

Isolation of a recombinant intracellular β -galactosidase by ammonium sulfate fractionation of cell homogenates

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Abstract

The *Escherichia coli* β -galactosidase (EC 3.2.1.23) expressed intracellularly as soluble, biologically active enzyme in the yeast *Saccharomyces cerevisiae* was recovered from clarified homogenates of the yeast cells by precipitation with crystalline ammonium sulfate. Effects of salt saturation (0–80%) of the homogenate, the initial total protein level (2–20 g·L⁻¹) and the processing pH (6–8) on the enzyme and protein recovery were investigated. As the ammonium sulfate concentration increased, the enzyme was precipitated preferentially and at 30% salt saturation nearly all had been recovered in the precipitate. In contrast, at this salt concentration only 25% of the total protein had precipitated. Preferential precipitation of β -galactosidase was associated with the hydrophobic nature of this large protein. Complete precipitation of the total protein required salt concentrations exceeding 70% of saturation. The salt concentration needed for complete recovery of the enzyme was not sensitive to the processing pH. Over the salt saturation level of 20–50%, the enzyme precipitation followed the Cohn equation. The salting-out constant was strongly affected by the initial protein level in the homogenate; higher values were observed at lower protein concentrations. The salting-out constant was unaffected by the processing pH; however, the Cohn parameter **B** was pH dependent in addition to being affected by the initial protein concentration. Within the β -galactosidase stability range of pH 6–8, pH variations alone (no added salt) proved ineffective in precipitating the enzyme.

Nomenclature

B	constant in equation (1), units·mL ⁻¹
c_i	concentration of the <i>i</i> th ion, mol·L ⁻¹
E	enzyme activity, units·mL ⁻¹
E_0	initial enzyme activity, units·mL ⁻¹
I	ionic strength, mol·L ⁻¹
K_s	salting-out constant, units·mol ⁻¹
OD ₄₂₀	optical density at 420 nanometres
P	total protein concentration, g·L ⁻¹
P_0	initial total protein concentration, g·L ⁻¹
S	solubility of protein or enzyme, mg·mL ⁻¹
t	incubation time, minutes
V	volume of reaction mixture, mL
z_i	ionic charge of the <i>i</i> th ion

Introduction

Enzymes, hormones and other proteins commonly need to be purified from complex mixtures containing other proteins and non-protein substances. Examples of such mixtures are plant and animal tissue extracts, blood plasma, fermentation and cell culture broths and homogenates of microorganisms. Protein recovery continues to be important in modern bioprocessing where many recombinant products occur intracellularly, necessitating purification from complex mixtures that are produced by cell disruption (Chisti and Moo-Young, 1986). Protein recovery from such mixtures is invariably a multistep process, with some form of precipitation often being used as one of the purification steps (Chisti and Moo-Young, 1994; Stavrinides et al., 1993). Precipitation methods alter the solubility of protein to remove it from solution, preferably with some

degree of selectivity to ensure purification. Purification factors of 3–10-fold that can typically be achieved are low compared with other high resolution separations such as the chromatographic methods (Bell et al., 1983; Chisti and Moo-Young, 1991). However, unlike many high resolution procedures, precipitation methods can inexpensively process large amounts of quite crude material. These methods achieve volume reduction that reduces the size requirements on the more expensive, higher resolution processing techniques further downstream (Chisti and Moo-Young, 1994). In addition, precipitation techniques are amenable to continuous flow operation (Chisti and Moo-Young, 1991; Higgins et al., 1978). Because of these advantages, precipitation methods are often used as an early step in the purification train.

Several means of altering the protein solubility are available: coagulation by heat may be used to precipitate unwanted thermolabile proteins in solutions of more stable ones; isoelectric precipitation may be used for pH stable, or biologically inactive proteins; alcohol or solvent fractionation may be employed to alter the dielectric properties of the solvent to cause precipitation; and salts may be added to alter solubility (Chisti and Moo-Young, 1991; 1994; Wheelwright, 1991). Salting-out is usually the most mild precipitation method for biologically active proteins; hence, it is widely used. Salting-out is brought about by gradual, step-wise addition of either solid salts or concentrated solutions to solutions of proteins to achieve fractionation. Precipitation of protein apparently occurs by a reduction in water activity or water availability for hydration of the molecule; however, the exact mechanism of the salting-out effect remains unclear (Bell et al., 1983). The most hydrophobic and, hence, least hydrated proteins tend to be the first to come out of solution.

The effectiveness of ions in protein precipitation is known to depend on the ionic charge, with multivalent ions being the most effective. In practice, ammonium sulfate is commonly preferred for salting-out because it is inexpensive, has high solubility at low temperatures and it is usually compatible with the process. Beyond certain low levels of salt concentration, protein precipitation generally follows the Cohn equation (Chisti and Moo-Young, 1991)

$$\ln S = \mathbf{B} - K_s I, \quad (1)$$

where S is the solubility of protein, K_s is the salting-out constant and I is the ionic strength of solution. The constant \mathbf{B} depends on the pH, temperature and

the protein, but is little affected by the salt (Chisti and Moo-Young, 1991). The ionic strength I in Eq. (1) is defined as

$$I = \frac{1}{2} \sum_{i=1}^n c_i z_i^2 \quad (2)$$

where c_i and z_i are the concentration and charge of the i th ion. While Eq. (1) is empirical, attempts have been made to express the constants K_s and \mathbf{B} in terms of the more fundamental physicochemical characteristics of proteins (Melander and Horvath, 1977; Przybycien and Bailey, 1989). These developments notwithstanding, salting-out remains a highly empirical process.

Despite common empirically developed usage, salting-out *per se* has been little investigated, particularly in purification of recombinant proteins from homogenates of the producing cells. This paper reports on ammonium sulfate fractionation of a recombinant enzyme, β -galactosidase (EC 3.2.1.23) or lactase, of *Escherichia coli* produced in the yeast *Saccharomyces cerevisiae*. The soluble, active enzyme is precipitated from clarified cell homogenate. The effects of salt concentration, the processing pH and the initial quantity of protein in solution on precipitation behaviour are reported. While many of the published procedures for recovery of β -galactosidase from *E. coli* have utilized ammonium sulfate fractionation as one of the step in the purification scheme (Gacesa and Hubble, 1987, p. 41), recovery from recombinant *S. cerevisiae* has not been investigated.

The enzyme is of commercial significance in processes for removing lactose from milk and milk products intended for consumption by lactose-intolerant individuals. Although the intestinal bacterium *E. coli* is a good producer of β -galactosidase, for safety reasons commercial preparations of the enzyme for food use have generally been obtained from the yeasts *Kluyveromyces fragilis* and *Saccharomyces lactis*. Other sources of β -galactosidase include fungi such as *Aspergillus niger*. In combination with chromogenic substrates (e.g., 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside or X-gal; *o*-nitrophenyl- β -D-galactoside or ONPG), β -galactosidase is also used in many enzyme-linked immunosorbent assay (ELISA) systems (Gacesa and Hubble, 1987, p. 72).

Materials and methods

Microorganism and fermentation conditions

The recombinant enzyme β -galactosidase was produced in a genetically engineered yeast, *Saccharomyces cerevisiae* YN124 (*cir*⁺ *alpha ura 3-52 try*₁₋₂₈₉ *gal 2*), containing the plasmid pLG669-Z (Guarente and Ptashne, 1981). The plasmid contained a section of the *Escherichia coli* β -galactosidase (EC 3.2.1.23) gene under the control of *S. cerevisiae* *CYC1* constitutive promoter. Markers for ampicillin resistance in *E. coli* and uracil auxotrophy in *S. cerevisiae* occurred on the plasmid. The enzyme was produced intracellularly in a soluble active form as distinct from inclusion body type production.

The culture procedure was similar to that employed by Garrido et al. (1994). The yeast was maintained at 4 °C on slants of a selective medium of yeast nitrogen base (YNB, Difco) supplemented with tryptophan to a final concentration of 20 mg·L⁻¹. Inoculum was grown in the selective medium in shake flasks held at 30 °C for 2-days on a rotary shaker (200 rpm). This assured a high initial fraction of plasmid-containing cells. A non-selective production medium, the yeast extract-peptone-glucose (YPG) medium, as reported by Barnett et al. (1983), was used for production of cell mass. The composition of the YPG-medium was (g·L⁻¹): yeast extract, 5; peptone, 10; and glucose, 20. The pH was adjusted to pH 5.2 with 15 mM citrate-phosphate buffer (Hardjito et al., 1992). Cells were grown in a 12 L (nominal) stirred tank fermenter (MBR Bioreactor AG, Switzerland) equipped with pH, temperature, dissolved oxygen and foam controllers. Presterilized YPG medium (10 L) at 30 °C, pH 5.2, was inoculated with 500 mL inoculum described earlier. The fermenter was agitated at 500 rpm, pH was controlled at pH 5.2 using acid (2 M hydrochloric acid) and alkali (2 M sodium hydroxide) additions. Dissolved oxygen was maintained at greater than 80% of air saturation by aeration at one volume of air flow per unit volume of broth per minute. The cells were harvested after 20-h of fermentation, centrifuged in small portions and washed twice with phosphate buffer (0.1 M, pH 7.0).

Cell disruption

The intracellular β -galactosidase was released by mechanical disruption of the cells. The harvested cells were resuspended to 25% wet weight (corresponding to about 50 g DW·L⁻¹, in phosphate buffer (0.1 M, pH

7.0). The cell slurry was fed to a continuous flow, high speed, bead mill (Sulzer Annu Mill 01, Sulzer Brothers Limited, Winterthur, Switzerland) loaded with glass beads (0.5 mm diameter; 2500 kg·m⁻³, density) to 80% of the chamber volume (210 mL). The flow rate of the slurry was 100 mL·min⁻¹. The agitation speed was set at 2000 rpm and the temperature was controlled at 10 °C by cooling through the jacket that surrounded the grinding chamber. The effluent from the mill was reprocessed through the grinding chamber for a total of six passes, giving a cumulative disruption time of 6.3 min. This figure was calculated using the 80% bead loading (based on packed or settled volume) and the reported ratio of 0.625 (Garrido et al., 1994) between the actual volume of solids and the packed volume. The beads were the same as used by Garrido et al. (1994).

The disruption conditions used had previously been shown to achieve complete release of the enzyme (Garrido et al., 1994). The disrupted slurry was centrifuged (Model L7 Series ultracentrifuge; Beckman, Palo Alto, CA) at 15 000 rpm (about 380 g), 10 °C, for 30 min to remove the cell debris. The clarified homogenate was kept refrigerated at 4 °C until needed.

Enzyme fractionation

Eight equal portions (40 mL each) of the clarified crude homogenate were placed in separate beakers (100 mL) and incubated at 10 °C. The amounts of solid ammonium sulfate required to achieve discreet degrees of saturation of (0–80%) of the homogenate samples were calculated (Yu and Tang, 1991) and measured out. One measured batch each was added to the eight homogenate samples while stirring. Agitation was continued for 1 h. The precipitate formed was removed by centrifugation (Model L7 Series ultracentrifuge; Beckman, Palo Alto, CA) at 15000 rpm (about 380 g), 10 °C, for 30 min. The supernatants were assayed for total protein and for β -galactosidase. The precipitate samples were redissolved quantitatively in 0.1 M phosphate buffer, pH 7.0, to produce the same volume of solution as the original sample of the homogenate. The resulting solution was assayed for total protein and the enzyme activity. The total protein and enzyme were expressed respectively as milligram protein and units of activity per millilitre of the original crude homogenate.

In some experiments designed to test the effects of solution pH on salting-out, the pH of the clarified homogenate (pH = 7.0) was altered to either pH 6 or pH

8 with addition of acid or alkali. The precipitate formed was centrifuged and the resulting homogenate was used in salting-out work as detailed above. The effect of the initial total protein concentration on salting-out was investigated by diluting (0.1 M phosphate buffer, pH 7.0) the clarified homogenate ($20 \text{ g}\cdot\text{L}^{-1}$ total protein) 10-fold to a total initial protein concentration of $1.9 \text{ g}\cdot\text{L}^{-1}$ prior to use in salting-out experiments.

Total protein assay

The Coomassie brilliant blue method was used for determining the total protein (Bradford, 1976; Sedmak and Grossberg, 1977). A 0.1 mL aliquot of the unknown dilute protein sample was pipetted into a test tube, 5.0 mL of Coomassie protein assay reagent (Pierce, Rockford, IL; catalog no. 23200) was added and the resulting solution was mixed well. Absorbance of the mixture was read within 90 min at 595 nm (Turner, Model 330 spectrophotometer) against a deionized water blank. The absorbance reading was converted to protein concentration using a standard curve and the known dilution factor. The standard curve was made by measurements on known solutions of bovine serum albumin Fraction V (Pierce, Rockford, IL; catalog no. 23210) in the $0\text{--}1500 \mu\text{g}\cdot\text{mL}^{-1}$ protein concentration range. The measurements were not affected by ammonium sulfate.

β -Galactosidase assay

The β -galactosidase activity was assayed using a modification of the method recommended by Miller (1972). Thus, an aliquot of solution (clarified cell homogenate, resuspended precipitate or supernatant) was diluted in Z buffer (composition ($\text{g}\cdot\text{L}^{-1}$): $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$, 16.1; $\text{NaH}_2\text{PO}_4\cdot \text{H}_2\text{O}$, 5.5; KCl, 0.75; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.246 and β -mercaptoethanol, 2.7 mL; the pH was 7.0) to a final volume of 1.0 mL. The mixture was pre-incubated at 28°C , 0.2 mL of *o*-nitrophenyl- β -D-galactoside (ONPG, $4 \text{ mg}\cdot\text{mL}^{-1}$ in 0.1 M phosphate buffer, pH 7.0) was added and the time was noted. After sufficient yellow colour had developed, the reaction was stopped by addition of 1 mL of 0.5 M Na_2CO_3 solution. The reaction time, or incubation time (t) was noted. The optical density was recorded at 420 nm using a spectrophotometer (Turner, Model 330). A unit of enzyme activity was defined as the amount of enzyme which produced one nanomole of *o*-nitrophenol per minute at 28°C , pH 7.0. The enzyme concentration was calcu-

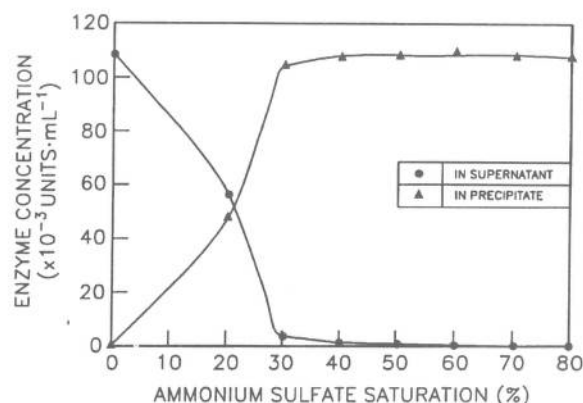


Fig. 1. Effect of degree of ammonium sulfate saturation on concentration of β -galactosidase in the precipitate and supernatant (pH = 7.0; $P_0 = 20.0 \text{ g}\cdot\text{L}^{-1}$). All concentrations are expressed in terms of the original volume of the homogenate solution.

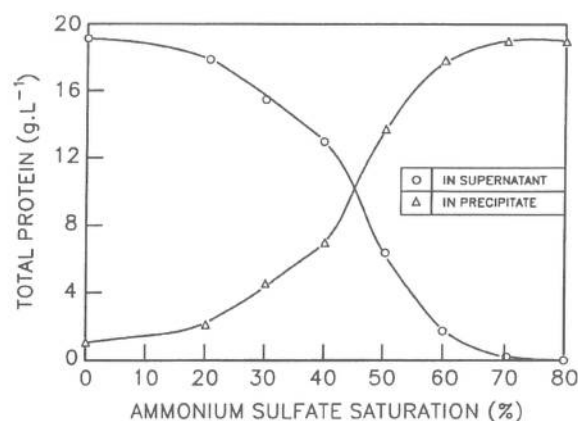


Fig. 2. Effect of degree of ammonium sulfate saturation on concentration of total protein in the precipitate and supernatant (pH = 7.0; $P_0 = 20.0 \text{ g}\cdot\text{L}^{-1}$). All concentrations are expressed in terms of the original volume of the homogenate solution.

lated as follows:

$$\text{Concentration (unit}\cdot\text{mL}^{-1}) = \frac{OD_{420}}{0.0022 \times t \times V}, \quad (3)$$

where t is the incubation period in minutes, V is the volume (mL) of the reaction mixture, OD_{420} is the optical density at 420 nm corresponding to the amount of *o*-nitrophenol produced and the constant 0.0022 is the optical density of one nanomole of *o*-nitrophenol at 420 nm.

Results and discussion

Effects of extent of salt saturation of homogenate

Effects of the degree of ammonium sulfate saturation of homogenate on salting-out were examined by adding different amounts of salt to separate portions of the same original bulk homogenate. The results are shown in Figs. 1 and 2 where concentrations of the enzyme and of total protein in the supernatant and the precipitate are presented. As shown in the figures, compared to total protein, the enzyme precipitated preferentially as the concentration of the salt increased. At 30% salt saturation, almost no enzyme activity remained in the supernatant as all had been recovered in the precipitate (Fig. 1). Consequently, for salt saturation of greater than 30%, the enzyme concentration isotherms were virtually flat (Fig. 1). In contrast, at 30% salt saturation, only about 25% of the total protein was precipitated (Fig. 2). Because of the presence of many different proteins (see for example Foster et al., 1976) with different hydration requirements to remain in solution, the total protein curves in Fig. 2 did not display a sharp concentration change at any specific salt saturation. Ammonium sulfate saturation of 70% or greater was necessary to precipitate most of the protein (Fig. 2).

The relatively faster precipitation of β -galactosidase that occurred at a relatively low salt concentration was consistent with the known hydrophobic nature of this unusually large protein which is an oligomeric enzyme consisting of four polypeptide chains with a combined molecular weight of 540,000 daltons (Loewy and Siekevitz, 1969). Thus, relatively small changes in water availability were sufficient to bring it out of solution. The percent enzyme recovery and the specific activity in the precipitate are shown in Fig. 3 which confirms greater than 95% recovery and highest specific activity at 30% salt saturation. Hence, an optimal salt saturation of 30% is indicated for the recovery of this enzyme from the recombinant yeast homogenate. This value is consistent with other reports on β -galactosidase recovery from *E. coli* cells (Higgins et al., 1978). Among the advantages of producing the enzyme in the yeast is that, unlike the nucleic acids laden homogenates of *E. coli* and other bacteria, the yeast homogenate is not particularly viscous and it does not require a DNA removal step. Hence, processing is simpler and less expensive.

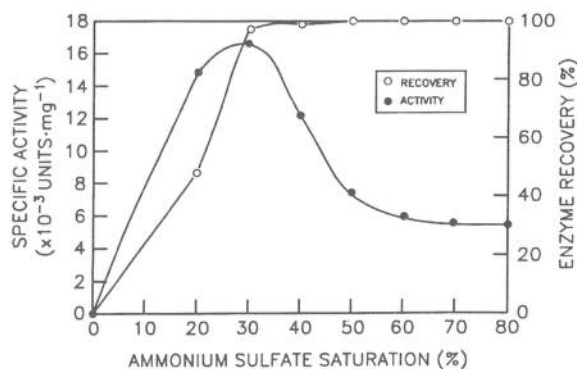


Fig. 3. Effect of degree of ammonium sulfate saturation on percent enzyme recovery and specific activity of the precipitate (pH = 7.0; $P_0 = 20.0 \text{ g}\cdot\text{L}^{-1}$).

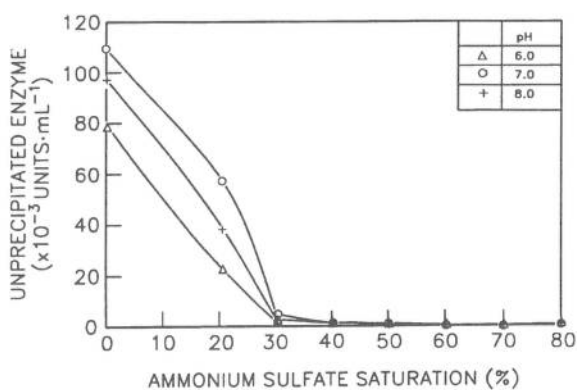


Fig. 4. Effect of solution pH on salting-out of the enzyme ($P_0 = 20.0 \text{ g}\cdot\text{L}^{-1}$).

Effects of pH

The pH range for activity and stability of *E. coli* β -galactosidase is known to be pH 6–8. Stability and

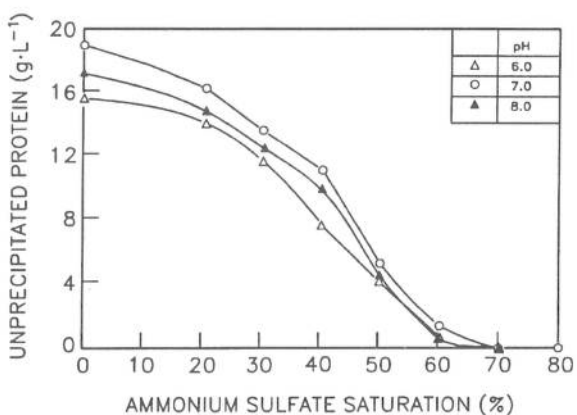


Fig. 5. Effect of solution pH on salting-out of the protein ($P_0 = 20.0 \text{ g}\cdot\text{L}^{-1}$).

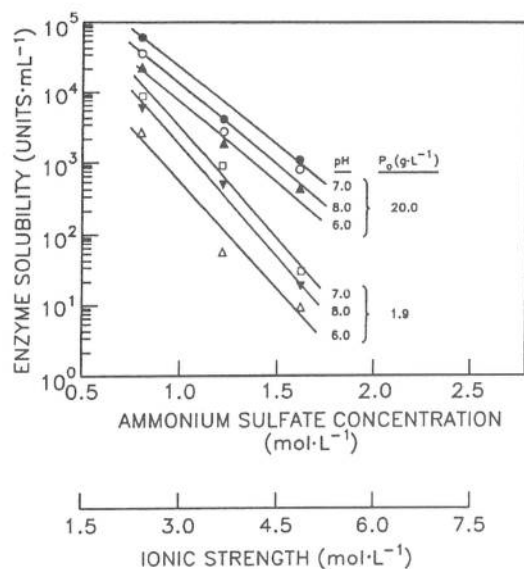


Fig. 12. Cohn equation plots for β -galactosidase recovery as functions of processing pH and initial protein concentration (P_0).

recovery of the total protein required salt saturation levels of 70% or higher. The preferential precipitation of the enzyme was associated with the hydrophobic character of this large, multichain molecule. The salt concentration needed for complete precipitation of the enzyme was not sensitive to the processing pH over the range of pH 6–8. Within this pH stability range of the enzyme, pH change alone (no added salt) was ineffective in recovering the enzyme or other proteins. The enzyme precipitation followed Cohn equation over 20–50% salt saturation levels. The salting-out constant depended on the initial total protein concentration, declining with increasing concentration, but was independent of pH. However, the Cohn parameter B was affected by pH as well as the initial total protein level. Precipitation from dilute solution enhanced the specific enzyme activity in the precipitate, but not sufficiently to compensate for the reduced overall recovery and the larger processing volume.

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