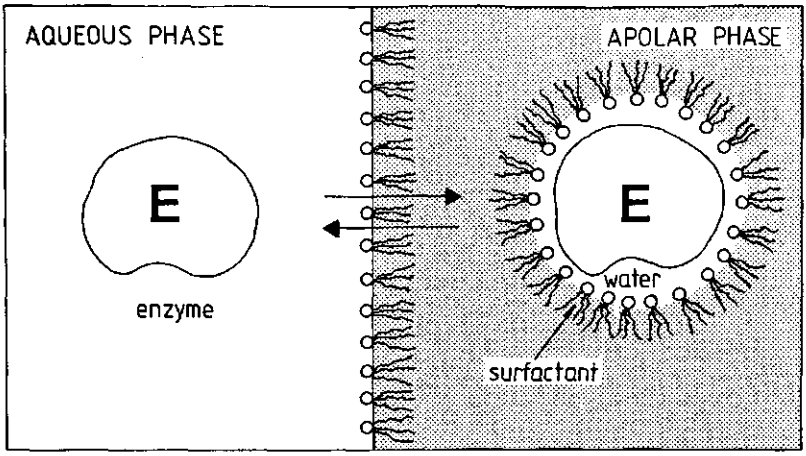

Fig. 1. Transfer of an enzyme between an aqueous phase and an apolar phase containing reversed micelles.

All these investigations have been done with either the anionic Aerosol OT (Meier *et al.*, 1984; Göklen and Hatton, 1985) or the cationic trioctylmethylammonium chloride (TOMAC) (Luisi *et al.*, 1979; Van 't Riet and Dekker, 1984) as surfactant. The influence of the apolar solvent, ionic strength and the pH on the transfer of proteins has been investigated. We have shown, in the case of the extracellular enzyme α-amylase that the concentration of the enzyme can be increased considerably by a forward extraction into a reversed micellar phase followed by a back extraction into a second aqueous phase of different composition (Van 't Riet and Dekker, 1984).

Here we report the performance of a double extraction process that is capable of transporting and concentrating α-amylase continuously from one aqueous phase to another via a reversed micellar phase (Figure 2). The results of this forward and back extraction could be described in a model using the experimentally determined distribution coefficients and mass transfer rates during both extractions.

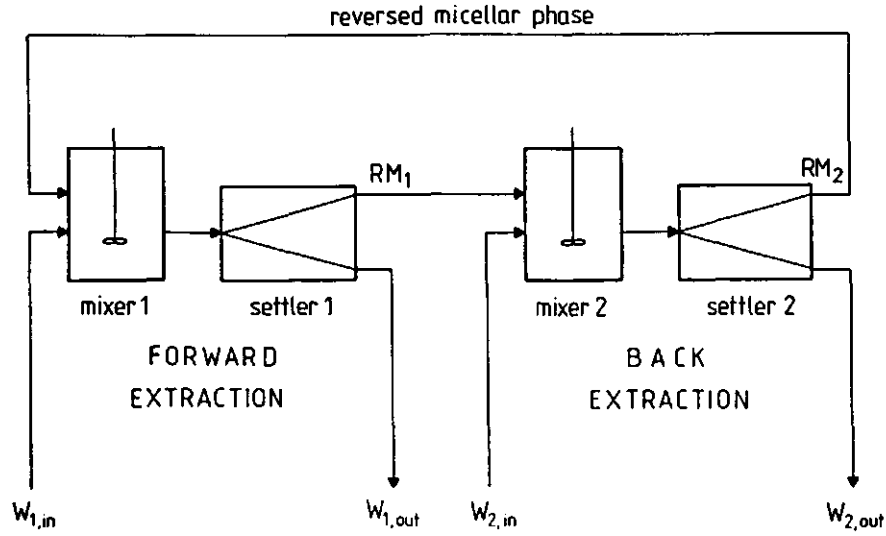

Figure 2: Flowsheet of a combined forward and back extraction, for two mixer/settler units with the reversed micellar phase circulating between the two extraction units.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals:

α-Amylase (EC 3.2.1.1, crystalline, 950 U mg^{-1} and crude, 50 U mg^{-1}) from *Bacillus subtilis* (correct name *Bacillus amyloliquefaciens* (Welker and Campbell, 1967)) was obtained from Sigma Chemical Co. Trioctylmethylammonium chloride (TOMAC) was obtained from Merck and contained 88% (w/w) of the quaternary ammonium salt and about 10% (w/w) of a mixture of octanol and decanol. Tris was obtained from Boehringer. All other chemicals were obtained from Merck and were of analytic grade.

2.2.2. α-Amylase activity:

The enzyme activity was determined according to the method described by Bernfeld, 1951. One unit is defined as the amount of enzyme that will liberate one mg of maltose from starch in three minutes at pH 6.9 at 20 °C.

2.2.3. Reversed micellar solution:

The reversed micellar solution contained 0.4% (w/v) TOMAC and 0.1% (v/v) octanol in isooctane.

2.2.4. Distribution of α-amylase between aqueous and reversed micellar phase:

The effect of the pH of the aqueous phase on the solubilization of α-amylase in the reversed micellar phase was determined as follows:
(1) To a 5 ml vial 2 ml of an aqueous phase (0.5 M tris adjusted to the desired pH with HCl) containing α-amylase (0.25 mg ml^{-1} pure or 1.0 mg ml^{-1} crude) and 2 ml of the reversed micellar phase were added. (2) The two liquid phases were mixed by rotating the vial 2.0 times per second. The time to reach equilibrium was found to be 4 to 5 minutes. (3) After this extraction the two phases were separated by gravity in 1 to 2 minutes. (4) Half of the reversed micellar solution was separated (1 ml) and added to another vial containing 1 ml of a second aqueous phase (0.5 M KH_2PO_4/K_2HPO_4, pH 6.9.). (5) A second extraction was performed, which was found to reach equilibrium within two minutes of shaking. (6) Both phases were separated by gravity and the α-amylase activity in the aqueous solution was determined.

The second extraction was found to be almost complete. This means that the α-amylase activity found in the second aqueous phase is a good approximation of the amount of active α-amylase solubilized in the reversed micellar phase.

2.2.5. Mass transfer in mixers:

The mixers were made of plexiglass (operating volume of 600 ml) and were baffled (Fig. 3(a)). The stirrer geometry is described in Fig. 3(b). This type of stirrer was used in order to obtain a homogeneous dispersion throughout the entire mixer. A standard Rushton turbine stirrer gave a very fine dispersion near the stirrer, but a strong coalescence in the top and bottom section of the mixer.

In a mass transfer experiment 200 ml of reversed micellar phase and 360 ml of aqueous phase (0.5 M tris·HCl, pH 9.85) without α-amylase were stirred in the mixer until the dispersion was homogeneous. Then 40 ml of aqueous phase (0.5 M tris·HCl, pH 9.85) containing α-amylase (10.0 mg ml^{-1}, crude) was added. Samples were taken and these were settled by gravity. The amount of α-amylase activity that was transferred to the apolar phase was determined as described above, starting at step 4.

To study the mass transfer rate during back extraction, 500 ml of reversed

micellar phase, containing α-amylase as a result of a first extraction with an aqueous α-amylase solution (1.0 mg ml^{-1} crude, in 0.5 M tris·HCl, pH 9.85), was stirred in the mixer. Then 50 ml of the second aqueous phase (1.0 M tris·HCl, pH 6.9) was added. After taking and settling samples the α-amylase activity was determined in the aqueous phase.

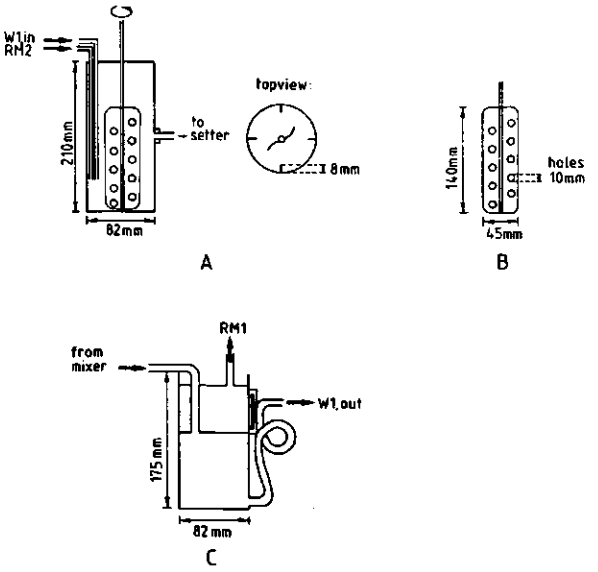

Fig. 3. Mixer and settler used for enzyme extraction. A: vessel; B: stirrer; C: settler.

2.2.6. Continuous extraction:

Continuous extraction of α-amylase was performed in two mixer/settler units, as shown in Fig. 2. The reversed micellar phase was circulated between the two extractors. The mixers are described above. The settlers (Figure 3(c)) were made of perspex (operating volume of 900 and 650 ml respectively). Peristaltic pumps with neoprene tubing (Watson-Marlow) were used.

The flows were set to: F_{W1} = 1.35 ml s^{-1}, F_{RM} = 0.67 ml s^{-1}, F_{W2} = 0.075 ml s^{-1}. The total volume of reversed micellar phase was 2 l.

W1,in contained 1.0 mg ml^{-1} of crude α-amylase in 0.5 M tris·HCl, pH 9.85. W2,in

contained 1.0 M tris·HCl, pH 6.9.

During the extraction experiments α-amylase activity was determined as described above in W1,out, W2,out, and in the apolar phase in both settlers.

2.3. EXTRACTION THEORY

A continuous forward and back extraction of an enzyme in two mixer/settler units (Fig. 2) with the reversed micellar phase circulating between the units can be described by the following equations:

2.3.1. mass transfer in mixers

For batch operation:

$$\frac{dC_{RM1}}{dt} = K_{ov,1}(m_1 C_{W1} - C_{RM1}) \qquad (1)$$

$$\frac{dC_{W2}}{dt} = K_{ov,2}(m_2 C_{RM2} - C_{W2}) \qquad (2)$$

For continuous operation:

$$C_{RM1} - C_{RM2} = K_{ov,1}\tau_1(m_1 C_{W1,out} - C_{RM1}) \qquad (3)$$

$$C_{W2,out} = K_{ov,2}\tau_2(m_2 C_{RM2} - C_{W2,out}) \qquad (4)$$

2.3.2. mass balances

$$F_{W1}(1 - C_{W1,out}) = F_{RM}(C_{RM1} - C_{RM2}) \qquad (5)$$

$$F_{W2}(C_{W2,out} - 0) = F_{RM}(C_{RM1} - C_{RM2}) \qquad (6)$$

When the value of the distribution coefficients, $m_1 = C_{RM}^*/C_{W1}$ and $m_2 = C_{W2}^*/C_{RM}$ and the overall mass transfer rate constants $K_{ov,1}$ and $K_{ov,2}$ are known, it is possible to calculate all the concentrations for a given set of flows and residence times.

If inactivation of the enzyme takes place during the extractions the concentrations in (1) to (6) have to be corrected for this effect.

2.4. RESULTS AND DISCUSSION

2.4.1. Distribution of α-amylase between aqueous and reversed micellar phase

The distribution of pure α-amylase between the aqueous and the reversed micellar phase was studied in relation to the pH of the aqueous phase. The amount of α-amylase activity transferred towards the reversed micellar phase, as a function of the initial pH in the aqueous phase is given in Fig. 4.

The results clearly indicate that the amount of active α-amylase that can be solubilized in the apolar phase by the reversed micelles is strongly pH dependent. At the optimal pH 9.90, 90 ± 2% of the α-amylase is solubilized in the reversed micellar phase. This would mean that the distribution coefficient of the enzyme in this system will have a value of about 10. In fact this value will be even higher, since some inactivation (5 ± 3%) of α-amylase takes place during these extractions.

When crude α-amylase was used (1.0 mg ml^{-1}) instead of crystalline α-amylase the optimal pH for solubilization shifted to pH 9.80 - 9.85, while the relative amount of solubilized α-amylase was less. The reason for this is not clear, impurities in the crude enzyme preparation might have their effect on the solubilization of the α-amylase in the reversed micelles.

The surfactant we used is a quaternary ammonium salt, which will give the interface of the reversed micelles a positive charge. Since α-amylase has a pI of around 5.2 (Stein and Fischer, 1960), the enzyme will have a net negative charge at pH 9.80 - 9.90, while the surfactant headgroups in the reversed micelle have a net positive charge. Maximum solubilization of α-amylase in reversed micelles seems to occur at a pH value at which the effective charge of the enzyme will be equal but opposite to the effective charge of the interface of the reversed micelle.

The buffering system used, tris·HCl (pKa = 8.3), will have an increasing ionic strength for decreasing pH values, since the unprotonated form has no net charge. This will cause less effective charge interactions between α-amylase and surfactant at lower pH values. The size of the reversed micelles will also be influenced by this effect.

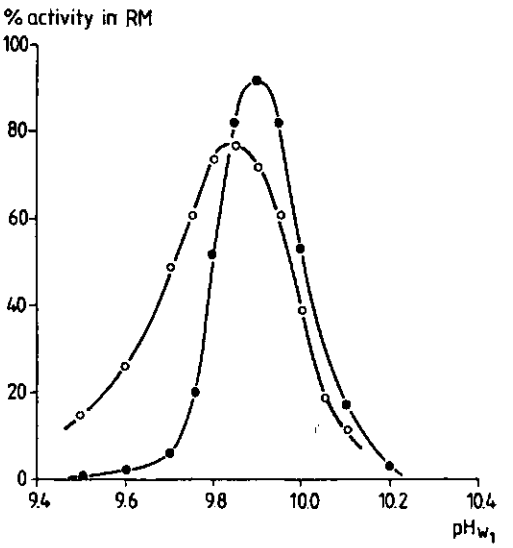

Fig. 4. Solubilization of pure (•) and crude (o) α-amylase in the reversed micellar phase in relation to the initial pH of the aqueous solution. The amount of the α-amylase activity, which is recovered from the reversed micellar phase is expressed as percentage of the initial α-amylase activity in the aqueous phase.

As can be seen from Fig. 4 the distribution coefficient of α-amylase over aqueous and reversed micellar phase will be very large at pH 6.9. A back extraction of a reversed micellar phase containing α-amylase with 1.0 M tris·HCl, pH 6.9 was performed. The distribution coefficient ($m_2 = C_{W2}^*/C_{RM}$) was determined by taking different volume ratios of aqueous to reversed micellar phase (Van 't Riet and Dekker, 1984). This resulted in a value of at least 250.

2.4.2. Mass transfer in mixers

To be able to perform a continuous extraction of α-amylase with a reversed micellar phase it is necessary to have data on the mass transfer rate during mixing. During the forward extraction of crude α-amylase (volume ratio RM/W1 = 0.5), the amount of α-amylase activity which was extracted in the reversed micellar phase was determined. The stirrer speed is set to the minimum value at which the dispersion was homogeneous throughout the entire mixer (2.8 s^{-1}), resulting in reversed micellar phase droplets with diameters of (2-5)×10^{-4} m (visually estimated).

Figure 5(a) shows the time course of the forward extraction of α-amylase. The observed optimum in the amount of α-amylase activity in the reversed micellar phase is due to the opposing effects of mass transfer and inactivation of α-amylase during mixing. This decrease in activity could be described by first order kinetics with a rate constant (k_i) of (2-4)×10^{-4} s^{-1}. By correcting the observed C_{RM1} values for this inactivation the overall mass transfer rate constant ($K_{ov,1}$) was determined by using equation (1) and the mass balance. This resulted in a value of (2-3)×10^{-3} s^{-1}.

The mass transfer rate of α-amylase during back extraction was also studied. During the extraction (volume ratio W2/RM = 0.1) the amount of α-amylase activity in the aqueous phase was determined. The stirrer speed necessary to obtain a homogenous dispersion, being 4.0 s^{-1}, was higher then during the forward extraction, resulting in water droplets with diameters of (5-10)×10^{-4} m (visually estimated). Figure 5(b) shows the time course of the back extraction of α-amylase. In contrast to the forward extraction of the enzyme no inactivation is observed during mixing. The overall mass transfer rate constant ($K_{ov,2}$) was determined by using equation (2) and the mass balance. This resulted in a value of (2-3)×10^{-4} s^{-1}.

The ratio of the mass transfer rate constants during forward and back extraction, $K_{ov,1}/K_{ov,2}$ = 10 can be explained by the ratio in surface areas during both extractions, $A_1/A_2 = (\epsilon_1 d_2)/(\epsilon_2 d_1)$ = 4 - 20. A difference in mass transfer rates during forward and back extraction has also been observed by other workers (Göklen and Hatton, 1985), they subscribe this difference to energetic effects of the phase transfer of the protein.

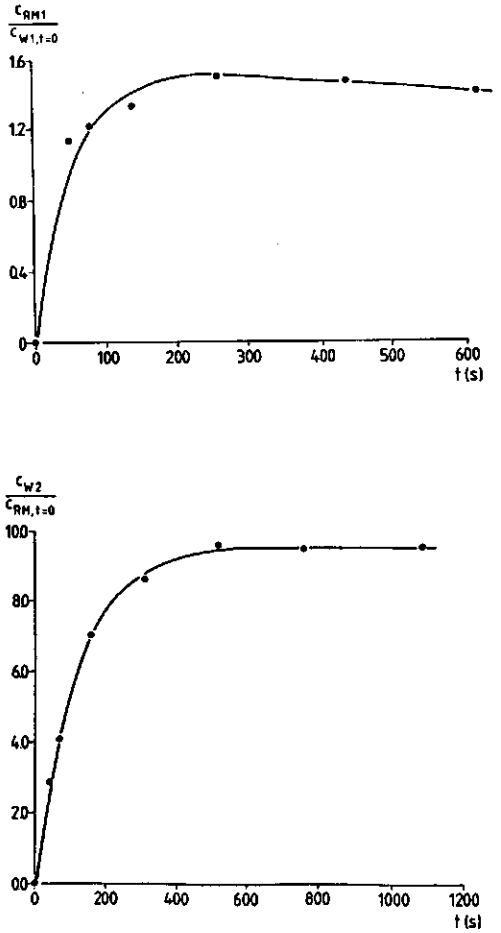

Fig. 5. A: Forward extraction of α-amylase (batch extraction). The relative concentration of active α-amylase transferred to the reversed micellar phase is plotted versus extraction time. (volume ratio RM/W1 = 0.5).
B: Back extraction of α-amylase (batch extraction). The relative concentration of active α-amylase transferred to the aqueous phase is plotted versus extraction time. (volume ratio W2/RM = 0.1).

2.4.3. Continuous extraction of α-amylase

Knowing the values of the distribution coefficients (m_1 and m_2) and the overall mass transfer rate constants ($K_{ov,1}$ and $K_{ov,2}$) during both the forward and back extraction it is possible to predict the concentrations in a continuous extraction process as described in Figure 2 by using equations (3) to (6).

We have performed these extractions in two mixer/settler units with the reversed micellar phase recirculating between the two extraction units (Fig. 2). The relative α-amylase activity in the aqueous and reversed micellar phases during the extractions are given in Figure 6. The steady state concentrations in the aqueous and reversed micellar phases are listed in Table 1.

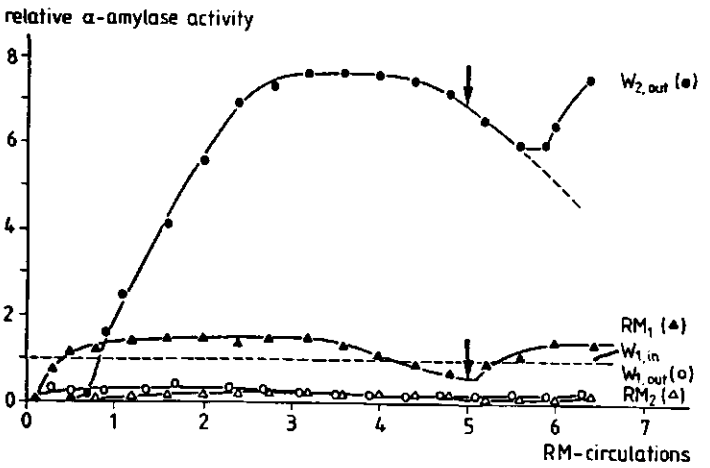

Fig. 6. Concentration of active α-amylase in W1,out, W2,out, RM1 and RM2 (all concentrations relative to W1,in), versus the number of circulations of the reversed micellar phase (one circulation takes 50 min). After 5 circulations an additional 0.2 % (w/v) TOMAC was added to the reversed micellar phase (arrow).

The extraction efficiency of the forward extraction is decreasing after 3.5 circulations of the reversed micellar phase, as can be seen by the decreasing α-amylase concentration in the reversed micellar phase (Fig. 6). After 5 circulations an additional

0.2% (w/v) of TOMAC was added to the reversed micellar phase. This restored the extraction efficiency, meaning that the surfactant is lost slowly (< 15% per circulation of the reversed micellar phase) during the extractions.

TABLE 1. Observed and calculated steady-state concentrations of active α-amylase during the continuous forward and back extraction experiment.

Relative α-amylase activity in	Observed	Calculated (corrected for inactivation)
W1,out	0.25	0.23 - 0.31
RM1	1.45	0.97 - 1.34
RM2	0.20	0.10 - 0.22
W2,out	7.80	6.50 - 9.60

During the extraction a precipitate is slowly building up at the interface between aqueous and reversed micellar phase. This might be a complex of denatured protein and surfactant, which is responsible for the decreasing surfactant concentration in the reversed micellar phase. This suggestion is supported by the fact that the protein balance shows a deficit of 10% - 20%.

In the steady state the recovery of enzymatic activity was about 45% in the second aqueous phase, while the first aqueous phase contained about 25% after the extractions. The amount of α-amylase which was inactivated during the extractions was thus 30%.

The concentrations, calculated by using equations (3) to (6), with correction for enzyme inactivation during the forward extraction and in the reversed micellar phase are also listed in Table 1. The observed concentrations are in good agreement with the calculated ones, if corrected for the inactivation.

2.5. CONCLUSIONS

The enzyme α-amylase can be extracted into a reversed micellar phase and *vice versa*. The distribution of the enzyme between aqueous and reversed micellar phase is strongly dependent on the pH and/or the ionic strength in the aqueous phase.

The distribution data of the enzyme has been used to perform a continuous forward and back extraction of α-amylase with a recirculating reversed micellar phase. The enzyme activity could be concentrated by a factor of eight by these extractions. Enzyme inactivation was about 30% during the extractions. The surfactant concentration in the reversed micellar phase is slowly decreasing during the extraction (< 15 % per circulation of reversed micellar phase). This might be due to the formation of complexes between the surfactant and denatured protein.

Because of the large increase in enzyme concentration, that can be obtained by the continuous forward and back extraction with a reversed micellar phase, the process might be very promising for application in the large scale recovery of industrial enzymes, although some problems (enzyme inactivation, surfactant loss) still have to be solved.

NOMENCLATURE

A	surface area	m^2
C	active concentration (relative to concentration in W1,in)	-
C^*	active concentration in equilibrium with other phase.	-
d	drop diameter	m
F	flow rate	$ml\ s^{-1}$
k_i	inactivation rate constant	s^{-1}
K_{ov}	overall mass transfer rate constant	s^{-1}
m	distribution coefficient of α-amylase	-
RM1	reversed micellar phase leaving the forward extractor	
RM2	reversed micellar phase leaving the back extractor	
t	time	s

W1,in	aqueous phase entering the forward extractor	
W1,out	aqueous phase leaving the forward extractor	
W2,in	aqueous phase entering the back extractor	
W2,out	aqueous phase leaving the back extractor	
ϵ	hold-up of disperse phase	-
τ	residence time in mixer	s

Subscripts:

1	during forward extraction
2	during back extraction

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CHAPTER 3

MODELING AND OPTIMIZATION OF THE REVERSED MICELLAR EXTRACTION OF α -AMYLASE

ABSTRACT

Enzymes can be concentrated by a continuous forward and back extraction with a reversed micellar phase. In this process the enzymes are transferred from one aqueous phase to another via a circulating organic solvent containing reversed micelles.

During the extraction of α-amylase, enzyme inactivation and surfactant loss is observed. This inactivation is found to be caused mainly by complexation of the surfactant with the enzyme in the first aqueous phase. This mechanism is also responsible for the surfactant losses during the extraction.

This chapter is based upon the publications:

Dekker, M., J.W.A. Baltussen, K. Van 't Riet, B.H. Bijsterbosch, C. Laane and R. Hilhorst (1987) In *"Biocatalysis in Organic Media"*, (Eds. C. Laane, J. Tramper and M.D. Lilly), Elsevier, 285.

Dekker, M., K. Van 't Riet, J.W.A. Baltussen, B.H. Bijsterbosch, R. Hilhorst and C. Laane (1987) In *"Proc. 4thEur. Congr. Biotechnol."*, Amsterdam, Vol II, 507.

Dekker, M., K. Van 't Riet, B.H. Bijsterbosch, R.B.G. Wolbert and R. Hilhorst (1989) *AIChE J.*, **35**, 321.

In this chapter a model description of the two extraction steps is formulated containing the inactivation rate and the mass transfer rate of the enzyme during the different stages of the extraction process.

Since enzyme inactivation mainly takes place in the first aqueous phase, reducing the steady state enzyme concentration in this phase leads to an improvement of the extraction efficiency. This reduction can be achieved by increasing the distribution coefficient and the mass transfer rate coefficient of the enzyme during forward extraction.

As predicted by this model the enzyme recovery yield in the second aqueous phase can be increased to 85% and the surfactant losses can be reduced to 2.5% per circulation of the reversed micellar phase. The model predicts that further improvement should be possible with modified extraction techniques.

3.1. INTRODUCTION

In biotechnology there is a need for new protein recovery processes, which combine a high selectivity for the desired product with substantial concentration and easy scale-up. In this context, liquid-liquid extraction of an aqueous solution with an organic solvent containing reversed micelles presents itself as a promising process for the selective recovery of proteins from a fermentation broth. Another important application of reversed micelles in the field of biotechnology is the bioconversion of apolar substrates (Luisi and Laane, 1986; Laane and Dekker, 1986; Laane *et al.*, 1987).

A reversed micelle consists of a spherical aggregate of surfactant molecules in an apolar solvent surrounding an inner core of water (Fig. 1a). The polar environment inside such a micelle enables polar compounds, such as proteins, to be solubilized in a largely apolar solvent (Fig. 1b).

It has been demonstrated that under certain conditions proteins can be transferred from an aqueous phase towards a reversed micellar phase and back (Luisi *et al.*, 1979; Van 't Riet and Dekker, 1984; Göklen and Hatton, 1985).

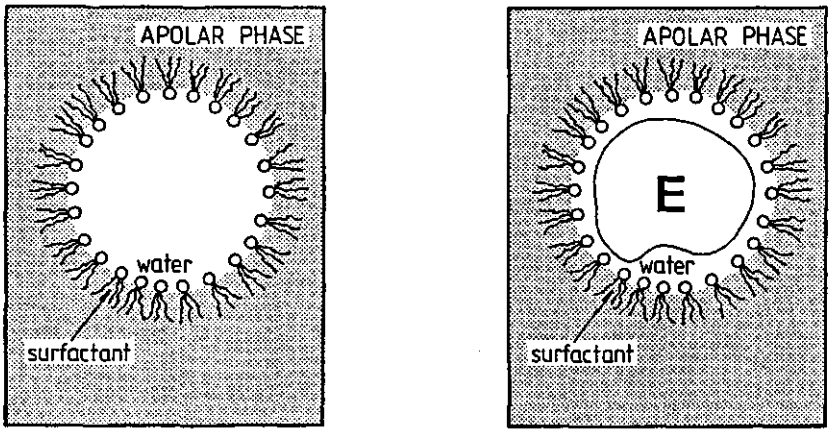

Fig. 1. a: Schematic representation of a reversed micelle.
b: Enzyme solubilized in the water core of the reversed micelle.

The partitioning of proteins between a reversed micellar phase and an aqueous phase depends on several factors, among which interactions between the protein and the reversed micelle. These interactions can be of an electrostatic nature involving charged groups of the protein and the surfactant head groups, or of a hydrophobic nature involving hydrophobic parts of the protein and the micellar interface or the apolar solvent. The fact that electrostatic interactions play an important role is demonstrated by the effect of the pH and the ionic strength of the aqueous phase on the partitioning of proteins between the reversed micellar and aqueous phases (Göklen and Hatton, 1986; Dekker *et al.*, 1987[b]; Göklen and Hatton, 1987). Thermodynamic descriptions of the distribution of proteins between a reversed micellar and an aqueous phase, based on electrostatic effects have already been introduced (Fraaije, 1987; Maestro and Luisi, 1986).

Differences in distribution behaviour have been used to separate a mixture of three proteins showing that selective extraction is possible (Göklen and Hatton, 1987).

To apply the reversed micellar extraction method for the recovery of proteins, a

continuous forward and back extraction process can be used. Previously we have investigated the performance of this process in two mixer/settler units as shown in Fig. 2 (Dekker *et al.*, 1986).

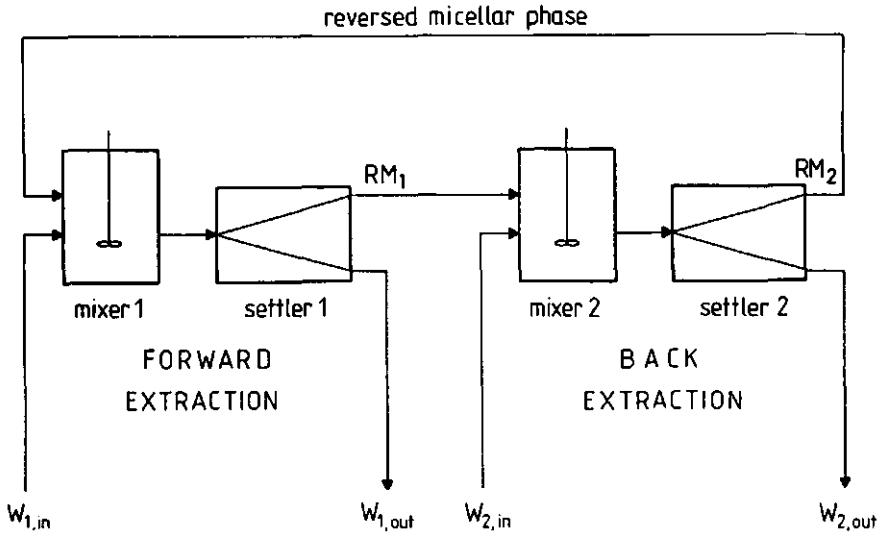

Fig. 2. Flowsheet of the combined forward and back extraction for two mixer/settler units, with the reversed micellar phase circulating between the two extraction units (see Dekker *et al.*, 1986 for construction details).

A reversed micellar phase of the surfactant trioctylmethylammonium chloride (TOMAC) in isooctane was used to concentrate the enzyme α-amylase by performing the forward extraction at a pH value of 9.9, where the distribution coefficient is maximal, and the back extraction at a much lower pH value. In this way the α-amylase concentration was increased eight times. During the extraction a loss of 30% of the enzyme activity was observed; 45% of the initial amount of α-amylase was present in the second aqueous phase, while 25% remained in the first aqueous phase.

In this chapter the mechanism of the enzyme inactivation during the extraction and the modelling of the extraction is described. As predicted by this model, activity recovery can be improved by increasing the distribution coefficient and the mass transfer rate during the forward extraction.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals:

α-Amylase (EC 3.2.1.1, crystalline, 1000 U mg^{-1} and crude, 90-100 U mg^{-1}) from Bacillus amyloliquefaciens was obtained from Sigma Chemical Co. The insoluble impurities of the crude enzyme preparation were removed by microfiltration of the enzyme solutions. Trioctylmethylammonium chloride (TOMAC) was obtained from Merck and contained 88% (w/w) of the quaternary ammonium salt, 10 % (w/w) of a mixture of octanol and decanol and 2% (w/w) of water. Rewopal HV5 (nonylphenol-pentaethoxylate) was obtained from REWO Chem. Group. All other chemicals were obtained from Merck and were of analytic grade.

3.2.2. Temperature:

All experiments were performed at a temperature of 20 ± 0.5 °C.

3.2.3. α-Amylase activity and concentration:

The enzyme activity was determined at pH 6.9 with an auto-analyzer (SKALAR), with soluble starch as a substrate. The increase in the concentration of reducing sugargroups was determined spectrophotometrically. Enzyme samples were diluted to the measurable range with 25 mM K_2HPO_4/KH_2PO_4, pH 6.9. One unit is defined according to Bernfeld (1951).

Protein concentration was determined with an auto-analyzer (SKALAR), using a biuret assay.

3.2.4. Reversed micellar phase:

The reversed micellar phase contained 0.40% (w/v) TOMAC, 0.088% (w/v) Rewopal HV5 and 0.1% (v/v) octanol in isooctane. The reversed micellar phase is saturated with buffer during extraction.

3.2.5. Distribution of α-amylase between aqueous and reversed micellar phase:

The effect of the pH of the aqueous phase on the solubilization of α-amylase in the reversed micellar phase was determined as described before (Dekker *et al.*, 1986).

The first aqueous phase consisted of 50 mM ethylenediamine (EDA) adjusted to the desired pH with HCl. The second aqueous phase was 0.5 M K_2HPO_4/KH_2PO_4, pH 5.5. The first and second extraction took 2 and 10 minutes respectively.

3.2.6. Inactivation of enzyme in the aqueous phase:

The inactivation of α-amylase in the presence of 0.1% (w/v) of either one of the components of the reversed micellar phase was studied in an aqueous phase (50 mM EDA, pH 10.0), under intensive stirring. Samples were taken at different time intervals and analyzed for enzyme activity.

3.2.7. Surfactant concentration:

The concentration of TOMAC in the reversed micellar phase was determined by dilution of 0.5 ml sample with 2.0 ml isooctane and extraction with 0.5 ml of an aqueous solution of colour reagent (1% w/v 3,5-dinitrosalicylic acid, 0.4 N NaOH and 30% w/v K,Na-tartrate). The amount of colourreagent extracted in the apolar phase was determined spectrophotometrically at 480 nm and showed a linear relation with the TOMAC concentration in the reversed micellar phase. This analysis took about 10 min.

The concentration Rewopal HV5 was determined by HPLC with a C8 reversed phase column which was eluted with 4:1 MeOH/H2O at a flow rate of 0.5 ml min^{-1}. Detection was by UV absorption at 280 nm.

3.2.8. Complexation between protein and surfactant:

Different concentrations of α-amylase (0 - 2.5 g l^{-1}; 1000 U mg^{-1}) in 2 ml samples of the aqueous phase (50 mM EDA pH 10.5) were extracted with 2 ml reversed micellar phase for one hour which always resulted in complete inactivation of the enzyme. After settling, the concentrations of surfactant and protein were determined in the reversed micellar phase and in the aqueous phase respectively.

The interfacial precipitate which was formed in the extraction process was isolated from the two liquids. To analyze this precipitate it was extracted for one hour in a two phase system consisting of isooctane with 0.1% (v/v) octanol and an aqueous phase with 0.5 M NaCl, 50 mM NaAc/HAc pH 6.0. After settling, the surfactant and protein concentrations were determined in the apolar and aqueous phase respectively.

3.2.9. Mass transfer rate measurement:

The measurement of the mass transfer rate coefficient was performed in a mixer/settler unit, as described by Dekker *et al.*, 1986, in continuous mode. In order to improve phase separation, the inlet of the settler was modified in such a way that the dispersion from the mixer enters the settler horizontally at the level of the interface between the two phases. The operating volume of the mixer was 750 ml. The α-amylase concentration (100 U mg^{-1}) in the aqueous phase (50 mM EDA, pH 10.0) of the mixer (W1) was 1 g l^{-1}, the reversed micellar phase contained no enzyme before the extraction. The flows of the two phases were: F_{W1} = 1.0 ml s^{-1} and F_{RM} = 0.5 ml s^{-1}. The concentrations C_{RM1} and $C_{W1,out}$ were measured as a function of time until a steady state was reached.

3.2.10. Continuous extraction:

The continuous extraction of α-amylase was performed in two mixer/settler units (Fig. 2) as described by Dekker *et al.* (1986). The flows were: F_{W1} = 1.0 ml s^{-1}, F_{RM} = 0.5 ml s^{-1} and F_{W2} = 0.05 ml s^{-1}. The total volume of the reversed micellar phase was 2.0 l. W1,in contained 1.0 g l^{-1} α-amylase (100 U mg^{-1}) in 50 mM EDA, pH 10.0, W2,in contained 0.5 M NaCl and 50 mM NaAc/HAc, pH 4.4. The concentration of TOMAC was analyzed every 100 minutes and adjusted if necessary.

3.3. MODELING OF THE EXTRACTION

In order to describe and optimize the extraction process, a mathematical model was formulated, which describes the time dependency of the concentration of active enzyme in all the phases. For each phase a differential equation is derived [equations 1-4]. The first term on the right hand side of each equation represents the concentration change due to flow into or out of the mixer, the second term the transfer of enzyme from or to the other phase in the mixer as driven by gradients in the chemical potential, and the third the inactivation of enzyme in that phase, by first order inactivation kinetics.

$$\frac{dC_{W1}}{dt} = \frac{C_{W1,in} - C_{W1}}{\tau_1} - \frac{K_{o1} \cdot A_1}{1 - \epsilon_1} \cdot (C_{W1} - C^*_{W1}) - k_{iW1} \cdot C_{W1} \qquad (1)$$

$$\frac{dC_{RM1}}{dt} = \frac{C_{RM2} - C_{RM1}}{\tau_1} + \frac{K_{o1} \cdot A_1}{\epsilon_1} \cdot (C_{W1} - C^*_{W1}) - k_{iRM} \cdot C_{RM1} \qquad (2)$$

$$\frac{dC_{W2}}{dt} = \frac{0 - C_{W2}}{\tau_2} + \frac{K_{o2} \cdot A_2}{\epsilon_2} \cdot (C_{RM2} - C^*_{RM2}) - k_{iW2} \cdot C_{W2} \qquad (3)$$

$$\frac{dC_{RM2}}{dt} = \frac{C_{RM1} - C_{RM2}}{\tau_2} - \frac{K_{o2} \cdot A_2}{1 - \epsilon_2} \cdot (C_{RM2} - C^*_{RM2}) - k_{iRM} \cdot C_{RM2} \qquad (4)$$

Equations 1-4, in combination with descriptions of the residence times of the phases in the settlers, can be used to describe the extraction process dynamically, by numerical solution, or to describe the steady state of the extraction, by analytical solution of the set of equations.

3.4. RESULTS AND DISCUSSION

3.4.1. Mechanism of inactivation

During the reversed micellar extraction of α-amylase inactivation of the enzyme takes place (Dekker *et al.*, 1986). This inactivation was observed mainly in the first mixer/settler unit during the forward extraction of the α-amylase to the reversed micellar phase.

To investigate in which phase inactivation takes place, α-amylase activity as a function of time was studied in an aqueous phase at the extraction conditions (50 mM EDA pH 10.0), but which had not been in contact with the reversed micellar phase, and in a reversed micellar phase, which was separated from the aqueous phase after

the extraction. In both phases no significant inactivation of the enzyme was observed ($k_i \leq 10^{-5}$ s^{-1}). Since also no inactivation is observed in the reversed micellar phase after the extraction, the inactivation has to take place by a component of the reversed micellar phase present in the aqueous phase during extraction.

To establish which component is responsible for the inactivation of the enzyme, the components were added separately to an aqueous enzyme solution. The effects of these additions on the enzyme stability are shown in Fig. 3.

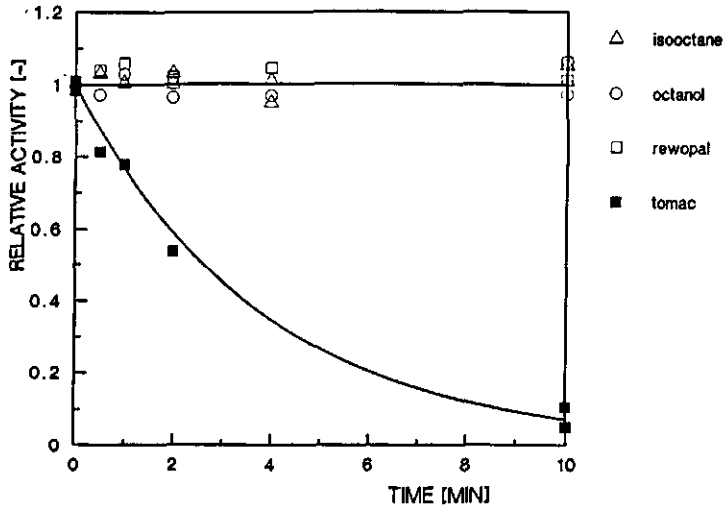

Fig. 3. Inactivation of α-amylase in an aqueous phase at pH 10.0 caused by the addition of 0.1% (w/v) of one of the reversed micellar phase components.

Only the cationic surfactant TOMAC is effective in inactivating the enzyme.

Although the equilibrium concentration of free TOMAC in the aqueous phase is very low, it can be continuously supplied by mass transfer out of the reversed micellar phase during extraction, when complexation between enzyme and surfactant occurs. This flow of surfactant out of the reversed micellar phase is visualized in Fig. 4, where the amount of TOMAC left over in the reversed micellar phase is shown as a function of the amount of α-amylase which has been inactivated in the aqueous phase.

A linear relation is observed up to a concentration of 1 g l^{-1}, indicating that 250 surfactant molecules are bound to one molecule of α-amylase. Literature data on complexation of the surfactant sodium dodecylsulfate with proteins show a similar ratio (210 - 260) of surfactant molecules to a protein molecule with the size of α-amylase (Takagi *et al.*, 1975). These results strongly suggest that the mechanism of enzyme inactivation during the reversed micellar extraction is constituted by the formation of complexes between TOMAC and enzyme in the aqueous phase. The observed ratio means that in the continuous extraction experiments an inactivation of 1% of the α-amylase goes along with a 0.17% loss of TOMAC from the reversed micellar phase per circulation of this phase.

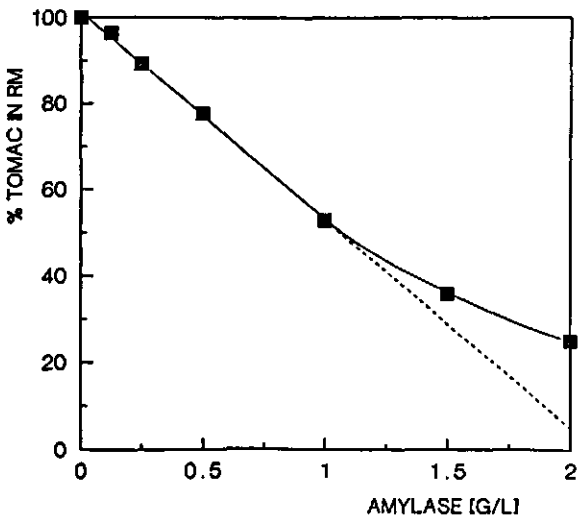

Fig. 4. TOMAC percentage remaining in the reversed micellar phase after extraction as a function of the inactivated amount of α-amylase. Extraction for one hour at pH 10.5 at 20 °C.

During the extraction process aggregates appear at the interface between the aqueous and reversed micellar phase. These could be partly dissociated by extraction with 0.5 M NaCl, 50 mM HAc/NaAc pH 6, in the presence of isooctane with 0.1% (v/v) octanol, to solubilize the dissociated TOMAC molecules. By performing this extraction 60% of both protein and surfactant could be recovered in the aqueous and

apolar phase respectively. Of the recovered protein only 20% was enzymatically active. This building up of insoluble complexes in the interface lends additional support to the conclusion that inactivation is caused by complex formation. It is worth mentioning that the formation of insoluble interfacial complexes is also observed for cytochrome c in reversed micellar systems with AOT (sodium di-2-ethyl- hexylsulfosuccinate) as anionic surfactant (Göklen and Hatton 1986).

The inactivation rate of α-amylase by TOMAC in the aqueous phase was found to be pH-dependent. In Table 1 the observed first order inactivation rate constants are shown for five pH values. These data explain why inactivation of the enzyme predominantly takes place during the forward extraction and not during the back extraction, which takes place at a much lower pH value in the aqueous phase.

TABLE 1. Inactivation rate coefficients of α-amylase in the aqueous phase in the presence of 1 g l^{-1} TOMAC for different pH values. (Buffers used: 0.5 M NaCl, 50 mM HAc/NaAc(1); 25 mM K_2HPO_4/KH_2PO_4(2); 50 mM EDA(3)).

pH	k_{iW1} (s^{-1})
4.4(1)	$5 \cdot 10^{-5}$
6.9(2)	$1 \cdot 10^{-5}$
9.5(3)	$8 \cdot 10^{-4}$
10.0(3)	$4 \cdot 10^{-3}$
10.5(3)	$> 2 \cdot 10^{-2}$

3.4.2. Optimization of the extraction efficiency

Because inactivation in the first aqueous phase can be described by a first order mechanism, the extraction efficiency is expected to increase by lowering the steady state enzyme concentration in this phase. This can be achieved by a high mass transfer rate and/or a high distribution coefficient of the enzyme between the reversed micellar phase and the aqueous phase.

Using equations 1-4 the steady state enzyme concentrations in all four phases can be calculated. In Fig. 5 results are shown of the simulation of the activity recovery

in W2 as a function of the mass transfer rate coefficient of the forward extraction for two values of the distribution coefficient.

As can be seen from Fig. 5, both an increase in the distribution coefficient, as well as an increase in the mass transfer rate coefficient result in a higher enzyme activity recovery in the second aqueous phase.

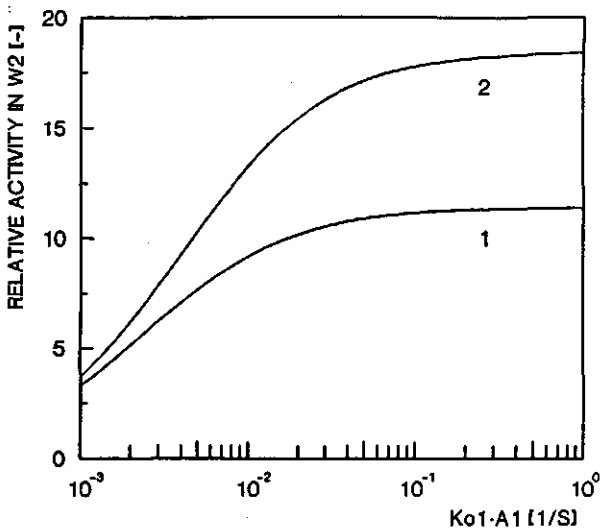

Fig. 5. Simulation of the effect of the mass transfer rate coefficient on the activity recovery in the second aqueous phase. The activity in W2 is relative to the initial activity in W1. 1: m_1 = 10; 2: m_1 = 100. Assumed values based on the experimental conditions: $k_{iW1} = 4 \cdot 10^{-3}$ s^{-1}, $k_{iRM} = 1 \cdot 10^{-5}$ s^{-1}, $k_{iW2} = 5 \cdot 10^{-5}$ s^{-1}, $m_2 = 10^{-3}$, $K_{o2} \cdot A_2 = 1.6 \cdot 10^{-2}$ s^{-1} and $F_{W1}:F_{RM}:F_{W2}$ = 20:10:1.

3.4.3. Effect of the distribution coefficient

Since the distribution behaviour of α-amylase depends on interactions between the protein and the reversed micelle, the composition of the reversed micellar phase will have an effect on the distribution coefficient.

The addition of a nonionic surfactant (Rewopal HV5) to the reversed micellar phase was found to cause an increase in both the distribution coefficient of α-amylase

as well as in the pH range in which solubilization occurs (Fig. 6). These effects could be caused by changes in the structure of the reversed micelles and in their adaptability in size and surface charge density due to the addition of the nonionic surfactant. More fundamental studies are necessary to elucidate this effect.

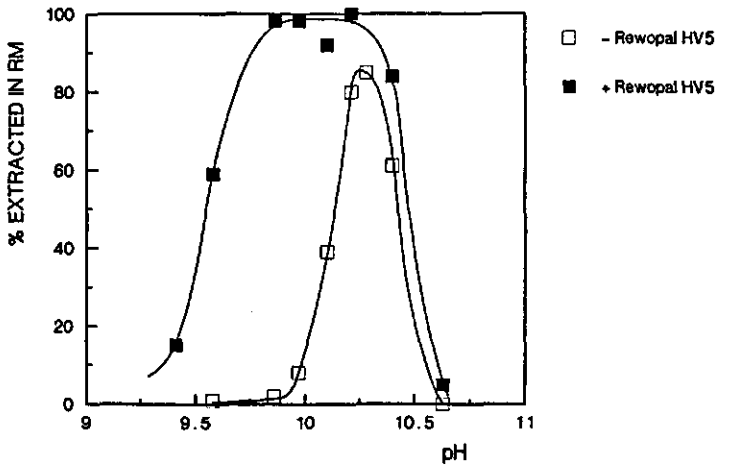

Fig. 6. Solubilization of α-amylase in a reversed micellar phase in relation to the pH in the aqueous phase. Effect of the addition of nonionic surfactant to the reversed micellar phase. (reversed micellar phase: 0.4% (w/v) TOMAC, 0.1% (v/v) octanol in isooctane +/- 0.088% (w/v) Rewopal HV5).

From Fig. 6 it can be concluded that in the presence of the nonionic surfactant almost complete extraction of the α-amylase from the aqueous phase can be obtained. Consequently the value of the distribution coefficient between reversed micellar phase and first aqueous phase, m_1, will be at least 100. This high value of the distribution coefficient also reflects that the equilibrium concentration in W1 (C^*_{W1}) will be virtually zero.

The increase in the extraction efficiency of the continuous forward and back extraction, predicted by this increase in the distribution coefficient, was observed indeed. The addition of the nonionic surfactant to the reversed micellar phase resulted in an increase in the activity recovery in the second aqueous phase from 45% to 65%,

reaching a concentration of 12 g/l (Fig. 7 (B)). Total activity recovery (W1 and W2) was 75%. The loss of TOMAC from the reversed micellar phase was 5% per circulation of the reversed micellar phase. The expected TOMAC loss at 25% enzyme inactivation, using the established ratio of 250 molecules of TOMAC per molecule of α-amylase is 4.3% per circulation of the reversed micellar phase. No loss of Rewopal HV5 was observed during the extractions.

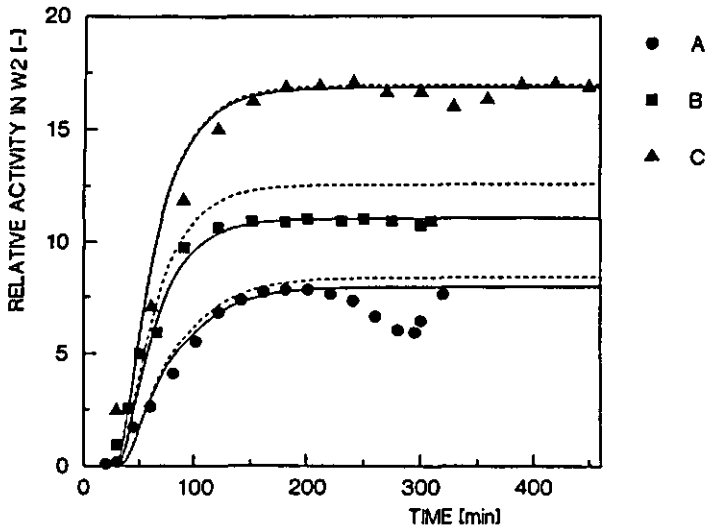

Fig. 7. Activity recovery in the second aqueous phase of the combined forward and back extraction. The activity in W2 is relative to the initial activity in W1.
A: N_1 = 2.8 s^{-1}, N_2 = 4.0 s^{-1}, without nonionic surfactant, (results from Dekker *et al.*, 1986, "dip" caused by TOMAC loss from reversed micellar phase); B: N_1 = 2.8 s^{-1}, N_2 = 4.0 s^{-1}, with nonionic surfactant; C: N_1 = 5.5 s^{-1}, N_2 = 4.0 s^{-1}, with nonionic surfactant.
(- - -) the model prediction by using the independently found values for the mass transfer and inactivation coefficients. (—) line fitted through steady state activities (fitted on k_{iW1}, values used: A: $4.6 \cdot 10^{-3}$, B: $6.4 \cdot 10^{-3}$, C: $4.3 \cdot 10^{-3}$ s^{-1})

The improvement of the continuous extraction upon addition of the nonionic surfactant is not solely due to an increase in the distribution coefficient during the

forward extraction. The addition of the nonionic surfactant also causes a 30% increase in the mass transfer rate, most likely by an effect on the dropsize distribution in the mixer.

3.4.4. Effect of the mass transfer rate

The mass transfer rate will depend on the stirrer speed in the mixer. When the mixer/settler extraction has reached a steady state both the mass transfer rate coefficient ($K_{o1} \cdot A_1$) as well as the inactivation rate constant in the first aqueous phase (k_{iW1}) can be calculated by using equation 1 and 2 ($k_{iRM} = 10^{-5}$ s^{-1}).

In Fig. 8 the results of the measurements of the mass transfer rate are given. The value of $K_{o1} \cdot A_1$ is proportional to $N_1^{2.3}$. This power of N_1 is in good agreement with the empirically expected value of 2.1 found for mass transfer controlled by diffusion in the continuous phase (Middleman, 1965; Van Heuven and Beek, 1971).

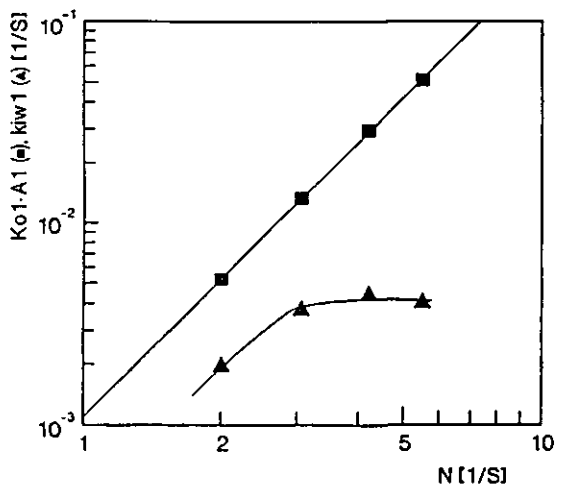

Fig. 8. Mass transfer rate and inactivation rate coefficient during forward extraction of α-amylase to the reversed micellar phase as a function of stirrer speed.

The inactivation rate constant in the aqueous phase was found to be $4 \cdot 10^{-3}$ s^{-1} and independent of the stirrer speed for $N_1 \geq 3$ s^{-1}. At $N_1 = 2$ s^{-1} a lower value ($2 \cdot 10^{-3}$ s^{-1}) was observed, this cannot be explained by the model, but may be due to limitation in the amount of surfactant in the aqueous phase. The mass transfer rate of both surfactant and enzyme will be reduced at lower stirrer speed. Because of the higher enzyme concentration in the aqueous phase, the disappearance rate of surfactant from the aqueous phase by complexation with the enzyme increases, while the mass transfer rate of the surfactant to the aqueous phase decreases. These two effects can cause the observed decrease in the inactivation rate constant in the aqueous phase at lower stirrer speed. This means that the inactivation rate can only be described by first order kinetics in case the surfactant concentration in the aqueous phase is not limiting. During the continuous forward and back extraction experiments the stirrer speed was sufficiently high to permit description of the inactivation by first order kinetics.

As predicted by the model, the total enzyme recovery increases with increasing stirrer speed, since the steady-state aqueous phase enzyme concentration, which is susceptible to surfactant inactivation, is lower.

For the back extraction a similar measurement of the mass transfer rate coefficient was performed at a stirrer speed (N_2) of 4.0 s^{-1}. This resulted in a calculated value of $1.6 \cdot 10^{-2}$ s^{-1} for $K_{o2} \cdot A_2$.

To show that the total extraction efficiency of the reversed micellar extraction is improved by a higher mass transfer rate, the combined forward and back extraction was performed at $N_1 = 5.5$ s^{-1} and $N_2 = 4.0$ s^{-1}. The results are shown in Fig. 7 (C). The total yield of active α-amylase in the second aqueous phase was about 85% giving a concentration of 17 g/l (17 times the initial concentration of the first aqueous phase). This concentration factor is in good agreement with the model prediction (Fig. 5). Only 3% of active enzyme remained in the first aqueous phase after the extraction. Surfactant losses were 2.5% per circulation of the reversed micellar phase (67 min). The expected TOMAC loss is 2.2% per circulation of the reversed micellar phase, using the ratio of 250 molecules of TOMAC per molecule of α-amylase.

3.4.5. Comparison between model and experiments

In Table 2 a summary of the experimental results and the model predictions of the steady state performance of the extraction is given. Both the experimental data of the steady state of the extraction (Table 2) and the observed dynamic behaviour of the extraction (Fig. 7) are in good agreement with the model predictions. This model offers the opportunity to predict the effect of changes in the process conditions (effect of residence times and mass transfer rate coefficients) and in the composition of the aqueous and reversed micellar phase (effect of inactivation rate constants and distribution coefficients) on the extraction efficiency.

TABLE 2. Comparison between experimental and predicted values for the concentration of enzyme in the second aqueous phase and the loss of TOMAC per circulation of the reversed micellar phase. Experimental conditions as described in Fig.7.

	m	$K_{o1} \cdot A_1$	C_{W1} [g l^{-1}]		TOMAC loss [%]		k_{iW1} [s^{-1}]
exp.	[-]	[s^{-1}]	exp.	model	exp.	model	fit
A	10	0.0075	7.8	8.2	n.d.	5-6.5	$4.6 \cdot 10^{-3}$
B	100	0.010	11.5	12.7	5.0	4.3	$6.4 \cdot 10^{-3}$
C	100	0.055	17.0	17.0	2.5	2.2	$4.3 \cdot 10^{-3}$

3.4.6. Further improvement of the yield of the extraction

The results presented above show that the extraction yield of active α-amylase can be improved by the increase of the distribution coefficient and the mass transfer rate coefficient during the forward extraction. A shorter residence time in the extractors in combination with a further increase in the mass transfer rate will give a higher yield of active enzyme in the second aqueous phase and will reduce the surfactant loss (see simulations shown in Fig. 9). The use of centrifugal separators or extractors might be valuable in this respect.

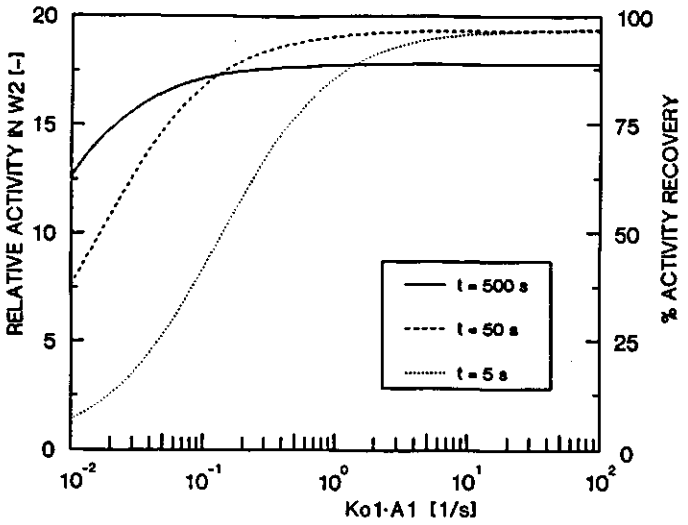

Fig. 9. Simulation of the effect of the mass transfer rate coefficient on the activity recovery in the second aqueous phase. The activity in W2 is relative to the initial activity in W1. The effect of the residence time in the mixers is shown. The used values for mass transfer and inactivation rates are the same as in Fig. 5.

The inactivation rate constant in the first aqueous phase is dependent on the temperature of extraction (Dekker and Den Ouden, unpublished results). Performing the extraction at lower temperatures will give an increase in the extraction yield.

Since enzyme inactivation mainly takes place by complexation with surfactant it is worthwhile to optimize the choice of surfactant used in the extraction system.

3.5. CONCLUSIONS

The recovery of enzymes by a combined forward and back extraction with a reversed micellar phase can be described by a theoretical model containing the mass transfer rate and inactivation rate of the enzyme during the extractions. The extraction performance can be optimized by using this model.

Enzyme inactivation during the reversed micellar extraction was found to take place mainly in the initial aqueous phase by complexation of the enzyme with the

cationic surfactant TOMAC. Reducing the steady state enzyme concentration in this phase should therefore lead to an improvement of the extraction efficiency.

Both an increase in the distribution coefficient, by changing the composition of the reversed micellar phase, as well as in the mass transfer rate coefficient, by increasing the stirrer speed, during the forward extraction have been demonstrated to improve the extraction efficiency.

The enzyme activity recovery in the second aqueous phase was increased to 85%, amounting to 17 times the initial concentration and surfactant losses were reduced to 2.5% per circulation of the reversed micellar phase.

Further improvement of the extraction yield might be obtained by a further increase in the mass transfer rate in combination with shorter residence times, by decreasing the temperature during extraction and by optimizing the nature of the surfactant.

ACKNOWLEDGEMENT

The authors would like to thank J.E. Sanders, F.W.C. Den Ouden and F.C.H.M. Roozen for their contributions to this research.

NOMENCLATURE

A_j	specific surface area in mixer j	$m^2\ m^{-3}$
C_j	concentration of active enzyme in phase j	$kg\ m^{-3}$
C^*_j	equilibrium concentration of active enzyme in phase j	$kg\ m^{-3}$
F_j	flow rate of phase j	$ml\ s^{-1}$
k_{ij}	inactivation rate constant in phase j	s^{-1}
K_{oj}	overall mass transfer rate constant in mixer j	$m\ s^{-1}$
m_j	distribution coefficient of α-amylase during extraction j	-
RMj	reversed micellar phase during extraction j	
t	time	s
Wj	aqueous phase during extraction j	
ϵ_j	hold-up of disperse phase in mixer j	-
τ_j	residence time in mixer j	s

Subscripts:

1	during forward extraction
2	during back extraction
in	entering extraction
out	leaving extraction

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CHAPTER 4

MASS TRANSFER RATE OF PROTEIN EXTRACTION WITH REVERSED MICELLES

ABSTRACT

The rate of mass transfer in the liquid-liquid extraction of the enzyme α-amylase between an aqueous phase and a reversed micellar phase has been investigated. Mass transfer rate coefficients have been measured in a mixer/settler and in a stirred cell.

The pH of the aqueous phase determines the distribution coefficient of the enzyme and thus the direction of transfer. Forward transfer of the enzyme from the aqueous to the reversed micellar phase (cationic surfactant) occurs at pH 10.0. The mass transfer rate of this process was found to be controlled by the diffusion of the enzyme in the aqueous phase boundary layer.

Back transfer from the reversed micellar phase to the aqueous phase occurs in the pH range 4 to 6. In contrast to the forward transfer, this process was found to be controlled by the interfacial process of enzyme release from the reversed micelles instead of the boundary layer diffusion.

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The same effects have been observed for the transfer of the enzyme ribonuclease A to and from a reversed micellar phase with an anionic surfactant.

A mechanism to explain the different mass transfer behaviour in forward and back transfer is suggested.

4.1. INTRODUCTION

A reversed micelle consists of a spherical aggregate of surfactant molecules in an apolar solvent surrounding an inner core of water. The internal polar environment enables polar compounds, such as proteins, to be solubilized in a largely apolar solvent.

It has been demonstrated that under certain conditions proteins can be transferred from an aqueous towards a reversed micellar phase and back (Luisi *et al.*, 1979; Van 't Riet and Dekker, 1984; Göklen and Hatton, 1985). The partitioning of proteins between the two phases depends on several factors. In general, proteins are only transferred to a reversed micellar phase at pH values at which their net sign of charge is opposite to that of the surfactant headgroups (Dekker *et al.*, 1989b). The difference between the pH value at which transfer occurs and the pI of the protein depends on the size of the protein molecule (Wolbert *et al.*, 1989). Because electrostatic interactions between the protein and the reversed micelle are important (Göklen and Hatton, 1987; Dekker *et al.*, 1987a), the distribution coefficient of proteins in these systems is determined by the pH and the ionic strength in the aqueous phase. Studies have been reported concerning the selectivity of the extractions (Wolbert *et al.*, 1989; Göklen and Hatton, 1987) and the thermodynamic modeling of the distribution behaviour (Fraaije, 1987; Caselli *et al.*, 1988; Bratko *et al.*, 1988).

Extraction of an aqueous protein solution with an organic solvent containing these reversed micelles presents itself as a promising process for the selective recovery of proteins from a fermentation broth (Rahaman *et al.*, 1988). To apply the reversed micellar extraction method for the recovery of proteins, a continuous forward and back extraction process can be used. Previously we have investigated the performance of this process in two mixer/settler units (Dekker *et al.*, 1986). By optimization of the distribution and mass transfer coefficients the enzyme α-amylase could be concentrated 17-fold with a recovery of 85% of enzyme activity (Dekker *et al.*,

1989a).

No quantitative data have been published concerning the characterization of the mass transfer processes during the transfer of a protein from an aqueous phase to a reversed micellar phase (forward extraction) and *vice versa* (back extraction). For a reliable scale up of the extractions more fundamental data are required. The objective of this work is to describe the mass transfer behaviour of the enzyme α-amylase during forward and back extraction with a reversed micellar phase of the cationic surfactant trioctylmethylammonium chloride (TOMAC) in isooctane. The results are compared with data for the transfer of the enzyme ribonuclease A from a reversed micellar phase of the anionic surfactant AOT in isooctane.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals:

α-Amylase (EC 3.2.1.1) from *Bacillus amyloliquefaciens* and ribonuclease A (EC 3.1.27.5) from bovine pancreas were obtained from Sigma Chemical Co. The insoluble impurities of the enzyme preparation were removed by microfiltration of the enzyme solutions. Trioctylmethylammonium chloride (TOMAC) was obtained from Merck and contained 88% (w/w) of the quaternary ammonium salt, 10 % (w/w) of a mixture of octanol and decanol and 2% (w/w) of water. Rewopal HV5 (nonylphenolpentaethoxylate) was obtained from REWO Chem. Group. All other chemicals were obtained from Merck and were of analytical grade.

4.2.2. Analysis:

α-Amylase activity and concentration were determined on an auto-analyzer with a colorimetric assay, as described previously (Dekker *et al.*, 1989a). Ribonuclease A concentration was determined by UV spectroscopy, as described by Göklen and Hatton, 1987.

4.2.3. Reversed micellar phase:

The reversed micellar phase used in the transfer experiments of α-amylase contained 0.40% (w/v) TOMAC, 0.088% (w/v) Rewopal HV5 and 0.1% (v/v) octanol

in isooctane. For the transfer of Ribonuclease A 50 mM Aerosol OT (sodium di-2-ethylhexylsulphosuccinate) in isooctane was used. The reversed micellar phases are saturated with buffer during extraction.

4.2.4. Temperature

All mixer/settler experiments were performed at 20 ± 0.5 °C. Stirred cell experiments during back extraction were performed at 20 ± 0.1 °C but during forward extractions at 10 ± 0.1 °C, in order to decrease the inactivation rate (Dekker *et al.*, 1989a).

4.2.5. Mass transfer rate measurements in the mixer/settler:

The mixer/settler units have been described previously (Dekker *et al.*, 1986). In order to improve phase separation, the inlet of the settler was modified in such a way that the dispersion from the mixer enters the settler horizontally at the level of the interface between the two phases. The operating volume of the mixer was 750 ml. For forward transfer the α-amylase concentration in the aqueous phase (50 mM EDA, pH 10.0) of the mixer (W1) was 1 g l^{-1} (10^5 U l^{-1}), the reversed micellar phase contained no enzyme before the extraction. The flows of the phases were: F_{W1} = 1.0 ml s^{-1} and F_{RM} = 0.5 ml s^{-1}. The α-amylase concentrations C_{RM1} and $C_{W1,out}$ were measured as a function of time until a steady state was reached.

For measurement of the back transfer rate two mixer/settler units, as described above, were used. In the first unit a forward extraction of the enzyme was performed at a stirring speed of 5.5 s^{-1}. In this way a reversed micellar phase with a constant concentration of α-amylase was obtained, which was reextracted in the second mixer/settler unit with an aqueous phase containing 0.5 M NaCl and 50 mM HAc/NaAc buffer. The flows of the phases were: F_{RM} = 0.5 ml s^{-1} and F_{W2} = 0.05 ml s^{-1}. The α-amylase concentrations C_{RM2} and $C_{W2,out}$ were measured as a function of time until a steady state was reached.

Back extraction rates for ribonuclease A from AOT reversed micelles were measured in rotating vials as described by Dekker *et al.* (1986). Forward extraction was performed from an aqueous phase containing 0.5 g l^{-1} enzyme, 0.2 M NaCl, and 25 mM EDA at pH 4.0. Back extraction was performed in the same buffer at higher pH values (8 - 11). Settling of the phases after the extraction was performed by centrifugation for one min at 5000 g.

4.2.6. Mass transfer rate measurements in the stirred cell:

The stirred cell used is shown schematically in Figure 1. The cell has been described previously (Scholtens *et al.*, 1979). Additional baffling is provided on the lower and upper draft tube in order to obtain a flat interface at all the applied stirring speeds. The volume of the cell is approximately 500 ml. The propellers were counterrotating, pumping the liquid in inward, mainly radial, direction along the interface. In order to achieve comparable hydrodynamics at both sides of the interface, equal values of *Re* for both phases were established ($N_W/N_{RM} = \nu_W/\nu_{RM} = 1.66$ (forward transfer, 10 °C) or 1.25 (back transfer, 20 °C)). In text and figures the value of the stirring speed in the aqueous phase (N_W) is given.

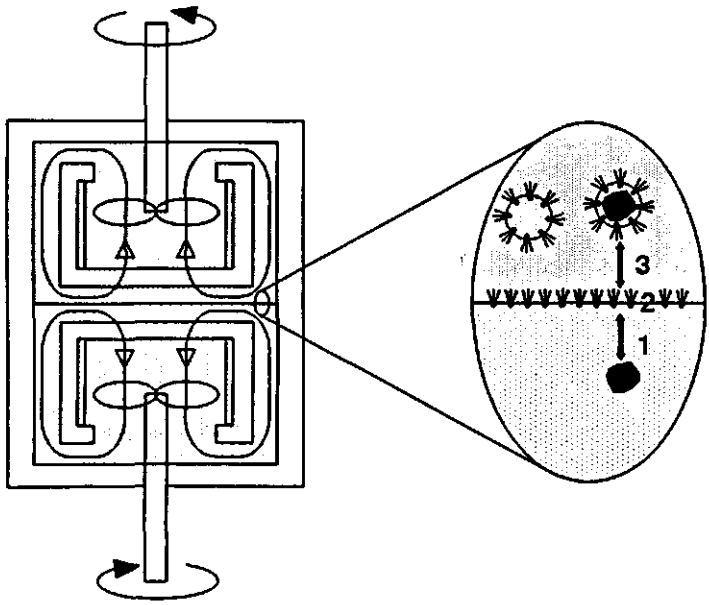

Fig. 1. Schematic representation of the stirred cell (left). Both stirrers are situated inside a draft tube, which is baffled on the vertical outsides and on the open side facing the interface (for details see Scholtens *et al.*, 1979). On the right part the transfer process is shown. 1: Diffusion of the enzyme in the aqueous phase to or from the interface; 2: Interfacial process of uptake or release of the enzyme in or from a reversed micelle; 3: Diffusion of the enzyme containing reversed micelle in the organic phase.

To measure the forward transfer, the aqueous phase containing 50 mM ethylene diamine (EDA) at pH 10.0, was first added to the cell. On top of this phase the reversed micellar phase was carefully layered. The two phases were equilibrated by stirring the system for two hours, which was found to be sufficient to saturate the reversed micellar phase with water (amount of water determined by Karl Fischer titration). The experiment was started by injecting a concentrated enzyme solution in the aqueous phase (final concentration 2.5×10^5 U l^{-1}). The α-amylase content of both phases was determined in small (1 ml) samples taken every 15 minutes, over a range of two hours.

The back transfer measurements were complicated by the fact that the water content of a reversed micellar phase is a function of the ionic strength of the conjugate aqueous phase; at higher ionic strength less water can be solubilized. If the reversed micellar phase containing enzyme from a forward extraction at 50 mM EDA was introduced in the stirred cell on top of the aqueous phase containing 0.5 M NaCl and 50 mM HAc/NaAc buffer, formation of a turbid reversed micellar phase occurred because of water expulsion from this phase. In this system no reliable measurements of the mass transfer coefficient could be made. To obtain an enzyme containing reversed micellar phase in equilibrium with an aqueous phase with the proper ionic strength, a continuous extraction in the mixer/settler units was performed with the reversed micellar phase circulating between the units. By performing the back extraction with an aqueous phase containing 0.5 M NaCl, 50 mM HAc/NaAc at pH 5.0 a considerable amount of enzyme is not transferred to the second aqueous phase, whereas the water content of the reversed micellar phase is adjusted to the ionic strength of the second aqueous phase. By using the reversed micellar phase obtained in this continuous back extraction in the stirred cell experiments, the phases remained clear during the experiments. The enzyme concentration in the reversed micellar phase obtained in this way was approximately 1.5×10^5 U l^{-1}. The enzyme content of both phases was determined as described for the forward transfer over a range of 5 hours.

4.2.7. Fitting procedure:

The mass transfer rate coefficients during the batch stirred cell extractions were calculated from the measured concentrations in both phases as a function of time using the non-linear fit algorithm of Marquardt, 1963.

4.3. MASS TRANSFER THEORY

The mass transfer rate during liquid-liquid extraction is in general determined by three resistances. During forward extraction an enzyme molecule has to diffuse from the bulk of the aqueous phase to the interface; at the interface the enzyme is to be encapsulated in a layer of surfactant molecules (formation of a protein filled reversed micelle); the filled reversed micelle has to diffuse from the interface into the bulk organic phase. During back extraction the reverse processes take place (Figure 2).

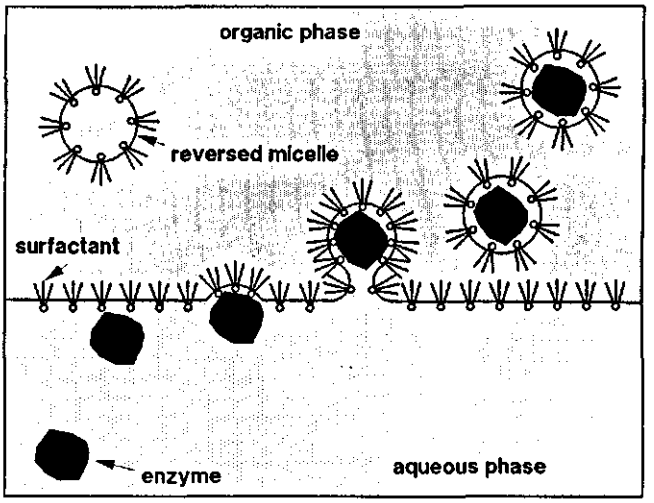

Fig. 2. Schematic representation of the transfer process of an enzyme between an aqueous phase and a reversed micellar phase. During forward transfer the enzyme is at the interface encapsulated in a layer of surfactant molecules, forming an enzyme containing reversed micelle (left to right). During back transfer the enzyme containing reversed micelle coalesces with the organic-aqueous interface and releases the enzyme in the bulk aqueous phase (right to left).

For most extraction systems the interfacial concentrations of the transferable compound are found to be in equilibrium. Therefore in these systems the mass transfer rate is only determined by the diffusional processes on either one or both

sides of the interface. In some cases, however, an interfacial reaction of the compound with a carrier molecule has been found to be rate limiting in the transfer process (Nitsch and Roth, 1978; Albery *et al.*, 1984).

Experiments in a stirred cell can elucidate whether a transfer process is limited by diffusion in the boundary layer in one of the phases or by an interfacial resistance. Increasing the stirring speed only results in a decrease of the boundary layer thickness, and will not affect the interfacial resistance.

The extraction process can be described by differential equations that are derived by combining the mass transfer equation with the mass balance of the enzyme and the inactivation kinetics of the enzyme in each phase (Dekker *et al.*, 1989a) (See appendix for nomenclature):

$$\frac{dC_1}{dt} = \frac{C_{1,in} - C_1}{\tau} - \frac{K_o A}{1-\epsilon}\left(C_1 - \frac{C_2}{m}\right) - k_{i1} C_1 \qquad (1)$$

$$\frac{dC_2}{dt} = \frac{C_{2,in} - C_2}{\tau} + \frac{K_o A}{\epsilon}\left(C_1 - \frac{C_2}{m}\right) - k_{i2} C_2 \qquad (2)$$

For a batch extraction the first term on the right hand side of equations (1) and (2) equals zero. Solving this set of linear differential equations (Morris and Brown, 1964) to obtain the time dependent concentrations in a batch extraction results in:

$$C_1(t) = \alpha_1 e^{\lambda_1 t} + \alpha_2 e^{\lambda_2 t} \qquad (3)$$

$$C_2(t) = \beta_1 e^{\lambda_1 t} + \beta_2 e^{\lambda_2 t} \qquad (4)$$

with:

$$\lambda_{1,2} = \frac{\gamma_1 + \gamma_4 \pm \sqrt{(\gamma_1 + \gamma_4)^2 - 4(\gamma_1\gamma_4 - \gamma_2\gamma_3)}}{2} \quad (5)$$

$$\alpha_1 = C_1(0) - \alpha_2 \quad (6)$$

$$\alpha_2 = \frac{C_2(0)\gamma_2 - C_1(0)(\lambda_1 - \gamma_1)}{(\lambda_1 - \lambda_2)} \quad (7)$$

$$\beta_1 = \frac{\alpha_1(\lambda_1 - \gamma_1)}{\gamma_2} \quad (8)$$

$$\beta_2 = \frac{\alpha_2(\lambda_2 - \gamma_1)}{\gamma_2} \quad (9)$$

$$\gamma_1 = -\frac{K_o A}{1 - \epsilon} - k_{i1} \quad (10)$$

$$\gamma_2 = \frac{K_o A}{(1 - \epsilon)m} \quad (11)$$

$$\gamma_3 = \frac{K_o A}{\epsilon} \quad (12)$$

$$\gamma_4 = -\frac{K_o A}{\epsilon m} - k_{i2} \quad (13)$$

The overall process is characterized by two time constants because the process of mass transfer and of enzyme inactivation occur simultaneously.

For the steady state of a continuous extraction (mixer/settler experiments) the terms on the left hand side of equations (1) and (2) equal zero. Solving these two linear equations simultaneously, with $C_{1,in} = 1$ and $C_{2,in} = 0$, results in the description of the steady state concentrations in the mixer as a function of mass transfer rate coefficient and inactivation rate constants:

$$C_1(\infty) = \frac{\zeta_3}{\zeta_1 - \frac{\zeta_2 \cdot \zeta_4}{\zeta_5}} \tag{14}$$

$$C_2(\infty) = \frac{\zeta_4 \cdot C_1}{\zeta_5} \tag{15}$$

with:

$$\zeta_1 = \frac{1}{\tau} + \frac{K_o A}{1-\epsilon} + k_{i1} \tag{16}$$

$$\zeta_2 = \frac{K_o A}{(1-\epsilon)m} \tag{17}$$

$$\zeta_3 = \frac{1}{\tau} \tag{18}$$

$$\zeta_4 = \frac{K_o A}{\epsilon} \tag{19}$$

$$\zeta_5 = \frac{1}{\tau} + \frac{K_o A}{\epsilon m} + k_{i2} \tag{20}$$

4.4. RESULTS

4.4.1. Forward transfer

4.4.1.1. Mixer/settler extraction

The mass transfer rate of the forward extraction of α-amylase in the mixer/settler unit has been measured as a function of the stirring speed in the mixer. Measuring of the concentrations in both phases before and after the extraction gives the opportunity to calculate an overall mass transfer rate coefficient (K_oA [s^{-1}]) as a function of the stirring speed (N [s^{-1}]) using equations (14) and (15) (Dekker *et al.*, 1989a). The results are shown in Fig. 3.

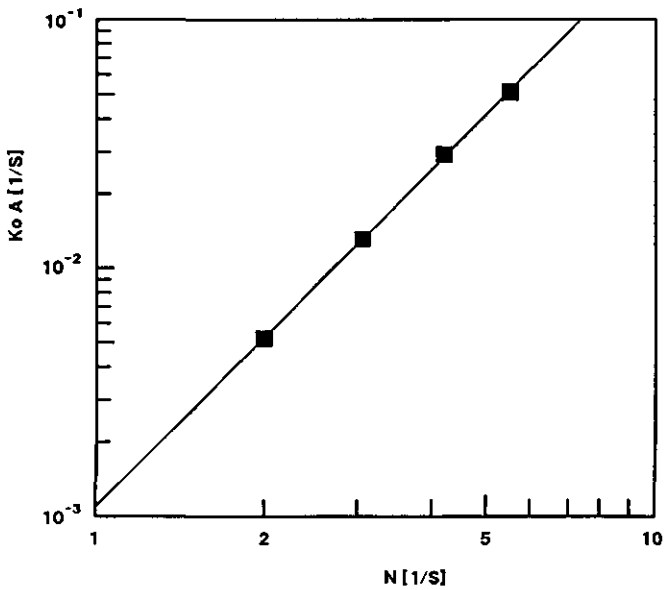

Fig. 3. Effect of the stirring speed on the overall mass transfer rate coefficient (K_oA) of α-amylase for forward extraction in the mixer/settler unit.

The mass transfer rate coefficient is found to be proportional to $N^{2.3}$. This relation is close to the expected empirical relation for mass transfer limited by diffusion in the continuous phase (K_oA proportional to $N^{2.1}$ (Middleman, 1965; Van Heuven and Beek, 1971)). No quantitative comparison with theoretical data on the mass transfer rate

could be made because of the difficult assessment of the specific surface area at the very low surface tension of the system (< 10^{-1} mN m^{-1}) and the non standard stirrer blades used.

4.4.1.2. Stirred cell extraction

By performing the forward extraction in the stirred cell, measurement of the overall mass transfer rate coefficient itself (K_o [m s^{-1}]) is possible, using equations (3) and (4). The observed relation between mass transfer rate coefficient and stirring speed in Fig. 4 illustrates a clear increase of the mass transfer rate coefficient with increasing stirring speed.

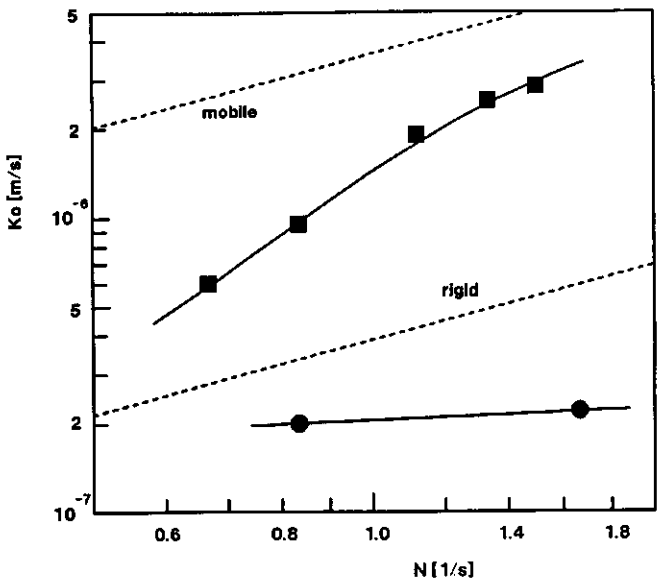

Fig. 4. Effect of stirring speed on the mass transfer rate coefficient (K_o) of α-amylase for forward extraction at pH 10 (—■—) and for back extraction at pH 5 (—●—) in the stirred cell. See Discussion for calculated (- - -) forward transfer rates.

4.4.2. Back transfer

4.4.2.1. Mixer/settler extraction

For the back extraction rate an unexpected effect of the pH, at constant stirring speed, was found (Fig. 5). Since the pH is very unlikely to have a significant effect on the diffusion coefficient or on the specific surface area an additional interfacial resistance seems to limit the back transfer rate.

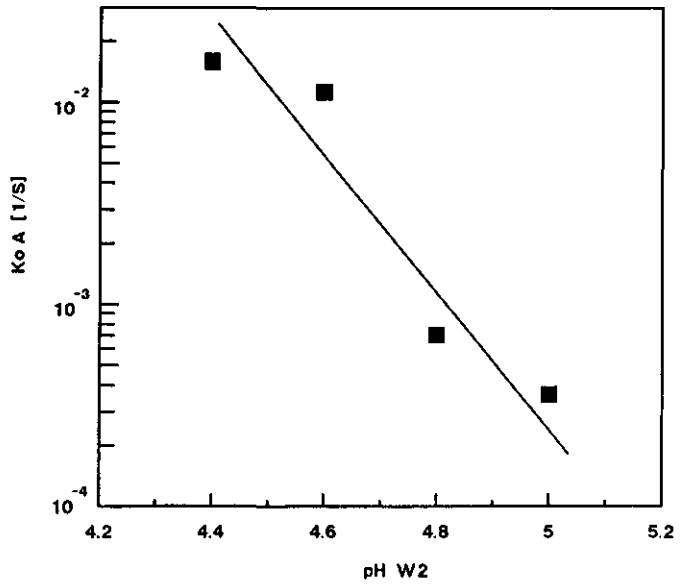

Fig. 5. Back extraction mass transfer rate coefficient (K_oA) of α-amylase in the mixer/settler unit as a function of the aqueous phase pH (N = 4 s^{-1}).

4.4.2.2. Stirred cell extraction

To eliminate any effect of the pH on the dropsize and thus on the specific surface area in the mixer (where K_oA is measured), the dependence of the α-amylase transfer on pH has also been measured in the stirred cell at constant stirring rate. The results are shown in Fig. 6.

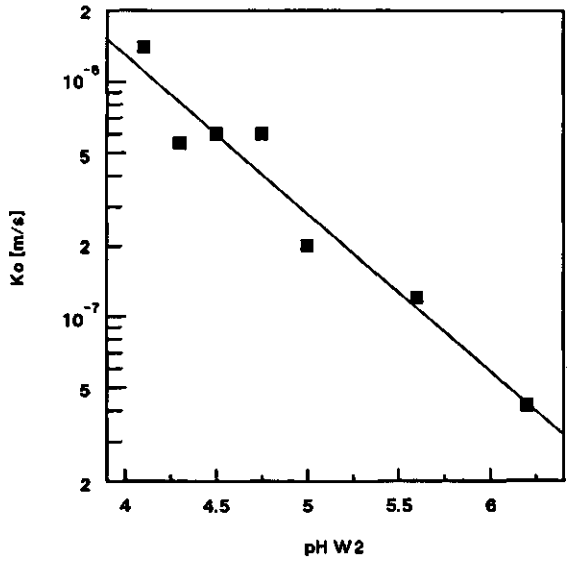

Fig. 6. Back extraction mass transfer rate coefficient (K_O) of α-amylase in the stirred cell as a function of the aqueous phase pH (N_W = 0.8 s^{-1}).

Again a large effect of the pH on the overall mass transfer rate coefficient (K_O) is observed; over a range of two pH units the mass transfer rate coefficient varies by a factor of 30.

Additional proof of the existence of a limiting interfacial resistance during back transfer was found in the dependence of the mass transfer rate coefficient on the stirring speed. Increasing the stirring speed from 0.8 s^{-1} to 1.6 s^{-1} (at pH 5 in the aqueous phase) resulted in an increase in the back transfer rate coefficient of only 10 % (Fig. 4). For the forward extraction over the same range of stirring speeds a tripling of the mass transfer rate coefficient was observed.

4.5. DISCUSSION

For the stirred cell extraction the mass transfer rate by diffusion across the laminar boundary layer parallel to the flat interface can be calculated using relations between the dimensionless numbers *Re* (Reynolds = vx/ν), *Sc* (Schmidt = ν/D), and *Sh* (Sherwood = $k_f x/D$). These relations are given in equations (24) and (25) for the case of a completely mobile or rigid interface respectively:

$$Sh = 1.13 \cdot Re^{0.5} \cdot Sc^{0.5} \qquad (21)$$

$$Sh = 0.664 \cdot Re^{0.5} \cdot Sc^{0.33} \qquad (22)$$

The diffusion coefficient can be estimated using the Stokes-Einstein equation for diffusion of spheres in a liquid:

$$D = \frac{kT}{6\pi\mu r} \qquad (23)$$

For forward transfer the diffusional mass transfer rate in the aqueous phase is calculated. Taking into acount the value of the distribution coefficient ($m_1 = C_{RM}/C_{W1} \geq 100$ (Dekker *et al.*, 1987b)) this resistance will be dominant over the diffusional resistance in the reversed micellar phase. For back transfer the diffusional mass transfer rate in the reversed micellar phase will be dominant, using the same arguments ($m_2 = C_{W2}/C_{RM} \geq 100$ (Dekker *et al.*, 1989a)). Equation (23) gives a value of 6.0×10^{-11} m^2 s^{-1} for the diffusion coefficient of α-amylase in water during forward extraction and 1.0×10^{-10} m^2 s^{-1} for the diffusion coefficient of a reversed micelle containing a molecule of α-amylase in isooctane during back extraction. The effective flow area is taken between r = 1 cm and r = 3 cm, giving an effective transfer area of 50% of the total interfacial area.

Equations (21) and (22) combined with the relation between v and N (Scholtens *et al.*, 1979) give that K_o is proportional to $N^{0.84}$. The calculated relations between K_o and N for a mobile and rigid interface during forward extraction are shown in Figure 4. The observed dependence of K_o with N indicates that in the stirred cell part of the interface behaves rigid and the other part mobile, depending on the stirring speed.

Such behaviour has been observed before, in the same range of stirring speeds, with adsorption of surface active molecules in the interface (Scholtens *et al.*, 1979).

For the back extraction, however, the observed K_o are much lower (Fig. 4), while the calculated ones are 30-50% higher than for forward transfer. The calculated diffusional K_o varies with the same slope with N as the forward K_o, the experimentally determined K_o, however, was found to be independent of N. As can be seen in Figure 5, below pH 4.7 the observed mass transfer rate coefficients are in the range of a diffusion limited transfer in the reversed micellar phase to a rigid interface. At higher pH values the observed mass transfer rate coefficients are significantly lower than this lower limit of the expected value for diffusion limited transfer.

The low value for the mass transfer rate coefficient during back extraction, together with the strong pH effect, and the absence of a significant influence of the stirring speed on the back transfer all lead to the conclusion that an interfacial resistance is limiting the back transfer.

The additional resistance is probably related to the coalescence of the protein filled reversed micelle with the organic/aqueous interface, thereby releasing the protein molecule to the aqueous phase (Fig. 2). This interfacial process requires the breakup of a small organic film between the reversed micelle and the bulk aqueous phase. In this process the surfactant molecules stabilizing the film need to be displaced in order to create a connection between the waterpool of the reversed micelle and the bulk aqueous phase. The stability of a surfactant-stabilized film depends on the phase in which the surfactant is best soluble (Caroll, 1976). During back transfer an isooctane film has to break, whereas during forward extraction breaking of an aqueous phase film is involved. Since in the present case the surfactant is better soluble in the isooctane, the organic film will be much more stable than the aqueous one.

This film stability mechanism can explain why back transfer is slower than forward transfer but does not elucidate the observed pH dependence of the mass transfer rate coefficient. This might be explained by the interactions of the protein molecule with the surfactant head groups which will affect the mobility of the surfactant molecules. Although in the pH range of back transfer, the equilibrium distribution (which is also influenced by electrostatic interactions between protein and surfactant (Dekker *et al.*, 1989[b])) is totally towards the aqueous phase, interactions between

α-amylase and a positively charged surface are still possible. The titration curve of α-amylase (Fig. 7) shows that the number of negatively charged amino acid residues per molecule varies from 20 at pH 4 to 90 at pH 6.

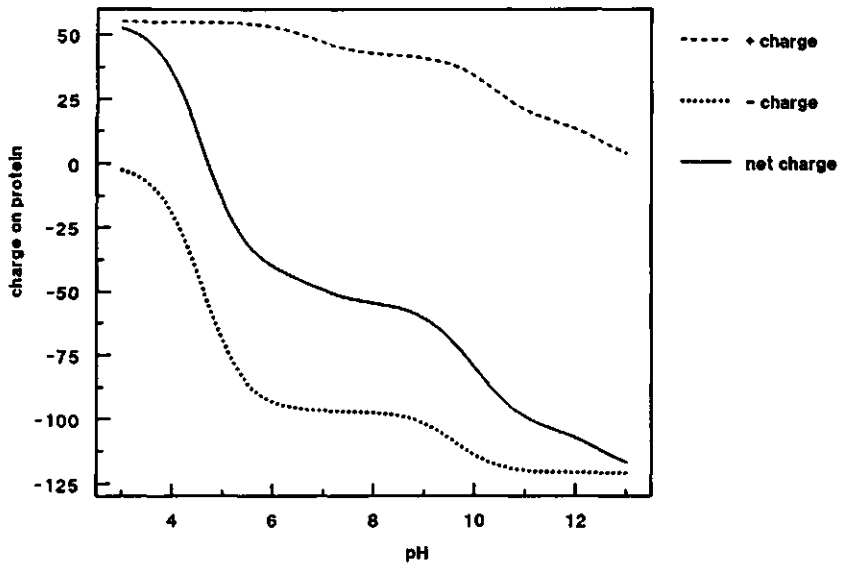

Fig. 7. Calculated titration behaviour of α-amylase; number of positively and negatively charged amino acid residues and net charge as a function of pH (Calculation based upon amino acid composition as reported by Junge *et al.*, 1959).

These negative charges can interact with the positively charged surfactant headgroups, reducing the mobility of the surfactant molecules which, according to the film rupture mechanism results in a retardation of the coalescence rate of the reversed micelles with the interface between the aqueous and organic phase.

Coalescence rates between empty reversed micelles have been found to be extremely fast (second order rate constant of 10^6-10^8 $M^{-1}s^{-1}$ (Fletcher *et al.*, 1985)). The rates of the coalescence of protein filled reversed micelles, could be much slower, according to the theoretical considerations mentioned above, depending on the ionization state of the protein compared to the surfactant charge. It has been shown that the nature of a solute in a waterpool can influence the exchange rate (Vos *et al.*, 1987).

In order to check whether the mass transfer mechanism holds for a protein/reversed micellar system with a different enzyme and an anionic surfactant as well, we also investigated the back transfer of ribonuclease A from AOT reversed micelles. This back transfer has been reported to be extremely slow (Woll, 1987) but no data with respect to pH of the aqueous phase have been given. Also for this protein has the titration curve been calculated (Fig. 8). Since we are now dealing with an anionic surfactant, it is relevant to consider the interaction of positively charged groups on the protein surface with the surfactant headgroups.

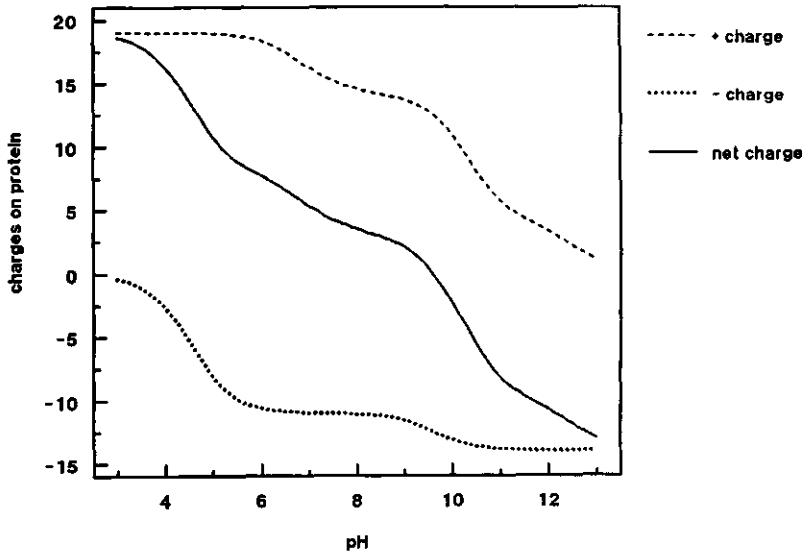

Fig. 8. Calculated titration behaviour of ribonuclease A; number of positively and negatively charged amino acid residues and net charge as a function of pH (Calculation based upon amino acid composition as reported by Tanford and Havenstein, 1956).

We have measured K_oA for the back transfer to an aqueous phase containing 0.2 M NaCl and 25 mM ethylenediamine. The results in Figure 9 show that also for ribonuclease A the pH of back transfer exerts a large effect on the mass transfer rate in the range in which the relative number of positively charged groups varies significantly. Again a low value of K_oA is obtained. Since visual observation of the dispersion during mixing does not reveal a decreased A as compared to the TOMAC reversed

micellar system, also for this system an interfacial resistance will be dominant. A further increase in the pH might eventually lead to a diffusion controlled process also for ribonuclease A, but problems with the stability of the enzyme will be encountered at such high pH values.

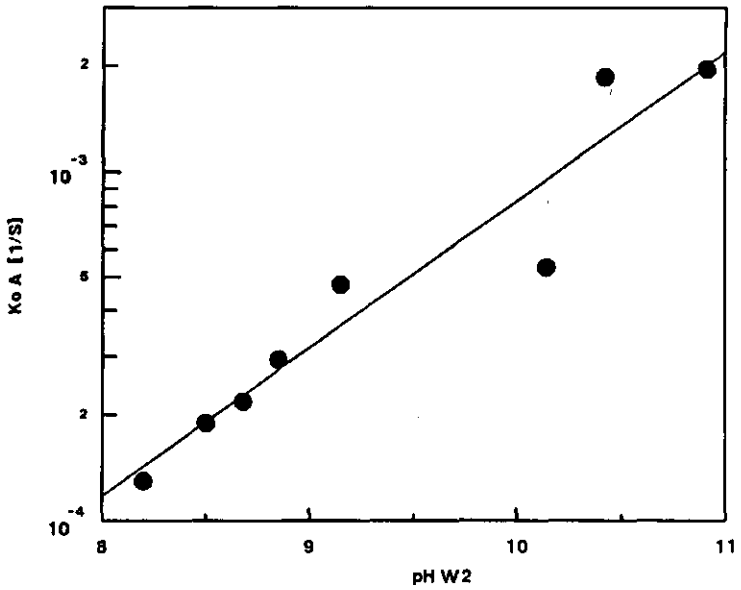

Fig. 9. Back extraction mass transfer rate coefficient (K_oA) of ribonuclease A from AOT reversed micelles as a function of the aqueous phase pH.

4.6. CONCLUSIONS

The forward and back extraction of the enzyme α-amylase between an aqueous phase and a reversed micellar phase of the cationic surfactant TOMAC in isooctane have different mass transfer resistances. The transfer rate of the enzyme from the aqueous phase to the reversed micellar phase (at pH 10.0) is controlled by the diffusion of the protein in the aqueous phase boundary layer. The transfer rate of the reverse process (at pH 4 - 6) is much lower and is found to be controlled by an interfacial resistance. This additional resistance is probably due to the low coalescence rate of a protein containing reversed micelle with the interface caused by interactions between the protein surface and the surfactant molecules. Decreasing

the pH resulted in a reduction of this interfacial resistance and thereby in an enhanced overall mass transfer rate, due to a decrease in protein interaction with the positively charged surfactant molecules. The same type of behaviour is observed for the back transfer of ribonuclease A from a reversed micellar phase of the anionic surfactant AOT in isooctane, in which case an increase in pH resulted in an enhanced back transfer rate.

ACKNOWLEDGEMENTS

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NOMENCLATURE

A	specific surface area	m^{-1}
C_j	concentration of active enzyme in phase j	kg m^{-3}
D	diffusion coefficient	m^2 s^{-1}
F_j	flow of phase j	ml s^{-1}
k	Boltzmann's constant ($1.3805 \cdot 10^{-23}$)	J K^{-1}
k_i	inactivation rate constant	s^{-1}
k_j	mass transfer rate coefficient in phase j	m s^{-1}
K_o	overall mass transfer rate coefficient	m s^{-1}
N	stirring speed	s^{-1}
m	distribution coefficient (C_2/C_1)	-
r	radius	m
Re	Reynolds number (vx/ν)	-
Sc	Schmidt number (ν/D)	-
Sh	Sherwood number (kx/D)	-
t	time	s
T	temperature	K
v	velocity	m s^{-1}
x	characteristic length	m
α_j	coefficient defined in equations 6 and 7	kg m^{-3}

β_j	coefficient defined in equations 8 and 9	kg m^{-3}
γ_j	coefficient defined in equations 10-13	s^{-1}
ϵ_j	hold up of receiving phase (phase 2)	-
ζ_j	coefficient defined in equations 16-20	s^{-1}
λ_j	coefficient defined in equation 5	s^{-1}
μ	dynamic viscosity	Ns m^{-2}
ν	kinematic viscosity	m^2 s^{-1}
τ	residence time	s

sub/superscripts

1	phase 1: supplying phase
2	phase 2: receiving phase
in	flowing into mixer
m	mobile interface
obs	observed value
out	flowing out of mixer
r	rigid interface
RM	reversed micellar phase
W	aqueous phase
W1	aqueous phase of forward extraction
W2	aqueous phase of back extraction
σ	interface

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CHAPTER 5

TEMPERATURE EFFECT ON THE REVERSED MICELLAR EXTRACTION OF ENZYMES

ABSTRACT

In "conventional" liquid/liquid extraction of protein solutions with a reversed micellar phase, the protein is transferred from an aqueous phase to the reversed micellar phase by forward extraction, and subsequently to a second aqueous phase during back extraction. This back transfer is a relatively slow process, due to a large interfacial resistance for mass transfer.

In this paper we report an alternative procedure for the back extraction, using the effect of temperature. By increasing the temperature of the reversed micellar phase, after it has been saturated with the aqueous phase during the forward extraction, a separate aqueous phase is formed in which most of the enzyme is concentrated. This excess aqueous phase can be separated easily from the reversed micellar phase by centrifugation. The enzyme can be recovered in this expelled phase at extremely high concentrations (up to 2000x the initial concentration). This process has been performed in a continuous way with two centrifugal separators, with the reversed micellar phase circulating between the two units. For α-amylase a recovery of 73 % of enzyme activity was obtained.

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5.1. INTRODUCTION

A reversed micelle consists of a spherical aggregate of surfactant molecules in an apolar solvent surrounding an inner core of water. The internal polar environment of these droplets enables polar compounds, such as proteins, to be solubilized in a largely apolar solvent.

It has been demonstrated that under certain conditions proteins can be transferred from an aqueous towards a reversed micellar phase and back (Dekker *et al.*, 1989[b]). In general, proteins are only transferred to a reversed micellar phase at pH values at which their net sign of charge is opposite to that of the surfactant headgroups. The difference between the pH value at which transfer occurs and the pI of the protein depends on the size of the protein molecule (Wolbert *et al.*, 1989). Because electrostatic interactions between the protein and the reversed micelle are important (Göklen and Hatton, 1987; Dekker *et al.*, 1987[a]), the distribution coefficient of proteins in these systems is not only determined by the pH but also by the ionic strength in the aqueous phase. Studies have been reported concerning the selectivity of the extractions (Göklen and Hatton, 1987; Wolbert *et al.*, 1989) and the thermodynamic modeling of the distribution behaviour (Fraaije, 1987; Caselli *et al.*, 1988; Bratko *et al.*, 1988; Fraaije *et al.*, 1989).

Extraction of an aqueous protein solution with an organic solvent containing reversed micelles presents itself as a promising process for the selective recovery of proteins from a fermentation broth (Rahaman *et al.*, 1988). To apply this reversed micellar extraction method, a continuous forward and back extraction process can be used. Previously we have investigated the performance of this process in two mixer/settler units (Dekker *et al.*, 1986; Dekker *et al.*, 1989[a]).

The back transfer rate for protein extraction has been reported to be relatively slow (Dekker *et al.*, 1989[c]). This slow mass transfer was ascribed to an interfacial resistance caused by the low coalescence rate of reversed micelles containing a protein molecule with the interface between the organic and aqueous phase. The aqueous phase pH was found to have a large effect on this interfacial resistance. Decreasing the number of groups on the protein with a charge opposite to that of the surfactant headgroups, resulted in an enhanced back transfer rate.

In this paper we describe an alternative process for the desolubilization of protein from the reversed micellar phase, based upon the effect of temperature on the phase behaviour of the latter. This procedure circumvents the disadvantageous effects of the decreased back extraction mass transfer rate.

5.2. MATERIALS AND METHODS

5.2.1. Chemicals

α-Amylase (EC 3.2.1.1) from *Bacillus amyloliquefaciens* was obtained from Sigma Chemical Co. The insoluble impurities of the enzyme preparation were removed by microfiltration of the enzyme solutions. Trioctylmethylammonium chloride (TOMAC) was obtained from Merck and contained 88% (w/w) of the quaternary ammonium salt, 10 % (w/w) of a mixture of octanol and decanol and 2% (w/w) of water. Rewopal HV5 (nonylphenolpentaethoxylate) was obtained from REWO Chem. Group. All other chemicals were obtained from Merck and were of analytical grade.

5.2.2. Analysis

α-Amylase activity and concentration were determined on a SKALAR auto-analyzer with a colorimetric assay, as described previously (Dekker *et al.*, 1989[a]). Surfactant concentrations in the organic phase were determined by a spectrophotometrical assay (Dekker *et al.*, 1989[a]). The water content of the reversed micellar phase was determined by Karl Fischer titration.

5.2.3. Temperature effect on phase behaviour

The reversed micellar phase (0.40% (w/v) TOMAC, 0.088% (w/v) Rewopal HV5 and 0.1% (v/v) octanol in isooctane) was equilibrated with the aqueous phase (50 mM ethylenediamine, EDA, adjusted to pH 10.1 with HCl) by mixing samples for two hours at temperatures ranging from 10 to 60 °C. Phase separation was achieved by centrifugation (5 min, 2500 g) at the same temperatures. The upper reversed micellar phase was collected and analyzed for water content and surfactant concentration.

5.2.4. Temperature effect on protein solubilization

The extraction - desolubilization procedure is shown schematically in Figure 1.

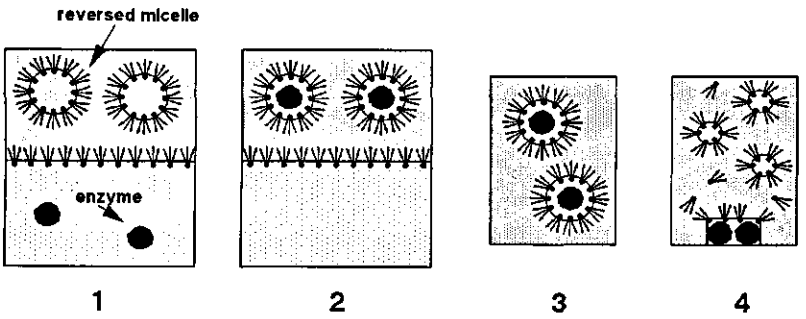

Figure 1: Schematic representation of the extraction/desolubilization procedure, showing the transfer of the enzyme from the aqueous phase to the reversed micellar phase at low temperature (1-2) and the recovery at high temperature (3-4).

A forward extraction has been performed at low temperature (10 °C) at pH 10.1 (aqueous phase: 1 g l^{-1} α-amylase, 50 mM EDA/HCl), as described by Dekker *et al.*, 1986. The reversed micellar phase from this extraction, containing the enzyme, was separated from the aqueous phase and incubated for 10 minutes at constant temperatures ranging from 10 to 60 °C. Subsequently the samples were centrifuged at 10000 g for 10 s in order to spin down the separate phase which had formed. The supernatant reversed micellar phase was removed and analyzed for water content. The expelled aqueous phase was solubilized in an aqueous phase (0.5 M NaCl; 50 mM HAc/NaAc at pH 5.0) and analyzed for enzyme activity.

5.2.5. Continuous centrifugal extraction

The continuous extraction was performed with two centrifuges (Westfalia Separator, type TA1) (Figure 2).

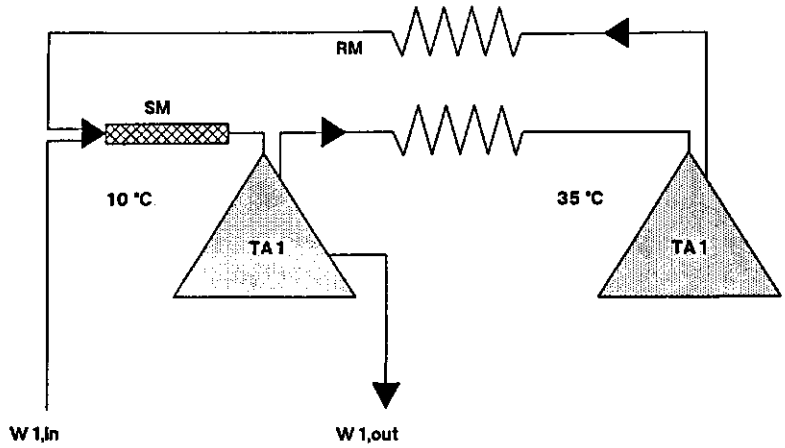

Figure 2: Flow sheet of the continuous centrifugal extraction. W1: Aqueous phase from which the enzyme is to be recovered; RM: reversed micellar phase; SM: static mixer; TA1: centrifuges.

The forward extraction was performed at 10 °C in a static mixer (internal diameter: 1 cm, length: 0.5 m) and in the mixing zone in the first centrifuge. The flow of aqueous phase was 60 l h^{-1} and of reversed micellar phase 30 l h^{-1}. After phase separation in the centrifuge the reversed micellar phase passed through a heating coil (internal diameter: 1 cm, length: 12 m, T = 35 °C) and into the second centrifuge to discharge the excess aqueous phase which had formed. The reversed micellar phase was subsequently cooled down to 10 °C and recirculated to the forward extraction unit. The second centrifuge was equipped with a solid bowl to collect the enzyme containing phase during the complete run (100 minutes). During the run samples of the reversed micellar phase and first aqueous phase were taken. At the end of the run the product in the bowl of the second centrifuge was collected and redissolved in 200 ml aqueous phase (0.5 M NaCl; 50 mM HAc/NaAc pH 5.0). In all samples the enzyme activity was determined.

5.3. RESULTS AND DISCUSSION

5.3.1. Temperature effect on phase behaviour

The maximum amount of aqueous phase which can be solubilized in the reversed micellar phase has been determined as a function of temperature (Figure 3). The amount of water solubilized is expressed as the molar ratio of water to surfactant in the organic phase (w_0 = [H_2O]/[surfactant]). As temperature increases the amount of solubilized water decreases. The TOMAC concentration in the organic phase was found to be constant (8 mM) in the tested temperature range.

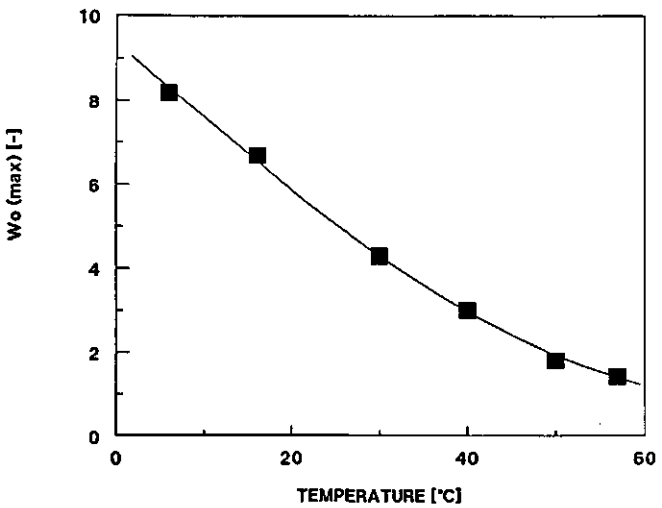

Figure 3: Maximum solubilization of water in the reversed micellar phase as a function of temperature.

A relation between the radius of the water core in the reversed micelle (r_c), the headgroup area of the surfactant molecules in the aqueous/organic interface (A_h), the volume of a water molecule (V_w), the fraction of surfactant molecules in the interface (α = [surfactant]$_{interface}$/[surfactant]$_{total}$) and w_0 can be derived, based on geometric considerations (Assih *et al.*,1982):

$$w_0 = \frac{r_c \cdot A_h}{3V_w} \cdot \alpha \qquad (1)$$

According to this equation the observed phase behaviour can be caused by several phenomena: a decrease of r_c, A_h, α, or an increase of V_w, or a combination of these changes. In the investigated range of temperature the changes in V_w will be less then 2%. For several surfactant systems no large effect of temperature on A_h has been observed (Zulauf and Eicke, 1979). The observed lowered capacity for solubilizing water will therefore mainly be due to a decrease in r_c and/or α.

5.3.2. Composition of expelled aqueous phase

From Figure 3 it is clear that heating the reversed micellar phase, which is equilibrated with the aqueous phase, causes expulsion of part of the solubilized water and leads to the formation of a separate phase (desolubilization). If protein is present in the reversed micellar phase, it will depend on the composition (pH and ionic strength) of this aqueous phase whether or not the protein will also be expelled from the reversed micelles.

For the determination of the composition of the expelled aqueous phase the reversed micellar phase contained 10 times the normal concentrations of surfactants and cosurfactant in order to obtain samples of expelled aqueous phase sufficiently large to be analyzed. It can be assumed that the micellar properties do not change significantly upon this increase in surfactant concentration (Eicke and Rehak, 1976). This organic phase was equilibrated at low temperature (10 °C) with the forward extraction buffer, by gentle mixing of the two phases with a magnetic stirrer for 2 hours. After phase separation and removal of the bulk aqueous phase the reversed micellar phase was heated to 35 °C (this temperature was chosen with respect to the enzyme recovery experiments as to be discussed below). After settling the expelled aqueous phase was analyzed for pH and buffer concentration.

TABLE 1. Composition of the aqueous phase in the forward extraction compared to that of the aqueous phase desolubilized from the reversed micellar phase.

Aqueous phase	T (°C)	pH	EDA (mM)
Forward extraction buffer	10	10.10	50
Forward extraction buffer	35	9.45	50
Desolubilized phase	35	8.80	32

In Table 1 the compositions of the aqueous phase in the forward extraction and the desolubilized aqueous phase are given. The expelled aqueous phase contains less EDA/EDA·HCl than during the forward extraction, which can be explained by the low concentration of surfactant co-ions (cations) which will be transferred to the reversed micellar phase (Leodidis and Hatton, 1989). The pH of the expelled aqueous phase is much lower than in the forward extraction buffer, the shift being much larger than the decrease in pH caused by the temperature rise only. These changes in composition of the aqueous phase can be caused by the redistribution of buffer molecules during both solubilization and expulsion. No data are available on the composition of the aqueous phase in the reversed micelles, so that no conclusions can be drawn with respect to the exact mechanism of these changes.

At 35 °C the distribution of α-amylase between the expelled aqueous phase and the reversed micellar phase is directed towards the aqueous phase. At this temperature and a buffer concentration of 32 mM, transfer towards the reversed micellar phase could only be observed at pH > 10. It can therefore be expected that during desolubilization at 35 °C the enzyme will be expelled together with the excess aqueous phase.

5.3.3. Temperature effect on protein recovery

The feasability of this temperature desolubilization method for recovering the enzyme from the reversed micellar phase was verified experimentally. The α-amylase was transferred to the reversed micellar phase by a forward extraction at 10 °C and

pH 10.1, which resulted in 95% transfer. The reversed micellar phase was subsequently heated and the expelled aqueous phase was removed by centrifugation (Fig. 1). In Figure 4 the percentage of active α-amylase in the expelled aqueous phase is shown as a function of the desolubilization temperature.

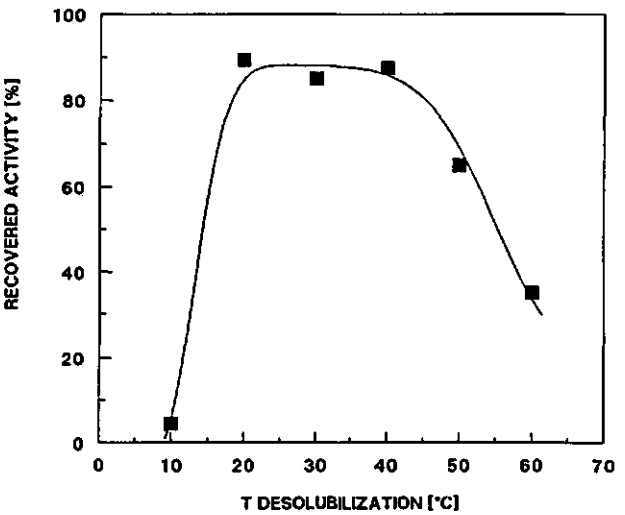

Figure 4: Recovery of enzyme from the reversed micellar phase by temperature desolubilization. Recovery of active enzyme expressed as % of starting activity in the aqueous phase.

Over 90% of initial enzyme activity could be recovered by raising the temperature to 20-40 °C. At higher temperatures inactivation of the α-amylase adversely affects the yield of active enzyme.

This desolubilization procedure thus offers an alternative to the conventional way of recovering proteins from the reversed micellar phase by a back extraction with a second aqueous phase. The latter was found to be much slower than the forward extraction, which was shown to be caused by an interfacial resistance towards coalescence of the protein-filled reversed micelles with the interface (Dekker *et al.*, 1989c). Desolubilization of the protein at a higher temperature is a much faster process, because of an enhanced fluidity of the interface combined with a reduced affinity of the surfactants for the interface.

5.3.4. Continuous centrifugal extraction

The combination of a forward extraction with a temperature desolubilization step to recover the enzyme α-amylase has been tested on a pilot scale using two Westfalia Separator centrifuges (type TA1). The reversed micellar phase was circulated between the two units (Figure 2). The aqueous phase leaving the forward extraction centrifuge (operated at 10 °C) retained 12% of the initial enzyme activity. From the small scale studies less than 5% was expected, indicating that the extraction in the static mixer and centrifuge (total mixing time was 5 s) was not complete.

In the second centrifuge (operated at 35 °C) the desolubilized aqueous phase was separated from the reversed micellar phase. The resulting enzyme concentrate was collected in the bowl of this centrifuge during the run. The reversed micellar phase was recirculated to the forward extraction unit. Less than 5% of active enzyme was detected in this phase. In the desolubilization step 83% of active enzyme was recovered, giving an overall yield of active α-amylase of 73%. Since the volume of the expelled phase is extremely small (ca. 35 ml) an enormous increase in enzyme concentration (ca. 2000x!) was obtained.

In conventional forward and back extraction a second aqueous phase with low pH is used for the recovery of the enzyme from the reversed micellar phase in the second centrifuge. Attempts to use this process (back extraction at the same temperature as the forward extraction) with the two centrifuges was not very successful. In this process the back extraction was incomplete (about 20-30% of the enzyme was re-extracted in the second aqueous phase during back extraction). Due to the high interfacial mass transfer resistance during back extraction the mixing time for back transfer was insufficient. This resulted in a build-up of enzyme in the recirculating reversed micellar phase causing a much lower extraction efficiency (In an extraction run of 100 l first aqueous phase, only 30-50% of active enzyme could be recovered in the second aqueous phase).

In Table 2 the results obtained with the two processes are compared. It can be concluded that the desolubilization of protein from the reversed micellar phase by increasing the temperature is a much more efficient way of recovering proteins from this phase.

TABLE 2. Comparison between the conventional forward and back extraction (FEBE) and the forward extraction with temperature desolubilization (FETD) both performed in Westfalia Separator centrifuges.

	FEBE	FETD
Initial activity	100%	100%
Loss at forward extraction	3%	12%
Overall recovery	30-50%	73%
Concentration increase	10×	2000×

5.4. CONCLUSIONS

The water solubilizing capacity of the reversed micellar phase of TOMAC/Rewopal HV5/octanol in isooctane is reduced with increasing temperature. This phase behaviour can be used to control the solubilization/desolubilization of enzymes and thus to create a successful recovery step for enzymes. The recovery of the enzyme α-amylase from the reversed micellar phase was found to be more efficient by using a temperature desolubilization step than by a back extraction with a second aqueous phase. Using continuous centrifuges for this process was found to be very efficient.

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CHAPTER 6

GENERAL DISCUSSION

The previous chapters have shown that by using reversed micelles enzymes can be selectively concentrated in a continuous liquid/liquid extraction process. In this chapter some potentials and limitations of the technique are discussed.

6.1. Concentration increase

The possibility to vary the distribution coefficient over a wide range enables a large concentration increase by using the combined forward and back extraction. The concentration increase which can be obtained will depend on the number of stages in each extractor, the flow ratios of the phases, and the allowed loss in the forward extractor. The theoretical maximum in concentration increase is given by the product of the distribution coefficient during the forward extraction (C_{RM1}/C_{W1}) and during the back extraction (C_{W2}/C_{RM2}).

Another important factor is the maximum solubility of the enzyme in the reversed micellar phase. This solubility depends on the surfactant concentration. A theoretical maximum in protein solubility is obtained if all surfactant is used to cover the surface of the protein in the organic phase:

$$n_e \cdot A_e = n_s \cdot A_h \qquad (1)$$

In which n_e is the number of enzyme molecules in the reversed micellar phase, A_e is the surface area for one enzyme molecule, n_s is the number of surfactant molecules

and A_h is the surface area of one surfactant headgroup. For this assumption equation 2 can be derived for the calculation of this maximum concentration (C_{max}) of a spherical protein in the reversed micellar phase:

$$C_{max} = 1.5 \cdot 10^{19} \cdot M_r^{1/3} \cdot [S] \cdot A_h \qquad [kg \cdot m^{-3}] \qquad (2)$$

In which M_r is the molecular weight of the enzyme, and [S] the surfactant concentration in the reversed micellar phase.

For α-amylase (M_r = 50 kDal) and a reversed micellar phase of 0.01 M TOMAC (assumed A_h = $5 \cdot 10^{-19}$ m^2) equation 2 results in a maximum solubility of 2.7 kg m^{-3}. This value agrees well with the experimentally found value of 2.5 kg m^{-3} (Van Der Pol, 1989). Since larger proteins have a lower surface/volume ratio than smaller ones the solubility increases with protein size (Figure 1).

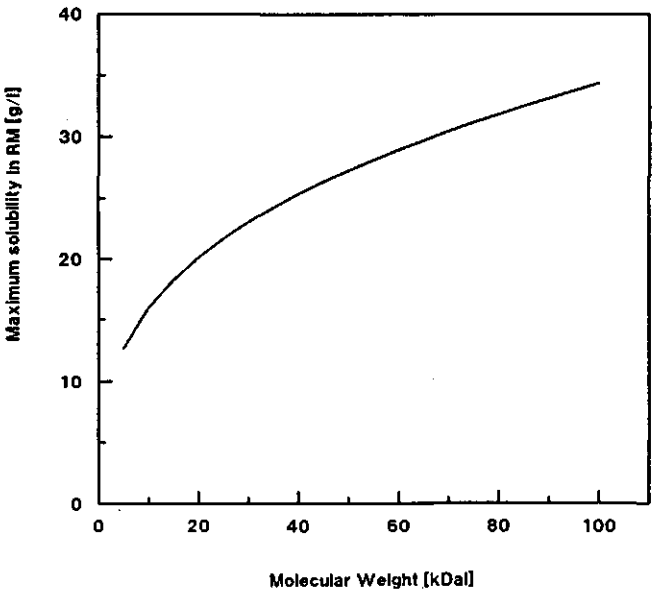

Fig. 1. Maximum solubility of proteins in a reversed micellar phase of 0.1 M surfactant with A_h = $5 \cdot 10^{-19}$ m^2. Line calculated with equation 2.

It should be noted that equation 2 and Fig. 1. predict only the maximum solubility and not the distribution coefficient of the protein, which will depend on many other

parameters as discussed in Chapter 1. However, if in an extraction system the maximum solubility of the protein is reached, the distribution coefficient becomes a function of the enzyme concentration in the aqueous phase.

6.2. Selectivity

The reversed micellar extraction with ionic surfactants has been shown to be selective for proteins. Each protein has its own optimum pH for transfer to the reversed micellar phase depending on the pI of the protein and its molecular weight. Since protein transfer is characterized by a pH range, given a certain pH other proteins might be transferred as well. For reversed micelles of TOMAC/Rewopal the observed pH dependency of the fraction of protein transferred to the reversed micellar phase (ϕ) can be described by the empirical equation:

$$\phi = \phi_{max} \cdot e^{-10(pH_{opt} - pH)^4} \qquad (3)$$

Using equation 3 the purification factor of the desired enzyme (the ratio of the amount of enzyme to the amount of total protein after the extraction divided by this ratio before the extractions) can be calculated for its extraction from a random mixture of proteins. In Figure 2 this theoretical purification factor is given as a function of the percentage desired enzyme in the starting protein mixture. It has been assumed that the number of proteins approaches infinity and that all proteins have $\phi_{max} = 1$, at equally distributed pH values between 0 and 14.

The calculated relation holds for a mixture containing a very large number of proteins. In the case that only two or three proteins are present the purification factor will be determined by the optimum pH of transfer for those particular proteins. The purification factor might therefore either be much larger or much smaller than predicted by Figure 2. It should be noted that in practice not all the maximum fractions of transferred protein will have a value of one.

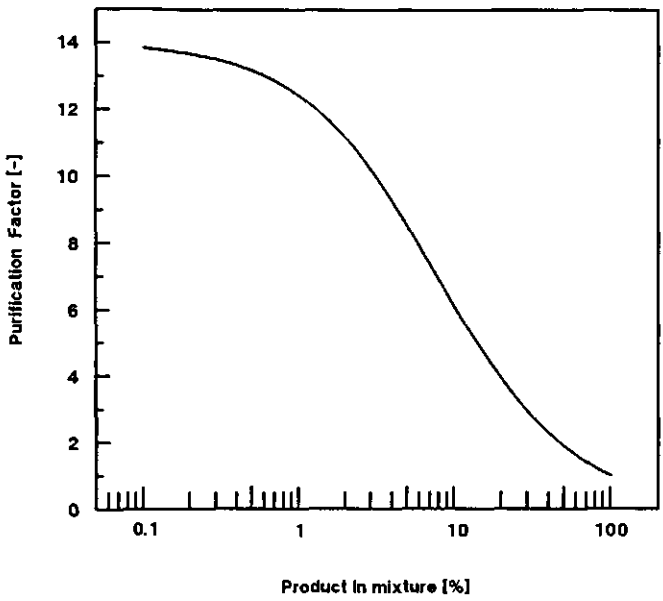

Fig. 2. Calculated purification factor for a desired enzyme out of a simulated protein mixture, with optimum pH values for transfer equally distributed between 0 and 14.

The selectivity of the extraction might be enhanced by using more specific interactions between surfactant and enzyme instead of electrostatic interactions. The use of affinity interactions with a substrate analogue coupled to an apolar tail has been shown to be feasible for the extraction of concanavalin A (Woll and Hatton, 1989). The affinity surfactant was added to a reversed micellar phase of an ionic surfactant, so that the transfer to the reversed micelles will be a combination of affinity and electrostatic interactions.

For α-amylase affinity surfactants of maltodextrins esterified to fatty acids have been used successfully in combination with a cationic surfactant (Van Der Zandt, 1989). Addition of the affinity surfactant enabled transfer of the enzyme at pH values at which normally no transfer occurred. As found for concanavalin A by Woll and Hatton (1989) this affinity extraction also appeared to be inhibited by the addition of free ligand in the aqueous phase, indicating that affinity interactions are responsible for the transfer indeed.

The use of an affinity surfactant in combination with a nonionic surfactant will result in a more selective extraction, since electrostatic interactions will no longer be possible.

Possible limitations of the reversed micellar extraction

6.3. Protein size limitation

The size of a protein determines the difference between the pI of the protein and the optimum pH of transfer (Wolbert *et al.*, 1989). The larger the protein that has to be transferred, the larger the difference between pH and pI. This relation limits the size of proteins that can be transferred depending on their pI and the surfactant used (Fig. 3).

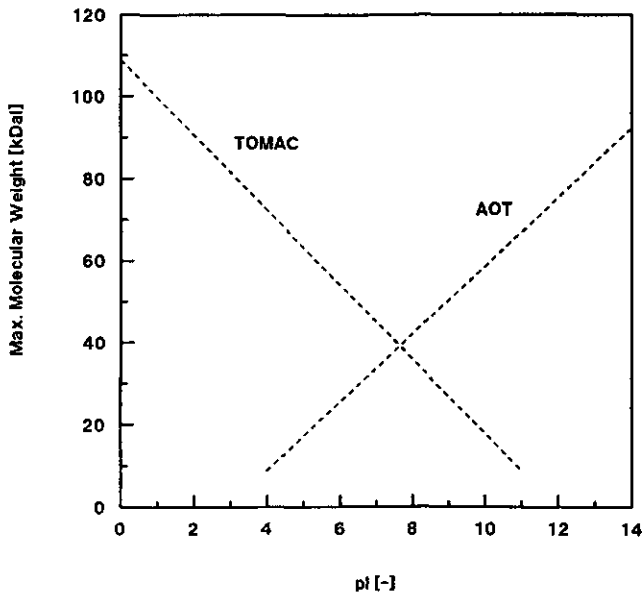

Fig. 3. Maximum molecular weight of a protein in order to be extractable between pH 4 and 11 as a function of the pI of the protein, given for the surfactants AOT and TOMAC.

The molecular weight limitations represented in Fig. 3 can only be used for the two surfactant systems tested. With other surfactants it might be possible to extract larger proteins in the reversed micelles, depending on their size and flexibility.

6.4. Salt limitation

In reversed micellar extraction with ionic surfactants electrostatic interactions proved to be most important. This implies that the ionic strength of the aqueous phase affects the pH at which the protein can be transferred to the reversed micellar phase. For the cationic surfactant TOMAC the pH of transfer shifts towards higher values (shift of ca. 1 pH unit per 100 mM NaCl, Dekker *et al.*, 1987). For the anionic surfactant AOT this shift is towards lower pH values (shift of ca. 1 pH unit per 150 mM NaCl, Leodidis and Hatton, 1989).

Using these shifts a maximum salt concentration in the aqueous phase can be calculated at which a protein can yet be transferred (assuming the transfer has to be between pH 4 and 11 considering the pH stability of the protein) depending on the pH of transfer without any additional salt (Fig. 4).

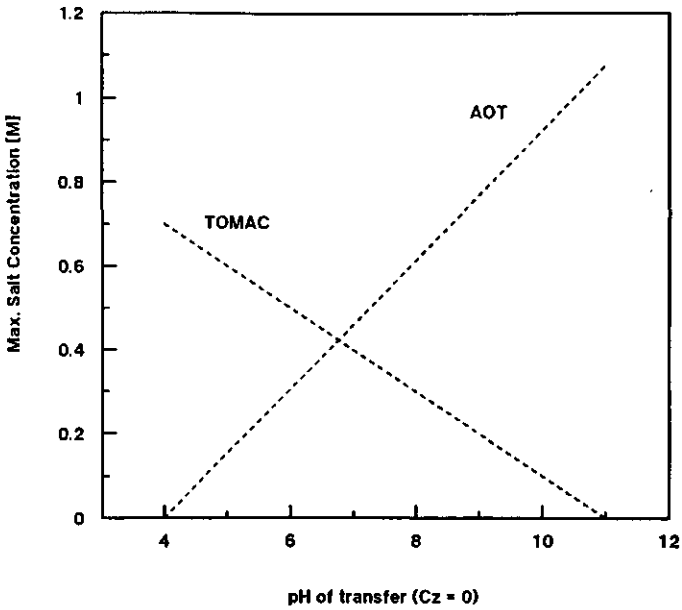

Fig. 4. Maximum allowable salt concentration in the aqueous phase as a function of pH of transfer without additional salt, given for the surfactants AOT and TOMAC.

The salt limitations depend on the type of salt which is used. Especially variation of the surfactant counter-ion can have a large effect on the extraction behaviour. In the TOMAC/isooctane system the addition of NaAc leads to a shift of the α-amylase

transfer to lower pH values as compared to NaCl. This effect can be explained by a five fold increase in the maximum water solubility as compared with the system having only chloride ions as surfactant counter-ion (Van Der Zandt, 1989).

The specific effects of different salt types make it difficult to predict the restrictions for the total ionic strength in extracting an enzyme from a fermentation broth. Rahaman *et al.* (1988) have shown that extraction of a protease from a fermentation broth with reversed micelles of the surfactant AOT in isooctane is possible. Their broth contained 10 kg m^{-3} sodium carbonate and trace amounts of other salts.

Further research with respect to the effect of various salts and other components present in fermentation broths on the reversed micellar properties and protein solubilization will probably give more information on the potentials of reversed micellar extraction for the recovery of enzymes from these broths.

6.5. Conclusion

It can be stated that the developments in reversed micellar extraction clearly demonstrate that this technology has potentials in biotechnology as a new large scale separation tool. The process has been shown to be selective. The extractions can be performed continuously, in a process which can be modelled adequately. The activity retention of enzymes recovered in this way can be high. Based upon two frequently used surfactant systems, some preliminary predictions can be made on the proteins and broths for which the extraction process is suited best. Further research will show for which proteins it is also economically feasible to use this type of technology in industrial processes.

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NOMENCLATURE

A	molecular surface area	m^2
C	concentration of protein	kg m^{-3}
M_r	molecular weight	Dal
n	number of molecules	-
[S]	concentration of surfactant	M
ϕ	fraction of protein in reversed micelles	-

sub/superscripts

e	enzyme
h	surfactant headgroup
RM	reversed micellar phase
W	aqueous phase
W1	aqueous phase of forward extraction
W2	aqueous phase of back extraction

SUMMARY

The objective of this study was to develop a liquid-liquid extraction process for the recovery of extracellular enzymes. The potentials of reaching this goal by using reversed micelles in an organic solvent have been investigated.

Reversed micelles are aggregates of surfactant molecules containing an inner core of water molecules, dispersed in a continuous organic solvent medium. The considerable biotechnological potential of these systems is derived principally from the ability of the water droplets to dissolve enzymes without loss of activity. Enzymes can be transported from a bulk aqueous phase to a reversed micellar phase and *visa versa*.

The distribution coefficient of an enzyme between a reversed micellar and an aqueous phase depends on the interactions which are possible between the enzyme and the reversed micelle. When ionic surfactants are used, electrostatic interactions have been shown to be the most important ones. The distribution can therefore be controlled by adjusting pH and ionic strength. The optimum pH for transfer depends on the size and titration behaviour of the enzyme. The extraction to a reversed micellar phase therefore shows enzyme selectivity.

Using the possibility to vary the distribution coefficient a continuous forward and back extraction process has been developed (Chapter 2). In two mixer/settler units the enzyme α-amylase is concentrated using a recirculating reversed micellar phase of the cationic surfactant trioctylmethylammonium chloride and the cosurfactant octanol in isooctane.

During the forward extraction some inactivation of the enzyme occurs by a complexation between the enzyme and the surfactant in the aqueous phase (Chapter 3). The extraction process has been modelled in terms of mass transfer and inactivation of the enzyme in all phases. As predicted by the model the extraction efficiency can be optimized by reducing the concentration of enzyme in the first aqueous phase

through increasing the distribution coefficient (by the addition of a nonionic surfactant to the reversed micellar phase) and by increasing the mass transfer rate during the forward extraction. The observed enzyme recovery values correlate quite well with the values predicted by the model.

An important parameter of the extractions is the mass transfer rate of the enzyme to and from the reversed micellar phase. During forward extraction the rate of mass transfer is controlled by diffusion in the aqueous phase. The back extraction rate, however, is governed by the interfacial process of coalescence of the reversed micelles with the bulk interface. This process is strongly dependent on the pH, probably due to interactions of the surfactant with charged groups on the enzyme (Chapter 4).

An alternative process for the recovery of the enzyme from the reversed micellar phase uses the temperature effect on the phase behaviour of the system (Chapter 5). By increasing the temperature some aqueous phase is expelled from the organic phase, enabling the enzyme to be recovered in this phase. This phenomenon was applied successfully in an extraction process with two centrifugal extractors.

The applicability of the process to fermentation broths has to be subjected to further investigation, but some established general features are discussed (Chapter 6). In conclusion it can be stated that reversed micellar extraction of enzymes is a selective process with an enormous potential to purify and concentrate proteins in one operation.

The study described in this thesis was performed in a partnership between the Departments of Food Science (Food and Bioprocess Engineering Group), Biochemistry and Physical and Colloid Chemistry. The project was financed by the Netherlands Technology Foundation (STW).

SAMENVATTING

Het doel van het in dit proefschrift beschreven onderzoek was een vloeistof-vloeistof extraktie proces te ontwikkelen voor de opwerking van extracellulaire enzymen. De mogelijkheden om dit doel te bereiken met een omgekeerde micellen bevattend organisch oplosmiddel zijn onderzocht.

Omgekeerde micellen zijn aggregaten van surfactant molekulen rond een kern van watermolekulen in een organisch oplosmiddel. De aanzienlijke biotechnologische mogelijkheden van dergelijke systemen zijn gebaseerd op het feit dat enzymen opgelost kunnen worden in de waterdruppeltjes zonder aktiviteitsverlies. Enzymen kunnen getransporteerd worden van een bulk waterfase naar een omgekeerde micelfase en *vice versa*.

De verdelingscoëfficient van een enzym hangt af van de interakties die mogelijk zijn tussen het enzym en de omgekeerde micellen. Bij het gebruik van ionogene surfactants zijn elektrostatische interakties het meest belangrijk gebleken. De verdeling kan daardoor gestuurd worden door de pH en de ionsterkte aan te passen. De optimale pH voor opname in de omgekeerde micelfase hangt af van de grootte en het titratiegedrag van het enzym. De extraktie naar de omgekeerde micelfase is hierdoor selektief voor het gewenste enzym.

Gebruikmakende van de mogelijkheid om de verdelingscoëfficient te sturen is een kontinu heen- en terugextraktie proces ontwikkeld (Hoofdstuk 2). In twee mixer/settler eenheden is het enzym α-amylase geconcentreerd met een recirculerende omgekeerde micel fase van het kationogene surfactant trioctylmethylammonium chloride en het cosurfactant octanol in isooktaan.

Bij de heenextrakties vindt inaktivering plaats van een deel van het enzym door een komplexatie tussen het enzym en het surfactant in de waterfase (Hoofdstuk 3). Het extraktie proces is gemodelleerd in termen van stofoverdracht en inaktivering van het enzym in alle fasen. De extraktie efficientie kan worden geoptimaliseerd door een

verlaging van de enzym concentratie in de eerste waterfase, door de verdelingscoëfficient te verhogen (door een niet-ionogeen surfactant aan de omgekeerde micelfase toe te voegen) en door de stofoverdrachtssnelheid tijdens de heen extraktie te vergroten. De waargenomen opbrengst aan aktief enzym komt goed overeen met de door het model voorspelde waarden.

Een belangrijke parameter van de extrakties is de stofoverdrachtssnelheid van het enzym naar en van de omgekeerde micel fase. Bij de heenextraktie is de stofoverdracht gecontroleerd door de diffusie in de waterige fase. De terugextraktie snelheid wordt echter bepaald door het proces van coalescentie van de omgekeerde micellen met het bulk grensvlak. Dit proces is sterk afhankelijk van de pH, waarschijnlijk door interakties die plaatsvinden tussen de surfactants en geladen groepen op het enzym (Hoofdstuk 4).

Een alternatief proces voor het terugwinnen van het enzym uit de omgekeerde micel fase maakt gebruik van het temperatuurseffekt op het fase gedrag van het systeem (Hoofdstuk 5). Door verhoging van de temperatuur wordt een deel van de waterige fase uit de omgekeerde micel fase uitgestoten, het enzym kan in deze extra waterfase worden teruggewonnen. Dit fenomeen is met succes toegepast in een extraktie proces met twee centrifugaal extraktoren.

De toepasbaarheid van het proces op fermentatie vloeistoffen is onderwerp van verder onderzoek, maar enkele algemene mogelijkheden en limitaties zijn genoemd (Hoofdstuk 6).

Concluderend kan gesteld worden dat de omgekeerde micel extraktie van enzymen een selektief proces is met een enorm potentieel om eiwitten te zuiveren en te concentreren in één stap.

Het onderzoek zoals beschreven in dit proefschrift is uitgevoerd in een gezamenlijk projekt van de vakgroepen Levensmiddelentechnologie (sektie Proceskunde), Biochemie en Fysische en Kolloïdchemie. Het projekt is gefinancierd door de Stichting voor de Technische Wetenschappen (STW).

CURRICULUM VITAE

Matthijs Dekker werd op 10 september 1961 geboren in St. Maartensbrug (gemeente Zijpe). In 1979 behaalde hij het diploma VWO-B aan de Rijksscholengemeenschap in Schagen. In datzelfde jaar begon hij met zijn studie aan de Landbouwuniversiteit in Wageningen.

In januari 1983 legde hij het kandidaatsexamen in de richting Moleculaire Wetenschappen met lof af. In de doctoraalfase van deze studie deed hij de hoofdvakken Proceskunde en Moleculaire Biologie en het bijvak Biochemie. Zijn stageperiode bracht hij door bij het Department of Chemistry, Bucknell University, Lewisburg, Pennsylvania, USA. In juli 1985 studeerde hij met lof af.

Van september 1985 tot september 1989 verrichtte hij bij de sektie Proceskunde van de Landbouwuniversiteit in Wageningen het onderzoek (gefinancierd door de STW) dat leidde tot dit proefschrift.

Hij werd onderscheiden met een Unilever Research Prijs (1985) en met een DSM Prijs voor Chemie en Technologie (1989).

Vanaf september 1989 werkt hij bij het Unilever Research Laboratorium in Vlaardingen.