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Partial Purification of Glutaryl-7-ACA Acylase from Crude Cellular Lysate by Reverse Micelles

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ABSTRACT

Glutaryl-7-ACA acylase was partially purified from the cellular lysate of *Pseudomonas* sp. NCIMB 40409 by means of reverse micelles-water two-phases extractions. The tetrameric enzyme can be solubilized inside the reverse micelles formed by anionic (Aerosol OT, AOT) and cationic (tetradecyltrimethylammoniumbromide, TDAB) surfactants with retention of the enzymatic activity. With TDAB reverse micelles system, the acylase was partially extracted from the aqueous phase and, after backward transfer into a second water phase, a twofold purification factor was achieved. On the other hand, with the AOT micellar system, in conditions were most of the proteins but acylase, were extracted by the organic micellar solution, a sixfold increase of the specific activity of the acylase remaining in the aqueous phase was obtained.

Index Entries: Reverse micelles; AOT; TDAB; glutaryl-7-ACA acylase; enzyme purification.

INTRODUCTION

Reverse micelles are surfactant-stabilized microdroplets of water dispersed in organic solvents. Inside the micellar aqueous core, hydrophilic macromolecules, such as proteins *(1–3)* and nucleic acids *(4)*, can be solubilized. The micelle hosting capability as well as the properties of the guest macromolecule (e.g., enzyme activity) are strongly influenced by some key physico-chemical parameters, such as the water content (better expressed as W_o, the water-to-surfactant molar ratio), pH, ionic strength, and type of

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ions of the water phase. Moreover, proteins can be selectively extracted or separated from mixtures of other macromolecule *(5–8)* by tailoring the properties of the micellar inner core and, in some cases, the extraction process was developed on a continuous basis *(9)*. By choosing different conditions, the preferential release of the same protein back from the reverse micelles water pool into a second aqueous phase (back-transfer process) can be readily obtained. In this way, successful single component purification with high recovery yield from protein mixtures has been achieved *(10,11)*. The main factors influencing interphase partitioning (protein isoelectric point, pI, net charge, dimensions and interaction with the surfactant molecules, and so forth) have been elucidated and constitute the basis of semitheoretical models able to explain to some extent the properties of the micellar multicomponents systems *(12,13)*.

Most of the studies concerning protein separation or partitioning by using reverse micelles (either liquid-liquid or solid-liquid processes) were carried out on mixtures of two or more components "artificially" prepared by mixing commercially available highly purified proteins. Protein separations were only marginally studied with reverse micelles in real complex systems, i.e., seed meals *(14)*, cell lysate and fermentation broths (in the case of extracellular enzymes) *(9,15–17)*.

In this work, the purification efficiency of the reverse micelles systems formed by two surfactants was assayed by studying the purification of glutaryl-7-ACA acylase (gl-7-ACA acylase) present in a crude cellular lysate preparation of the micro-organism *Pseudomonas* sp. NCIMB 40409 *(18)*. This enzyme has a great potentiality as biocatalyst at industrial level because it catalyzes the cleavage of the glutaryl group from glutaryl-7-amino cephalosporanic acid (glutaryl-7-ACA) or its 3-desacetoxy derivative (glutaryl-7-ADCA). The products of the deacylation reactions are the corresponding 7-amino cephalosporanic acids, 7-ACA and 7-ADCA, two key intermediates for the preparation of semisynthetic cephalosporanic antibiotics *(19)*.

MATERIALS AND METHODS

MATERIALS

Anionic surfactant bis(2-ethylhexyl) sodium sulfosuccinate (AOT) was obtained from Sigma (Munich, Germany), dried under reduced pressure at 70°C overnight and then used without further purification. Isooctane (2,2,4-trimethyl-pentane) and chloroform for UV spectroscopy were from Carlo Erba (Italy). Tetradecyltrimethylammoniumbromide (TDAB), tris(hydroxymethyl) aminomethane (tris) were from Sigma (Italy). Water was double-distilled. 7β-(4-carboxy-butanamido)desacetoxy cephalosporanic acid, 7-ADCA, dioxane, and acetic acid were purchased

from Carlo Erba. Boric acid, potassium iodate, and *p*-dimethylbenzaldehyde were from Fluka (Buchs, Switzerland). Glutaryl-7-ADCA and glutaryl-*p*-nitroanilide were synthesized as described elsewhere *(18)*. Isoelectric focusing pI protein standards were from BioRad (Italy).

METHODS

Preparation of the Cellular Lysate

Pseudomonas sp. NCIMB 40409 was cultivated as indicated elsewhere *(19)*. Ten to fifteen units per liter of fermentation broth were obtained. Cells were disrupted by ultrasonic treatment at 250 W (seven treatments, 1 min each, alternated with cooling). The temperature never exceeded 10°C. After centrifugation at 11,000g for 40 min, ammonium sulfate (70% saturation) was added to the supernatant at 0°C. The suspension was centrifugated after 5 h and the precipitated material was resuspended in water, dialyzed overnight, and freeze dried. The powder was stored at 4°C and then used to prepare the aqueous phases (hereafter referred to as lysate solutions).

Liquid–Liquid Forward and Backward Extractions

The extraction experiments were performed by using the reverse micelles formed by AOT 100 m*M* in isooctane and by TDAB 100 m*M* in chloroform:isooctane 1:1 v/v. In the forward transfers, the lysate solutions (40 mL) and the AOT or TDAB micellar solutions (40 mL) were gently stirred at 20 ± 1°C. The extraction was carried out at fixed Wo or by consecutively changing the micellar phase. In this case, the micellar solution in contact with the same water phase, was repeatedly substituted every 2 h (starting from Wo 3 and Wo 10 for the TDAB and AOT system, respectively) with freshly prepared organic solutions at increasing Wo value. The concentration of the proteins in the micellar and aqueous phase was controlled by UV spectrophotometry (absorbance at 280 mm) or by the method of Bradford *(26)*. Backward transfers from the TDAB micelles into water (1:1 v/v) were usually carried out in the presence of borate buffer solution 50 m*M*, pH 9, KI 1*M* (20 mL). In both phases, acylase activity was assayed as described below by using glutaryl-7-ADCA as substrate (*See* activity test).

Tetradecyltrimethylammonium bromide (TDAB) 100 m*M* was used as cationic surfactant to prepare reverse micelles in chloroform:isooctane 1:1 v/v instead of the more frequently used hexadecylammonium bromide (CTAB) 50 m*M*. CTAB reverse micelles did not withstand long-term (several hours) contact with an aqueous phase (1:1 volume) without development of turbidity and phase mixing. These effects were strongly dependent on Wo and temperature. No significant improvement was observed in the presence of salt (other than borate) in the water phase, such

as KI, KBr, phosphate, or NaCl. Conversely, TDAB micellar solutions were stable (from Wo 3 to Wo 20) after several hours of contact with the water phase at 20°C in the presence of KI or KBr. These salts may decrease the solubility of the surfactant in the aqueous phase, as it is the case for AOT *(20)*. Therefore, all the experiments with TDAB were performed in the presence of 30 m*M* KI. For the experiments at pH 6.5, a solution of KH_2IO_4 (50 m*M*) was used as buffer.

Activity Tests

Gl-7-ACA acylase activity was assayed by following the formation of the hydrolysis product, 7-ADCA, with the method of Balasinghan *(21)*. The final substrate concentration of the assay mixture was 2 m*M*. One-half milliliter of the micellar or aqueous reaction solution were added to 2 mL of a 2:1 (v/v) mixture of acetic acid (20% v/v in water) and 0.05*M* NaOH. Then, 0.5 mL of *p*-dimethylaminobenzaldehyde (PDAB) 0.5% w/v in methanol was added to the solution. The absorbance of the transparent aqueous phase was measured at 415 nm. No significant interference caused by AOT was observed, whereas with the TDAB micellar system, the method could not be applied because of the interfering presence of chloroform and TDAB. The units of acylase activity, U, were calculated by the equation: $U/mL = (\Delta A_{415})/(0.4xt)$, where ΔA_{415} is the difference in the absorbance at 415 nm observed after *t* (reaction time) and 0.4 is the extinction coefficient at 415 nm of the yellow-colored Schiff base formed between PDAB and the free amino group of 7-ADCA. One unit of gl-7-ACA acylase activity is the quantity of enzyme that produces 1 μmol of 7-ADCA per min at 37°C pH 7.5. Glutaryl-7-ADCA was used as substrate instead of glutaryl-7-ACA because the hydrolysis of the ester in position 3 of the β-lactam moiety by an esterase eventually present in the lysate would have interfered with the quantitative analysis of the reaction.

Unspecific acylase activity of the lysate solution was assayed with glutaryl-*p*-nitroanilide as model substrate. One hundred microliters of solution was mixed with 10 μL of a stock solution of glutaryl-*p*-nitroanilide (20 mg/mL) and 1 mL of borate buffer 50 m*M* pH 8. The reaction mixture was incubated at 37°C and the absorbance of the hydrolysis product, *p*-nitroaniline, was measured at 386 nm (extinction coefficient $13{,}000M^{-1}$ cm^{-1}) *(22)*.

Glutaryl-7-ACA Acylase Activity Measurements in AOT Reverse Micelles

A preparation of pure glutaryl-7-ACA acylase (gℓ-acylase) was used. The protein was purified as described elsewhere *(23)*. A concentrated protein solution (20 mg/mL) was exhaustively dialyzed against tris buffer 30 m*M*, pH 7.5, and then injected directly into dry micelles formed by AOT 200 m*M*

in isooctane. Two independent micellar solutions containing the enzyme and the substrate (at different concentrations), respectively, were prepared at a given Wo. After few minutes of temperature equilibration, the reaction was started by mixing the two micellar solutions (1:1 volume ratio). The same enzyme micellar solution was used to prepare all the samples at a given W_o in order to minimize the difference in enzyme concentration. Below W_o the solubility of the protein decreased significantly. The reaction was carried out at 37°C for 20–30 min. Control experiments showed that no solubilization of gl-7-ADCA occurred in isooctane or isooctane/chloroform in the absence of surfactants. Because of its polar character, after solubilization the substrate can be considered entirely confined inside the micelles. Curve fitting and kinetic analysis of the activity data in aqueous and micellar solutions were performed with the program ENZFITTER (Biosoft, UK) according to Michaelis-Mentel model of enzyme kinetics.

Isoelectric Focusing

Isoelectric focusing experiments were performed with a flat-bed unit FBE 3000 (Pharmacia, Uppsala, Sweden) by using Anpholine PAG plates (polyacrylamide gels pre-cast on polymer support film from Pharmacia). The pH range of the gels was 3.5–9.5. The protein was focused at 30 W for 1.5 h (1300 V). Phosphoric acid and sodium hydroxide 1*M* were used as anode and cathode solutions, respectively. Gels were stained with Coomassie Blue R250. The pI of the samples were calculated by comparing their migration distances from the cathodic edge of the gel with those of the pI markers.

RESULTS AND DISCUSSION

TDAB Micellar System

Gl-7-ACA acylase is a tetrameric enzyme whose activity is pH independent between pH 6.0 and pH 10.0 *(24)*. Below pH 6, the enzyme looses progressively its activity, probably because it dissociates into two inactive dimers *(23)*. Consequently, the extraction experiments were limited in the pH range above pH 6.5. Isoelectric focusing experiments on the purified protein showed the presence of two bands, corresponding to the isoelectric point, pI, of 4.9 and 4.4, respectively, probably associated with the two different subunits which form the dimer. This suggests that at pH values above pH 6.5, where the enzyme is fully active, the macromolecule bears a net negative charge. Therefore, the extraction was first carried out with the micellar system formed by TDAB, a cationic surfactant.

Liquid-liquid extraction experiments were performed by changing the organic phase (TDAB 100 m*M*) step by step, and keeping the same aqueous solution. The amount of water present in the organic phase was progressively increased at each step starting from Wo 4. The purpose was

Table 1
Two-Phases Extraction with TDAB Reverse Micelles[a]

Wo	gl-acylase activity mU/ml (%)		
	pH 6.5	pH 8	pH 9
aqueous solution*	7.2	4.5	8.8
4	3.5(48)	4.4(97)	7.0(79)
10	2.5(34)	0.8(18)	4.4(50)
15	0.14(2)	0.9(20)	0.9(10)
20	0	0	0.15

*pH 6.5, potassium iodate buffer 50 m*M*, KI 30 m*M*.
pH 8 and pH 9, potassium borate buffer 50 m*M*, KI 30 m*M*.
[a]Aqueous phase activities after consecutive extractions with micellar phases at increasing Wo as a function of pH.

to extract quantitatively the gl-7-ACA acylase and, possibly, to determine the most efficient Wo range. The lysate in borate buffer 50 m*M*, KI 30 m*M*, and iodate buffer 50 m*M*, KI 30 m*M* were used as aqueous phase at pH 8.0, 9.0 and pH 6.5, respectively. The results are summarized in Table 1. As it can be seen, the gl-7-ACA acylase remaining in the water phase steadily decreased at each extraction step, suggesting an apparent quantitative solubilization of the enzyme in the organic micellar phase. At Wo 10, more than 65% of the activity (average value among the pHs) was already solubilized. It should be noted that the relative amount of enzyme extracted after three steps is roughly independent on pH.

Is it possible to reverse the solubilization process (from water to micelles) and transfer back the enzyme into an aqueous phase? Is the micelles-mediated double-transfer useful to enhance the enzyme specific activity? In order to answer these questions, back extraction from micelles to water was studied as a function of the organic-phase initial water content. After several trials (at different concentrations of KI or NaCl and buffer), the best composition of the second aqueous phase was borate 50 m*M*, pH 8, KI 1*M*. The results are summarized in Table 2, where the activity and the protein content present in the initial and final (after back-transfer) aqueous solutions, respectively, are listed as a function of Wo. Each Wo value was controlled independently in order to optimize the transfer process. G1-7-ACA acylase was only partially extracted into the micellar phase at each Wo. The largest transfer was around Wo 5–10. From micelles back to water, the transfer had a maximum around Wo 5 (12%). Some of the proteins were also solubilized into the micelles (average value over Wo, about 15%), but only part of them were released into a new aqueous phase in these conditions (KI 1 *M*, pH 8). Nevertheless,

Table 2
Two-Phases Extraction with TDAB Reverse Micelles at Different Wo and Back-Transfer to Aqueous Phase

Wo	%gl-acylase activity		% protein concentration		acylase specific activity mU/mg[e]
	residual[a]	back-transfer[b]	residual[c]	back-transfer[d]	
-	-	-	-	-	3.2[f]
3	71.	1.5	85.	4.1	1.1
5	60.	12.0	83.	6.0	6.5
10	62.	7.1	86.	3.0	0.97
15	82.	4.1	84.	2.1	0.82
20	90.	0.	98.	0.7	-

[a]Activity remaining in the initial aqueous phase, borate buffer 50 m*M*, KI 30 m*M* pH 9. Initial activity: 0.011 U/mL.

[b]Back-trasfer from the micellar phase with buffer solution (1:1 v/v borate 50 m*M*, KI 1*M*, pH 8) for 2 h at 25°C. Calculated from $O.D._{280}$ of the micellar phase.

[c]Proteins remaining in the initial aqueous phase. Initial protein concentration: 3.37 mg/mL.

[d]Proteins after back-transfer from micelles into water (1:1 v/v borate buffer 50 m*M*, pH 8, KI 1*M*, 2 h at 25°C). Percentage calculated from the absorbance at 280 mm.

[e]Specific activity in the aqueous solution after back-transfer.

[f]Initial specific activity.

the specific activity of the gl-7-ACA acylase recovered from the micellar phase at Wo 5 was doubled with respect to that present in the initial aqueous solution.

The enzyme recovery after the back-transfer process did not fully account for the decrease of the acylase activity in the initial aqueous solution. This observation, as long as the results of the step-by-step continuous extraction experiments (*see* Table 1), where almost no residual activity was found in the aqueous solution after four-steps extraction, suggests that part of the acylase activity is irreversibly loss during the double-transfer cycle.

AOT Micellar System

Since the pI of gl-7-ACA acylase is in the acid range (pI 4.9), solubilization by AOT reverse micelles, an anionic surfactant, should be unfavorable at pH above 4.9. In general, proteins are not solubilized by AOT at pH above their pI, although other factors (i.e., the molecular dimensions), beside the electrostatic ones, contribute to the free energy associated with the transfer process (*5*). Therefore, extraction conditions were chosen where most of the proteins but gl-7-ACA acylase could be extracted. The purpose was to improve the purity of the crude lysate acylase preparation by removal of the maximal amount of contaminating proteins.

Table 3
Two-Phases Extraction by Consecutive Changes of the Micellar Phase at Different Wo (volume ratio 1:1)[b]

Wo	gl-acylase activity (mU/ml)	protein concentration (mg/ml)	gl-acylase specific activity (mU/mg)	acylase activity[a] (U/ml)
-	5.3	1.4	3.78	1.8
10	4.7	1.0	4.7	1.8
20	4.3	-	-	1.7
30	4.0	0.4	10.	1.5
40	3.7	-	-	0.91
50	3.6	0.15	24.0	0.7

[a]Unspecific acylase activity on glutaryl-*p*-nitroanilide.

[b]Aqueous phase: cellular lysate in borate buffer 50 m*M* pH8.0; micellar phase: AOT 100 m*M* containing borate 50 m*M*, pH 8.0.

The extraction experiments consisted on monitoring the protein uptake from the same lysate solution into the AOT organic phase at pH 8.0. The micellar phase was consecutively changed stepwise (1:1 v/v). At each step, Wo was progressively increased. As it is shown in Table 3, after four consecutive extraction steps at increasing Wo, 68% of the initial gl-7-ACA acylase activity was retained in the aqueous phase (column 2) and was not extracted. Conversely, the total protein concentration decreased of about 90% (column 3), most of the proteins being solubilized by the micellar phase. Consequently, gl-7-ACA acylase specific activity increases more than six-fold. No gl-7-ACA acylase activity was detected in the micellar phase at any purification step. Nevertheless, at the end of the stepwise purification procedure, part of the enzymatic activity was lost (32%). This may be a result of the irreversible enzyme inactivation at the liquid-liquid interface. Trace amount of AOT dissolved in the water phase did not have any consequence on the enzyme activity. Control experiments showed that the concentration of AOT in aqueous solution can be raised from 0.001 m*M* up to 0.1 m*M* (at pH 7 and pH 8, 30°C) without any change of the gl-7-ACA acylase activity.

The selectivity of the method towards gl-7-ACA acylase may be tested by following the activity of other enzymes. For example, glutaryl-*p*-nitroanilide is a substrate not only of gl-7-ACA acylase, but also of other unspecific acylases or proteases present in the cellular lysate. Unlike gl-7-ACA acylase, more than 60% of the enzymatic activity on glutaryl-*p*-nitroanilide was extracted (Table 3, column 5) into the micellar solutions up to Wo 50.

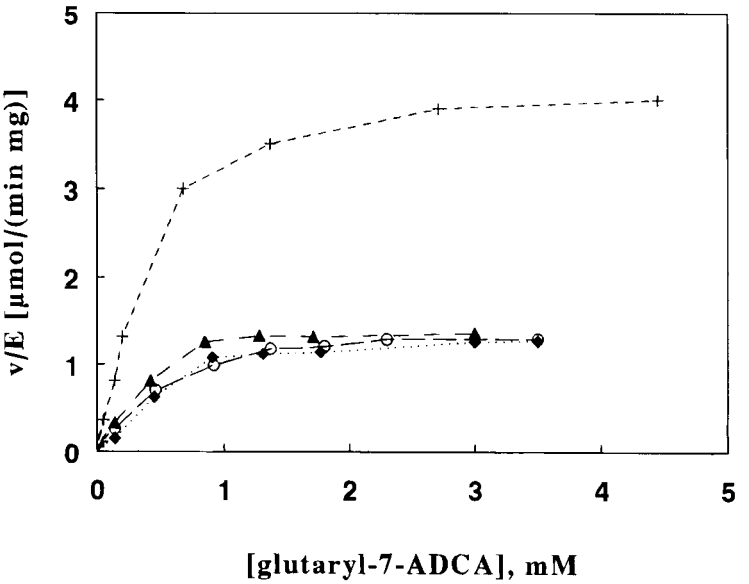

Fig. 1. Glutaryl-7-ACA acylase activity in aqueous and micellar solution. Normalized velocity (E = enzyme concentration, 0.05–0.08 mg/mL, final concentration) as a function of substrate concentration. Substrate: glutaryl-7-ADCA. (+) borate buffer 50 m*M*, pH 7.5; the same buffer solubilized in AOT 100 m*M* in isooctane at Wo 15 (○), Wo 22.5 (◆) and Wo 30 (▲).

Although extraction of gl-7-ACA acylase in the liquid-liquid two phases system did not occur in the pH range where the enzyme is active, solubilization of gl-7-ACA acylase inside the AOT micellar system could be achieved when pure micellar solutions were used. After injection of a small amount of a protein aqueous solution into the organic phase, solubilization readily occurred, provided that the water content was high enough (> Wo 7) to create a suitable micellar water pool. In this way it was possible to investigate whether the apparent loss of activity during the extraction experiments were caused by the AOT denaturating action at interface or/and by the change of enzyme intrinsic properties inside the micellar core (if indeed partial solubilization occurred).

The activity of gl-7-ACA acylase solubilized in the AOT reverse micelles was, therefore, studied by using a highly purified enzyme preparation. After solubilization inside the micellar water pool, the enzyme retained its catalytic activity. In Fig. 1 the rate of hydrolysis of glutaryl-7-ADCA is plotted as a function of substrate concentration at different Wo (AOT 200 m*M*, borate buffer solution 30 m*M* pH 7.5 as aqueous microphase inside the micelles). The kinetic parameters calculated from the curves are listed in Table 4. In the whole Wo range studied, the apparent rate constant, K_{cat}, is lower than in water at any Wo. Moreover, the enzyme looses its catalytic efficiency, i.e. K_{cat}/K_M, with respect to water.

Table 4
Kinetic Parameters of the Hydrolysis of Glutaryl-7-ADCA by Glutaryl-7-ACA Acylase in Aqueous Solution and in the Reverse Micelles Formed by AOT 200 m*M* in Isooctane at 37°C

conditions	V_{max} μmol/min mg	k_{cat} sec^{-1}	K_M $mol^{-1} x10^{-4}$	k_{cat}/K_M mol/sec x 10^4
tris 30 mM pH 7.5	4.41	37.	3.71	9.9
Wo 15.	1.6	13.4	5.86	2.28
Wo 22.5	1.61	14.	6.20	2.2
Wo 30.	1.79	15.	6.40	2.34

The rate constant marginally depends on the amount of water present in the micellar system and, consequently, on the size of the water pool, at least between Wo 15 and Wo 30. Although the exact dimensions of gl-7-ACA acylase molecule (MW 140,000 Daltons) are not known, by analogy based on the molecular weights, its molecular volume should be close to that of yeast alcohol dehydrogenase (YADH, MW 150.000 Daltons). Since the volume of YADH (molecular radius: 36Å) is matched by the size of the AOT micelle water pool at Wo 22–23 *(25)*, it is reasonable to assume that the size of the gl-7-ACA acylase molecule would be equal to the water pool volume at slightly smaller Wo. The values of V_{max} and K_M are in good agreement with the literature values (4.66 μmol min^{-1}, mg^{-1}, and 0.1 $mmol^{-1}$, respectively, pH 7.0, 37°C) *(24)*.

In conclusion, the extraction of gl-7-ACA acylase by reverse micelles was studied by using the crude ammonium sulfate precipitate (70% saturation) as starting material. The lysate is a complex mixture including, besides proteins, several soluble cellular components, such as oligosaccharides, some nucleic acids and glycoconjugates. With the AOT micellar system, a sixfold purification factor and an overall 65% retention of activity was achieved within few hours. On the other hand, selective extraction of gl-7-ACA acylase from the lysate solution was achieved with the reverse micelles formed by the cationic TDAB followed by backward extraction into a second water phase. The purification factor was two and the overall yield was only 12%. In this case the efficiency of the process was not very satisfactory, but in principle it can be still applied as a separation technique.

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