Modeling and Optimization of the Reversed Micellar Extraction of α-Amylase

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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. A reversed micelle consists of a spherical aggregate of surfactant molecules in an apolar solvent surrounding an inner core of water. The polar environment inside such a micelle 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 phase towards a reversed micellar phase and back (Van 't Riet and Dekker, 1984; Göklen and Hatton, 1985). The partitioning of proteins between a reversed micellar phase and an aqueous phase depends on several factors, one being electrostatic interactions between the protein and the reversed micelle (Göklen and Hatton, 1987; Dekker et al., 1987b).

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). In this way the enzyme α-amylase was concentrated eight times. During the extraction, a loss of 30% of the enzyme activity and a slow loss of surfactant were observed.

In this paper, the mechanism of the enzyme inactivation during the extraction and the modeling of the extraction, is described. As predicted by this model, activity recovery and surfactant loss can be optimized considerably.

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Experimental

Extractions

Extractions were performed at a temperature of 20 ± 0.5°C, as described before (Dekker et al., 1986). The reversed micellar phase contained 0.40% (w/v) TOMAC (trioctylmethylammonium chloride), 0.088% (w/v) Rewopal HV5 (nonylphenolpentaethoxylate, obtained from Rewo Chem. Group, FRG) and 0.1% (v/v) octanol in isooctane. The aqueous phase during the forward extraction of α-amylase contained 50 mM ethylenediamine (EDA) at pH 10.0; during back extraction, 0.5 M NaCl and 50 mM NaAc/HAc at pH 4.4.

The flows, F, during the continuous extraction were 1.0 mL · s^{-1} for the first aqueous phase, W_1, 0.5 mL · s^{-1} for the reversed micellar phase, RM, and 0.05 mL · s^{-1} for the second aqueous phase, W_2. The reversed micellar phase was circulating between the two extraction units, with a circulation time of 65 min. The concentration of TOMAC in this phase was analyzed every 100 minutes and adjusted if necessary.

Analysis

α-Amylase activity and concentration was determined with an auto-analyzer, SKALAR, using a starch degradation and biuret assay, respectively.

The concentration of TOMAC in the reversed micellar phase was determined by extraction of a 2.5 mL diluted sample with 0.5 mL of an aqueous solution of color reagent (1% w/v 3,5-

dinitrosalicylic acid, 0.4 N NaOH and 30% w/v K,Na-tartrate). The absorption at 480 nm was linearly related to the TOMAC concentration.

The concentration of Rewopal HV5 was determined by HPLC with a C8 reversed phase column which was eluted with 4:1 MeOH/H_2O at a flow rate of 0.5 mL/min. Detection was by UV absorption at 280 nm.

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, based on the flow, mass transfer, and first-order inactivation kinetics. For each phase, a differential equation is derived:

$$\frac{dC_{W1}}{dt} = \frac{C_{W1,in} - C_{W1}}{\tau_1} - \frac{K_{o1} \cdot A_1}{1 - \epsilon_1} \cdot (C_{W1} - C^*_{W1}) - k_{iw1} \cdot C_{W1} \quad (1)$$

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

$$\frac{dC_{W2}}{dt} = \frac{0 - C_{W2}}{\tau_2} + \frac{K_{o2} \cdot A_2}{\epsilon_2} \cdot (C_{RM2} - C^*_{RM2}) - k_{iw2} \cdot C_{W2} \quad (3)$$

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

where

A_j = specific surface area in mixer j, m^2/m^3
C_j = concentration of active enzyme in phase j, kg/m^3
k_{ij} = inactivation rate constant in phase j, s^{-1}
K_{oj} = overall mass transfer rate constant in mixer j, m/s
t = time, s
ϵj = hold-up of dispersed phase in mixer j
τ_j = residence time in mixer j, s

Results and Discussion

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. Since no inactivation ($k_i < 10^{-5}\ s^{-1}$) is observed in the aqueous phase before and in the reversed micellar phase after the extraction, the enzyme must be inactivated by a component of the reversed micellar phase, present in the aqueous phase during extraction. Separate addition of these components to an aqueous enzyme solution showed that only the cationic surfactant TOMAC is effective in inactivating the enzyme (Figure 1).

Since the equilibrium concentration of free TOMAC in the aqueous phase is very low, it has to be supplied continuously from the reversed micellar phase during extraction, when the

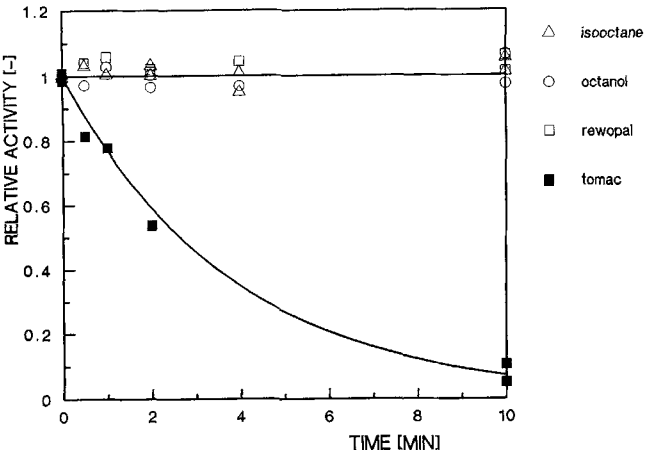

Figure 1. α-Amylase in an aqueous phase (50 mM EDA, pH 10.0) inactivated by adding 0.1% (w/v) of a reversed micellar phase component.

decrease in the aqueous phase occurs due to complexation between enzyme and surfactant. The transfer of surfactant is visualized in Figure 2. The initial slope of this plot indicates that 250 surfactant molecules are bound to one enzyme molecule. Data on complexation of the surfactant sodium dodecylsulfate with proteins, show a similar ratio (210–260) for a protein of the same size as α-amylase (Takagi et al., 1975).

During the inactivation experiments, aggregates of the surfactant/enzyme complex appear at the interface between the aqueous and the reversed micellar phases. From these aggregates, 60% of both protein and surfactant could be recovered by adding salt and lowering the pH. Insoluble interfacial complexes are also reported for the reversed micellar extraction of cytochrome-c, with AOT 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. At pH values below 7, no inactivation is observed ($k_i < 10^{-5}\ s^{-1}$), which explains why inactivation of the enzyme predominantly takes place during the

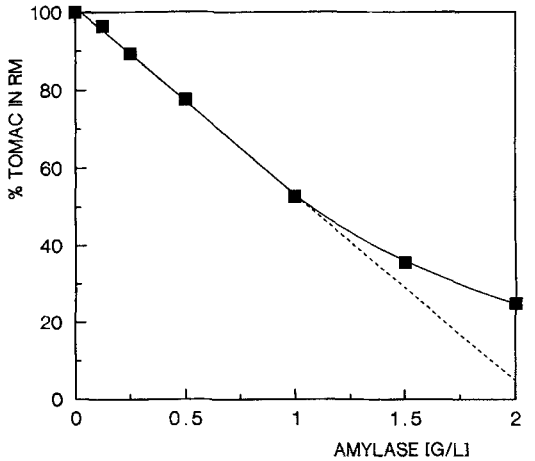

Figure 2. TOMAC percentage remaining in the reversed micellar phase after extraction vs. inactivated amount of α-amylase.
Extraction for one hour at pH 10.5

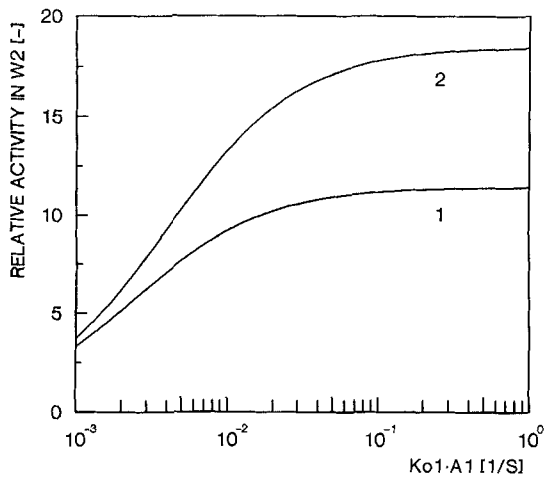

Figure 3. Simulated effect of the mass transfer rate coefficient on the activity recovery in the second aqueous phase.

The activity in W_2 is relative to the initial activity in W_1: 1. $m_1 = 10$; 2. $m_1 = 100$.
Assumed values based on 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}; F_{W1}:F_{RM}:F_{W2} = 20:10:1.

forward extraction (pH 10.0) and not during the back extraction (pH 4.4).

Optimization of the Extraction Efficiency

Because inactivation in the first aqueous phase can be described by a first-order mechanism, the extraction efficiency

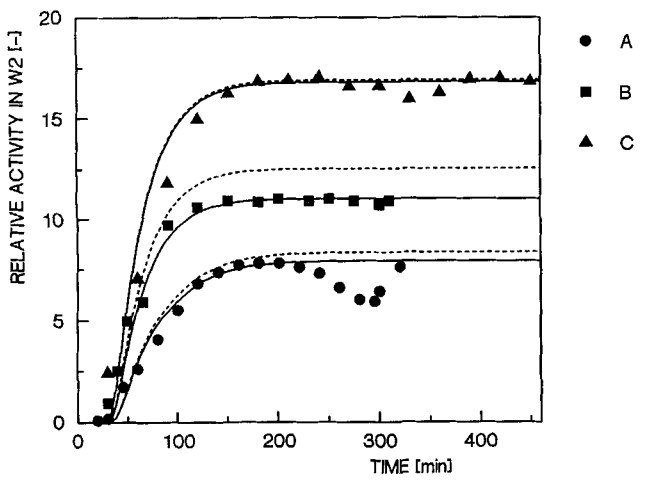

Figure 4. Activity recovery in the second aqueous phase of the combined forward and back extraction. The activity in W_2 is relative to the initial activity in W_1.

A. $N_1 = 2.8$ s^{-1}; $N_2 = 4.0$ s^{-1}; without nonionic surfactant Dekker et al., 1986; "dip" caused by TOMAC loss from reserved 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)
----, model predicted by independently found values for the mass transfer and inactivation coefficients —, 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}

is expected to increase with a lower steady-state enzyme concentration in this phase. This lowering 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 Eqs. 1–4, the steady-state enzyme concentrations in all four phases can be calculated. The results in Figure 3 confirm the expected trend.

Effect of 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 ($m_1 = C_{RM}/C_{W1}$ increases from 10 to at least 100), as well as in the pH range in which solubilization occurs (Dekker et al., 1987a). These effects could be caused by changes in the structure of the reversed micelles and in changes in their adaptability in size and surface charge density due to the addition of the nonionic surfactant.

Using this improved reversed micellar phase for the continuous forward and back extraction of α-amylase resulted in an increase in the activity recovery in the second aqueous phase from 45% to 65%, reaching a concentration of 12 g/L, Figure 4(B). Total activity recovery (W_1 and W_2) was 75%. The loss of TOMAC from the reversed micellar phase was 5% per circulation of the reversed micellar phase. No loss of Rewopal HV5 was observed during the extractions.

Effect of the mass transfer rate

When the forward extraction in the mixer/settler has reached a steady state, both the mass transfer rate coefficient, $K_{o1} \cdot A_1$, and the inactivation rate constant in the first aqueous phase, k_{iw1}, can be calculated by using Eqs. 1 and 2.

In Figure 5, the results of these measurements are given as a function of stirring speed, N s^{-1}. 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).

The value of k_{iw1} was found to be $4 \cdot 10^{-3}$ s^{-1} and independent of N_1 for $N_1 \geq 3$ s^{-1}. At $N_1 = 2$ s^{-1} a lower value ($2 \cdot 10^{-3}$ s^{-1})

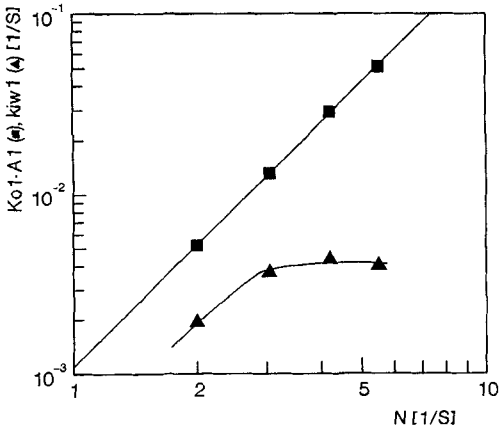

Figure 5. Mass transfer rate and inactivation coefficient during forward extraction of α-amylase to the reversed micellar phase as a function of stirrer speed.

Table 1. Experimental vs. Predicted Values for Enzyme Concentration in Second Aqueous Phase and TOMAC Loss per Circulation of Reversed Micellar Phase*

Exp.	m	$K_{o1} \cdot A_1$ [s^{-1}]	C_{W2} [g/L] Exp.	C_{W2} [g/L] Model	TOMAC Loss [%] Exp.	TOMAC Loss [%] Model	k_{iW1} [s^{-1}] 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	$4.3 \cdot 10^{-3}$

*Experimental conditions as described in Figure 4.

was observed, which may be due to limitation in the transfer of surfactant to the aqueous phase. During the continuous forward and back extraction experiments the stirrer speed was sufficiently high to permit description of the inactivation by first-order kinetics.

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 Figure 4 (*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). Only 3% of the active enzyme remained in the first aqueous phase after the extraction. TOMAC loss was reduced to 2.5% per circulation of the reversed micellar phase.

Model predictions

The experimental data of the steady state of the extraction, Table 1, and the observed dynamic behaviour of the extraction, Figure 4, are in good agreement with the model predictions. This model offers the opportunity to predict the effect of changes, both 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.

A shorter residence time in the extractors, in combination with an increase in the mass transfer rate will give a further improvement of the yield of active enzyme in the second aqueous phase and will further reduce the surfactant loss. The use of centrifugal separators or extractors might be valuable in this respect.

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