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Reverse Micellar Extraction of β-Galactosidase from Barley (*Hordeum vulgare*)

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Abstract The reverse micellar system of sodium bis(2-ethylhexyl)sulfosuccinate (AOT)/isooctane was used for the extraction and primary purification of β-galactosidase (EC 3.2.1.23) from the aqueous extract of barley (*Hordeum vulgare*) for the first time. The process parameters such as the concentration of the surfactant, the volume of the sample injected, and its protein concentration, pH, and ionic strength of the initial aqueous phase for forward extraction, buffer pH, and salt concentration for back extraction are varied to optimize the extraction efficiency. Studies carried out with both phase transfer and injection mode of reverse micellar extraction confirmed the injection mode to be more suitable for β-galactosidase extraction. The extent of reverse micellar solubilization of proteins increased with an increase in protein concentration of the feed sample. However, back extraction efficiency remained almost constant (13–14.4%), which indicates the selectivity of AOT reverse micelles for a particular protein under given experimental conditions. β-Galactosidase was extracted with an activity recovery of 98.74% and a degree of purification of 7.2-fold.

Keywords Reverse micellar system · Barley · β-Galactosidase · Phase transfer · Injection mode

Introduction

The milk sugar lactose is hydrolyzed to its components D-glucose and D-galactose by β-galactosidase. It is present in microorganisms, plants, and animals [1]. In this respect, the source of the enzyme is of critical importance, as it is one of the factors in addition to downstream processing methods that decide the viability of the commercial production. Barley seems to be an inexpensive source of β-galactosidase whose ready availability is an additional advantage. Among the seeds of a number of different plants examined, barley

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has the highest hydrolytic capacity when lactose was used as a substrate [2]. Its application in the food industry includes the production of products such as sweet syrups, ice creams, dairy desserts, and confectionery of low lactose content and/or increased sweetness [3]. Many of the plant β-galactosidase including barley was isolated and purified using a series of column chromatographic techniques such as diethylaminoethyl–blue sepharose–carboxymethyl cellulose–sephadex G100 chromatography and ammonium sulfate precipitation followed by affinity chromatography on lactosyl sepharose [2, 4–7].

Reverse micellar extraction (RME) is an attractive liquid–liquid extraction technique for downstream processing of biomolecules. Reverse micelles provide mild separation conditions for enzyme recovery in an active form [8–13]. By appropriate manipulation of process parameters such as solution pH, ionic strength, and phase volume ratio, it is possible to obtain the purification and concentration of the desired enzyme in a single step [14]. Proteins are solubilized into reverse micelles by various modes of RME such as phase transfer, injection, or powder modes. The extraction of proteins into reverse micelles is controlled by steric, electrostatic, and hydrophobic interactions between proteins and micelles [15]. Generally, large monomeric and oligomeric proteins are rather difficult to be extracted into reverse micelles. Such proteins are likely to be excluded by steric interactions with micelles or irreversibly denatured in the reverse micellar system due to electrostatic interaction [16, 17]. Hydrophilic bulky proteins with larger molecular weights can be easily solubilized into a microwater pool of sodium bis(2-ethylhexyl)sulfosuccinate (AOT) reverse micelles by the injection mode [18]. The injection mode takes a low time for extraction and ensures a high enzyme activity. However, a very few studies on extraction have been made using the injection mode for the solubilization and separation of proteins by reverse micelles [17, 19]. In the present study, the effect of various process parameters on both forward and back extractions of β-galactosidase by the injection mode of RME was investigated to optimize the extraction efficiency of the AOT/iso-octane reverse micellar system.

Materials and Methods

Materials

Enzyme Source

Barley used in the present study was purchased from the local market.

Chemicals

AOT was from BDH Laboratory Supplies (Poole, England), and iso-octane (high-performance liquid chromatography grade) was purchased from Merck (Mumbai, India). *n*-Hexane (extrapure, AR), *o*-nitrophenyl-β-galactopyranoside, and *o*-nirophenol were purchased from SRL (Mumbai, India). All other chemicals used for the experiments and analyses were of analytical grade. All the chemicals were used without further purification.

Enzyme Extract

Fifty grams of barley was soaked in 300 ml of 0.1 M, pH 6.0 ammonium acetate buffer for 5 h, and it was homogenized in a blender for 10 min. The resulting slurry was filtered

through a double layer of cheesecloth, and the filtrate was centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatant obtained was subjected to precipitation at 60% ammonium sulfate saturation. The precipitate was dissolved in 0.1 M, pH 5.0 ammonium acetate buffer and was extensively dialyzed against 10 mM, pH 8.0 Tris–HCl buffer at 6–8 °C for 24 h with mild agitation on a magnetic stirrer. The dialysate (2–4 mg/ml protein concentration) thus obtained was used for further experiments.

Reverse Micellar Extraction

Forward extraction was carried out by slowly injecting a known (400–1,000 μl) volume of dialysate into 10 ml of the AOT/iso-octane solution and by stirring the solution until a clear phase was obtained (10 min). For phase transfer experiments, 10 ml of dialysate was mixed with 10 ml of the organic phase (30 min). Back extraction of β-galactosidase from reverse micelles was carried out by contacting 10 ml of the clear reverse micellar solution with 5 ml of the buffer of known pH and salt concentration for 30 min. Then, the solution was centrifuged at 2000×*g* for 5 min for the separation of the phases. The aqueous phase was collected, and aliquots of this were analyzed for enzyme activity and protein content. All the experiments were carried out at 25±2 °C, in duplicate, and an average value is reported.

Enzyme Assay

β-Galactosidase was assayed in the presence of 0.1 M sodium citrate buffer, pH 4.0, using *o*-nitrophenyl-β-galactopyranoside (6 mM) as the substrate. The absorbance of *o*-nitrophenol formed by the enzyme reaction was measured in an alkaline medium at 410 nm using a spectrophotometer (Shimadzu UV-160A, Japan). One unit of β-galactosidase activity is defined as the amount of enzyme that hydrolyzes 1 nmol of substrate per minute under the standard conditions of 37 °C and pH 4.0 for 15 min. Activity recovery and degree of purification are calculated using Eqs. 1 and 2, respectively.

$$\text{Activity recovery (\%)} = \frac{(\text{Activity of } \beta-\text{galactosidase after back extraction} \times \text{total volume})}{(\text{Activity of } \beta-\text{galactosidase in feed} \times \text{volume of feed injected})} \times 100 \quad (1)$$

$$\text{Degree of purification (fold)} = \frac{(\text{Specific activity of } \beta-\text{galactosidase after back extraction})}{(\text{Specific activity of } \beta-\text{galactosidase in feed})} \quad (2)$$

Protein Content

Protein content in feed and extracted aqueous phase was determined by measuring the absorbance at 280 nm using bovine serum albumin as the standard. The sample analyses were performed against respective blank solutions. Protein concentration readings were

taken in triplicate, and an average value was used for the calculation of extraction efficiency.

$$\text{Back extraction efficiency (\%)} = \frac{(\text{Protein concentration in aqueous phase} \times \text{total volume})}{(\text{Protein concentration in feed} \times \text{volume of feed injected})} \times 100 \quad (3)$$

Water content (W_0)

Water content in the organic phase after forward extraction was measured by a Karl–Fischer coulometer (Metler Toledo DL32, Germany). Water content (W_0) is defined as the molar ratio of solubilized water to the surfactant.

Results and Discussion

In the present study, β-galactosidase (75 kDa oligomeric protein with subunits of 42 and 33 kDa) solubilization by both phase transfer and injection modes of RME was attempted. Forward extraction of β-galactosidase by phase transfer mode was carried out at various pH values (3–6.5), 200 mM AOT, and a salt concentration of 0.1 M NaCl. The resulted aqueous phase after forward extraction was turbid with the precipitate at the interface, while the organic phase was clear. The back extraction was carried out with pH 8.0 Tris buffer containing 0.1 M KCl. The activity recovery obtained was in the range of 12–33%. The reason for lower recovery may be due to the inactivation and precipitation of the enzyme. Large-molecular-weight oligomeric proteins are difficult to extract by phase transfer mode, and back-extracted enzymes were strongly inactivated due to the change in surface hydrophobicity of proteins and strong electrostatic interactions [15].

Protein transfer from an aqueous phase into a reversed micellar phase has been shown to depend on the composition of both the phases and properties of the protein under investigation. Since the recovery was lower with the phase transfer mode of RME, the injection mode was used for further study of β-galactosidase extraction. Forward and back extraction conditions were varied to maximize the activity recovery and degree of purification.

Effect of Feed pH

The effect of feed pH was studied on activity recovery, back extraction efficiency, and degree of purification of β-galactosidase at 200 mM AOT and 800 μl feed volume. The feed pH determines the protein net charge. When pH was increased from 4.0 to 7.5, the activity recovery and degree of purification increased, whereas the back extraction efficiency decreased as shown in Fig. 1. This could be attributed to the decrease in the net charge on other proteins with an increase in feed pH. On the other hand, β-galactosidase extraction increased with an increase in pH. In particular, the extraction at feed pH lower than p*I* (4.95–5.7) resulted in interface precipitation. This may be due to the denaturation of the enzyme by electrostatic interaction with AOT. Hence, in further studies, forward extraction was carried out above p*I* of β-galactosidase. Though the extraction behavior of oligomeric proteins is complicated, very strong electrostatic interaction between

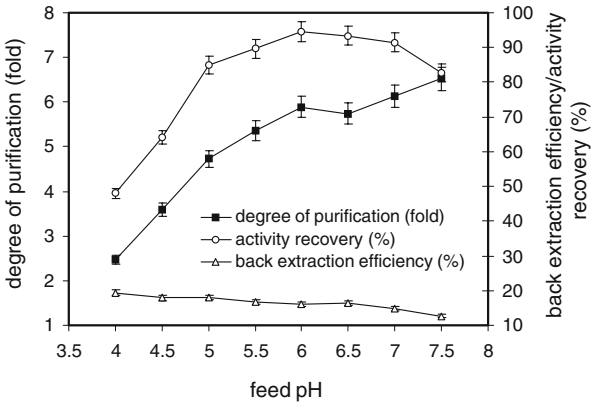

Fig. 1 Effect of feed pH on activity recovery, back extraction efficiency, and degree of purification. Forward extraction: feed volume 800 μl, 200 mM AOT. Back extraction: 0.1 M KCl, 10 mM, pH 8.0 Tris buffer

the proteins and AOT is undesirable for effective extraction. It causes a structural change of proteins and aggregate formation in the extraction process [20].

Effect of Feed Volume Injected

The solubilization of β-galactosidase in the AOT/iso-octane reversed micellar system was studied as a function of feed volume injected at 200 mM AOT concentration and feed pH 7.0. As the feed volume increased from 400 to 1000 μl, W_0 increased from 10.01 to 20.52. The reverse micellar phase obtained was transparent in all the feed volume tested. Above 1,000 μl of feed volume, the reverse micellar phase became turbid indicating excess water than that could be solubilized by reverse micelles under the given experimental conditions. As the feed volume increased, both activity recovery and degree of purification decreased as shown in Table 1. This may be due to a lower effective micelle concentration (concentration of micelles larger than the protein size) compared to the concentration of protein to be solubilized in the micelles at a higher injection volume of the feed. This will cause protein denaturation due to the steric exclusion of the excess protein from micelles into the organic solvent [21, 22].

Effect of Feed Protein Concentration

The effect of feed protein concentration was studied at constant feed volume of 800 μl (W_0= 17.26) and 200 mM AOT concentration. As the feed protein concentration increased, the

Table 1 Effect of feed volume injected on water content (W_0), activity recovery, and degree of purification.

Forward extraction: 200 mM AOT, feed pH 7.0, protein concentration 2.19 mg/ml, and activity 199.98 U/ml. Back extraction: 0.15 M KCl, 10 mM, pH 8.0 Tris buffer, protein concentration 0.031–0.106 mg/ml

Feed volume injected (μl)	Water content	Activity ecovery (%)	Degree of urification (fold)
400	10.01	92.21	5.19
500	11.77	92.31	4.42
600	13.06	88.36	3.85
700	15.39	85.06	3.27
800	17.26	83.44	3.10
900	19.6	81.41	2.99
1,000	20.52	76.71	3.18

extraction of proteins increased (Fig. 2), but back extraction efficiency remained almost constant (13–14.4%). β-Galactosidase activity recovery also remained almost constant (89–92%). This indicates the selectivity of AOT reverse micelles for particular protein under given experimental conditions.

Effect of Surfactant Concentration

Surfactant concentration was varied at a constant feed volume of 500 μl. The AOT concentration of 50 mM gave a cloudy micellar phase, so W_0 was not determined. At a concentration range of 75–200 mM AOT studied, a clear transparent phase was obtained. This indicates that whatever feed volume was injected, it is solubilized in the reverse micelles at the given surfactant concentration range. Since surfactant concentration was increased at a constant feed volume, the water content decreased (17.47 to 11.71), and back extraction efficiency also decreased, whereas activity recovery remained almost constant as shown in Table 2. This shows that the concentration of effective reverse micelles/water content (reverse micellar size) is sufficient for β-galactosidase extraction in the AOT concentration range studied. In the case of AOT, the properties of the solubilized water depend upon W_0. Below a W_0 of 10, the water pool water has properties that are substantially different from that of normal water. Conformational change and/or activity decrease in the biomolecule will mainly occur in this low-water content region [23].

Effect of Solvent

The solubilization of water in AOT reverse micelles depends on the solvent used for micelle formation. In the present study, iso-octane and *n*-hexane was used at various feed volumes. The activity recovery in the iso-octane/AOT system was higher compared to the *n*-hexane/AOT system at all the feed volumes injected as shown in Fig. 3. This may be due to a higher water content of the iso-octane system compared to the hexane system. At 400 μl feed volume with the hexane system, the activity recovery drastically decreased, and this may be due to low $W_0<10$. The solubility of water depends on the penetrating power of the solvent into the surfactant interface in reverse micelles. As the penetrating power of solvent increases, W_0 also increases. If there are no specific solvent–surfactant head group

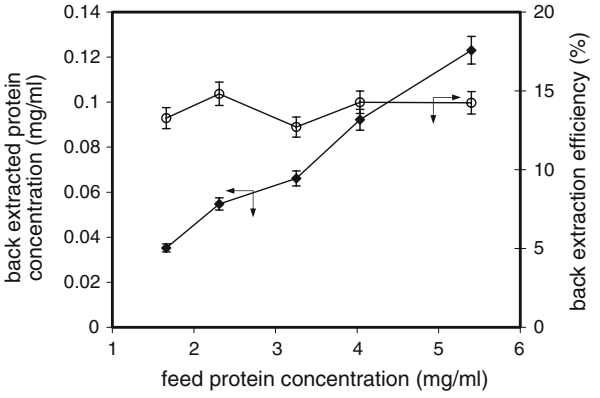

Fig. 2 Effect of feed protein concentration on back extraction efficiency. Forward extraction: feed volume 800 μl, 200 mM AOT, feed pH 7.0. Back extraction: 0.1 M KCl, 10 mM, pH 8.0 Tris buffer

Table 2 Effect of surfactant concentration on water content (W_0), back extraction efficiency, and activity recovery.

Forward extraction: feed volume 500 μl, feed pH 7.0, protein concentration 2.27 mg/ml, and activity 199.43 U/ml. Back extraction: 0.1 M KCl, 10 mM, pH 7.5 Tris buffer

Surfactant concentration (mM)	Water content	Back extraction efficiency (%)	Activity recovery (%)
75	17.47	18.39	90.49
100	17.75	16.96	89.12
125	15.48	15.89	89.81
150	14.58	14.10	89.11
175	12.02	13.03	88.43
200	11.71	12.67	88.43

interactions, the penetration will depend on the molecular similarity of the solvent and surfactant tails [24].

Effect of Salt Concentration

During forward extraction in the injection mode of RME, salt is not added so as to maintain higher water content within the reverse micelles. During back extraction, potassium chloride was added at different concentrations (0.1–1.0 M). Back extraction efficiency increased with an increase in salt concentration because as salt concentration increases, reverse micelles size decreases and proteins are easily expelled out of the micelles. However, the maximum activity recovery and degree of purification were obtained at 0.1 M KCl as shown in Fig. 4. At a higher salt concentration, the activity recovery decreased drastically, and this may be due to the denaturation of β-galactosidase at a higher ionic strength. Large-molecular-weight proteins are likely to be denatured by association with the ionic surfactant mediated by salt, since high salt concentration increases the surface hydrophobicity of enzymes [25].

Effect of Buffer pH

Effect of buffer pH (used for back extraction) on activity recovery and the degree of purification was studied at 0.1 M KCl concentration. pH was varied from 6.0 to 8.0, which is above the iso-electric point, so as to have a repulsive interaction between AOT and β-galactosidase. This helps in the easy recovery of enzyme from reverse micelles. At pH 6.0,

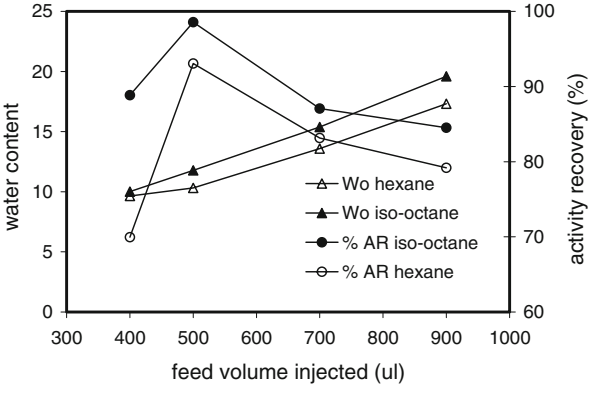

Fig. 3 Effect of different solvent on water content and activity recovery. W_0 Water content, *AR* activity recovery. Forward extraction: 200 mM AOT, feed pH 7.0. Back extraction: 0.1 M KCl, 10 mM, pH 8.0 Tris buffer

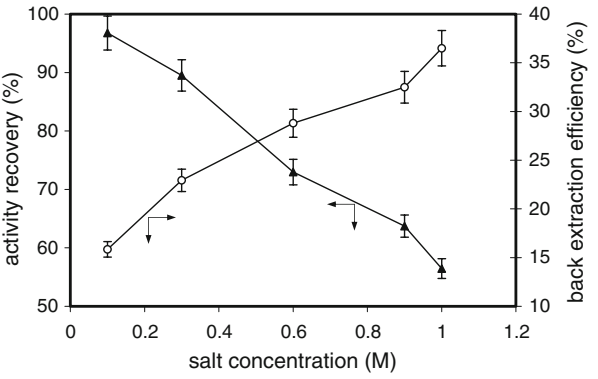

Fig. 4 Effect of salt concentration on back extraction efficiency and activity recovery. Forward extraction: 200 mM AOT, feed pH 7.0, feed volume injected 500 μl. Back extraction: 10 mM, pH 8.0 Tris buffer

almost all the β-galactosidase was extracted into the buffer with an activity recovery of 98.74% and a degree of purification of 7.21-fold. As the buffer pH increased, the activity recovery remained almost constant up to pH 7.5 and then slightly decreased at pH 8.0, whereas the degree of purification decreased drastically as shown in Fig. 5. This may be due to the extraction of contaminant proteins whose p*I* may be higher than that of β-galactosidase by electrostatic repulsion with AOT.

SDS-PAGE Analysis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli [26], and the gel was stained using Coomassie brilliant blue R-250. The β-galactosidase extracted using the AOT reverse micellar system was dialyzed overnight at 4 °C and lyophilized. The concentrated sample (1 mg/ml with an activity of 154.7 U/ml) was loaded to 12% gel along with feed and marker protein. The SDS lane pattern of the RME sample showed sharp 45-kDa and diffused 33-kDa bands corresponding to a dimeric nature [2, 7, 27] of barley β-galactosidase (Fig. 6).

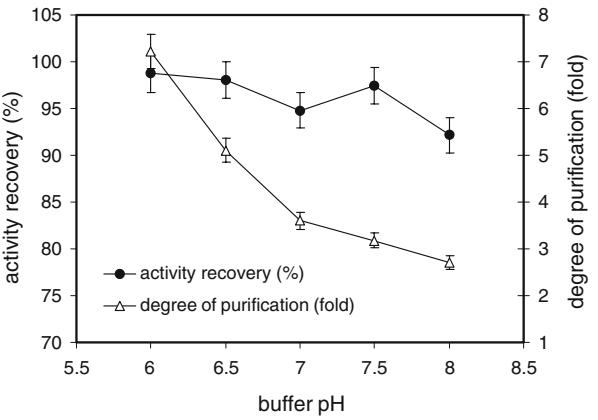

Fig. 5 Effect of buffer pH on activity recovery and degree of purification. Forward extraction: 200 mM AOT, feed pH 5.5, feed volume injected 500 μl. Back extraction: 10 mM buffer (pH 6.0–7.0 phosphate buffer, pH 7.5 and 8.0 Tris buffer), 0.1 M KCl

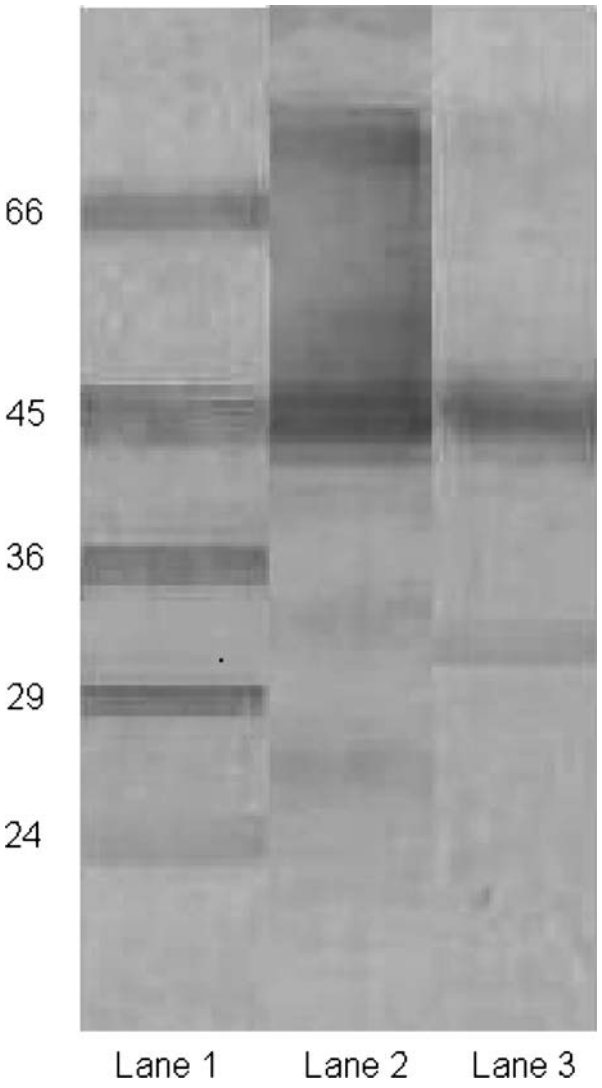

Fig. 6 SDS-PAGE pattern of RME sample. *Lane 1*: marker, *lane 2*: crude sample, *lane 3*: RME sample

Conclusions

β-Galactosidase was solubilized into AOT reverse micelles by the injection mode of RME and back extracted selectively with an activity recovery of 98.74% and a degree of purification of 7.21-fold. Both forward and back extractions carried out above the isoelectric point have given good extraction results in terms of activity recovery and degree of purification because bulky oligomeric proteins are easily denatured by electrostatic interaction with AOT below the isoelectric point. The reverse micellar extraction of β-galactosidase was found to depend on feed pH, water content, buffer pH, and salt concentration used. By controlling these parameters effective separation and purification of β-galactosidase from other contaminant proteins can be achieved in a single step. The present findings indicated that reverse micellar extraction could be used as an efficient primary purification step for the recovery of β-galactosidase.

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