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Single-step purification of a recombinant thermostable α-amylase after solubilization of the enzyme from insoluble aggregates

Anni Linden, Frank Niehaus, Garabed Antranikian*

Department of Technical Microbiology, Technical University Hamburg-Harburg, Denickestrasse 15, 21071 Hamburg, Germany

Abstract

The expression of the gene encoding a thermostable α-amylase (EC 3.2.1.1) (optimal activity at 100°C) from the hyperthermophilic archaeon *Pyrococcus woesei* in the mesophilic hosts *Escherichia coli* and *Halomonas elongata* resulted in the formation of insoluble aggregates. More than 85% of the recombinant enzyme was present within the cells as insoluble but catalytically active aggregates. The recombinant α-amylase was purified to homogeneity in a single step by hydrophobic interaction chromatography on a phenyl superose column after solubilization of the enzyme under nondenaturing conditions. The enzyme was purified 258-fold with a final yield of 54%. © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Purification; *Pyrococcus woesei*; *Escherichia coli*; *Halomonas elongata*; Aggregation; Inclusion bodies; Solubilization; Enzymes; α-Amylase

1. Introduction

The expression of recombinant genes in *Escherichia coli* often results in the accumulation of insoluble and inactive protein aggregates [1–3]. These aggregates — which are known as inclusion bodies — are generally homogeneous complexes with a high density and are predominantly composed of the recombinant protein derived from aggregation of specific folding intermediates [4,5]. The strategies for the extraction of recombinant proteins include a common basic set of steps [6]. The separation of inclusion bodies from the bulk of soluble host cell proteins by centrifugation is usually the first step in target protein purification followed by the solubilization of the isolated aggregates. Inclusion body solubilization requires rather strong denaturing agents such as chaotropic salts, organic solvents, detergents or extreme acidic–alkaline solutions. Afterwards, the denatured protein is renatured by the removal of the denaturant [1]. Although this strategy is established it exhibits a number of disadvantages: especially the use of detergents limits the application of various chromatographic matrices. The detergent may either bind to the matrices or may change the chromatographic behaviour of a protein due to the size of formed micelles. Furthermore, no general rules exist for renaturation, making renaturation a rather difficult and time consuming task. In principle, optimal renaturation conditions have to be determined for every single protein often by trial and error. In addition, the renaturation procedure may lead to the further loss of target protein due to irreversible aggregation. In some cases, however, inclusion body formation can be desirable when the target protein is protected against proteolysis [7].

*Corresponding author. Tel.: +49-40-7718-3117; fax: +49-40-7718-2909.

E-mail address: antranikian@tu-harburg.de (G. Antranikian)

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The expression of the gene encoding a thermostable α-amylase (EC 3.2.1.1) from the hyperthermophilic archaeon *Pyrococcus woesei* in the mesophilic hosts *E. coli* and *Halomonas elongata* resulted in the formation of insoluble aggregates (>85%) [8–10]. The recombinant, native enzyme, which has an apparent molecular weight of 110 kDa, is optimally active at 100°C and is present as catalytically active aggregates.

Recent results have shown that the expression of the same enzyme from the hyperthermophilic archaeon *Pyrococcus furiosus* in *E. coli* in a soluble form could be achieved when thioredoxin was coexpressed in this mesophilic host at 18°C. Compared to these results, however, higher levels of recombinant α-amylase (3-fold) from *P. woesei* were obtained as insoluble aggregates when *E. coli* was cultivated at 37°C under the conditions previously reported [10].

Although inclusion body formation was predominant, the aggregates exhibit high amylolytic activity indicating a correct folding of the target enzyme rather than the aggregation of specific folding intermediates. Based on these results a simple solubilization and purification protocol for the isolation of the recombinant pyrococcal α-amylase that are present within inclusion bodies was developed.

2. Experimental

2.1. Strains, plasmids and media

E. coli strain BL21(DE3) [11] (Novagene, Madison, WI, USA) was used as a host strain. Plasmid pPwA, a pET-15b derivative that encodes the *P. woesei* α-amylase gene under the control of the T7*lac* promotor was constructed as described elsewhere [10]. The fed-batch cultivation was conducted in a 2-l membrane reactor (Bioengineering AG, Wald, Switzerland) with automated temperature and pH control containing 1 l of mineral medium according to Märkl et al. [12] with 20 g/l glucose, 10 g/l casamino acids and 5 mg/l thiamine–HCl. The gene expression was induced at the late logarithmic growth phase with 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested 4 h after induction by centrifugation at 7000 *g* for 10 min. The recombinant *H. elongata* (ATCC 33174) strain, harboring plasmid pHS15 with the appropriate α-amylase coding region which is designated here as pHS15 PwA was kindly supplied by Dr S. Frillingos, Section of Organic Chemistry and Biochemistry, University of Ionnina (Ionnina, Greece). The cultivation was performed at 37°C in 1-l Erlenmeyer flasks containing 400 ml of modified SWYE medium (SW-3) [13] in which the final percentage of total salt concentration was decreased to 3% (w/v). Cells grown overnight on a rotary shaker at 210 rpm were harvested at 10 000 *g* for 10 min.

2.2. Protein aggregate isolation and α-amylase purification

All procedures were carried out at 4°C unless otherwise stated: 6.5 g of harvested recombinant *E. coli* cells were resuspended in 0.05 *M* sodium acetate, pH 6.0, and lysed by sonication on ice. The lysates were centrifuged at 54 000 *g* for 30 min to collect the insoluble protein aggregates. The pellet (protein aggregates and cell debris) was washed with 20 ml 0.05 *M* sodium phosphate (pH 7.2) including 3 m*M* EDTA and 1% (v/v) Triton X-100. After centrifugation at 54 000 *g* for 1 h the recombinant α-amylase was solubilized and extracted by repeated resuspension in 0.05 *M* sodium acetate (pH 6.0) containing 3 m*M* EDTA and 20% (v/v) glycerol (buffer A) followed by stirring at room temperature for 2–4 h and subsequent centrifugation at 54 000 *g* for 1 h until no amylolytic activity was detectable in the pellet. The supernatant fractions with the glycerol–amylase extract were pooled and purified as described in Section 2.3. The isolation and purification scheme of the recombinant amylase is shown in Fig. 1 and was the same for the enzyme expressed in *H. elongata* cells.

2.3. Purification of the solubilized recombinant α-amylase

The amylase was purified by hydrophobic interaction chromatography on a prepacked phenyl superose HR 5/5 column (5×50 mm; Amersham Pharmacia, Freiburg, Germany). The column liquid chromatography was performed at room temperature on a fast protein liquid chromatography (FPLC) system

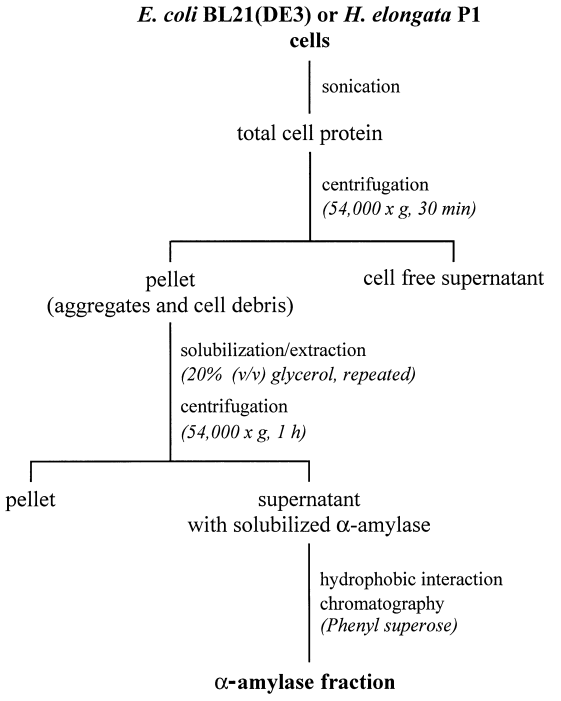

Fig. 1. The flow chart for the purification of *P. woesei* α-amylase from recombinant *E. coli* BL21(DE3) and *H. elongata* P1 cells.

equipped with two pumps (P-500) and a GP-250 PLUS gradient programmer. The glycerol–amylase extract (0.23 mg/ml protein) was centrifuged without further treatment at 15 000 *g* for 15 min prior to sample loading. Sample volumes of 3–10 ml were applied on the phenyl superose column previously equilibrated with 10 ml of buffer A by a 50-ml superloop (Pharmacia). After sample injection the column was washed with 10 ml of buffer A. The protein desorption was performed with an increasing linear gradient (20 ml) of 0.05 *M* sodium acetate (pH 6.0) containing 50% (v/v) ethylene glycol (buffer B) at a flow-rate of 0.1 ml/min. The fractions exhibiting amylase activity were pooled and analyzed by SDS- and native gradient-PAGE.

2.4. Electrophoresis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed under reducing conditions with 12% gels using the Bio-Rad Mini-PROTEAN II electrophoresis unit (Bio-Rad, München, Germany). Samples were treated in denaturing buffer at 95°C for 5 min before being loaded. The SDS low-molecular-weight standard mixture (Pharmacia) was used in order to determine the apparent molecular weight of the samples.

The native gradient-PAGE (4–20%) was conducted under nondenaturing and nonreducing conditions using pre-cast gradient Tris–glycine gels (Novex, Frankfurt, Germany) according to the manufacturer. Native high-molecular-weight standard mixture (Pharmacia) was used to determine the apparent molecular weight of the native proteins in the sample. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 following the method of Weber and Osborn [14].

For activity staining after gel electrophoresis the gel was incubated in 0.1 *M* sodium acetate (pH 6.0) containing 1% (w/v) soluble starch at room temperature for 1 h. Afterwards, the gel was incubated in 0.1 *M* sodium acetate, pH 6.0 at 90°C for 3–5 min. The degradation of starch by active protein was visualized by soaking the gel in iodine–potassium iodide (0.15–1.5%) after incubation at 90°C.

2.5. Enzyme assay and protein determination

Amylase activity was determined by measuring the amount of reducing sugar released during enzymatic hydrolysis of starch using a modified assay of Bergmeyer and Grassl [15]. In a standard assay 10 μl of enzyme solution was added to 90 μl of 0.1 *M* sodium acetate (pH 6.0) containing 1% (w/v) soluble starch and incubated at 94°C for 4 min. The reaction was terminated by cooling in ice water. One unit of amylase is defined as the amount of enzyme that liberates 1 μmol of reducing sugar per minute under the assay conditions with maltose as a standard.

The protein concentration was determined by the method of Bradford, modified by Stoschek [16].

2.6. Chemicals

Soluble starch, glycerol, ethylene glycol, iodine, and potassium iodide were obtained from Merck (Darmstadt, Germany). Triton X-100 was purchased from Serva (Freiburg, Germany) and IPTG from Roche (former Boehringer, Mannheim, Germany).

3. Results and discussion

After expression of the thermostable recombinant *P. woesei* α-amylase gene in *E. coli* more than 85% of the enzyme activity was detected as insoluble protein aggregates formed inside the cells. A similar result was obtained when the moderate halophile *H. elongata* was used as the expression host. In this case 70% of the recombinant enzyme were found as insoluble protein aggregates. Obviously, the formation of inclusion bodies is not unique to *E. coli* and it has also been documented in other genetically engineered microorganisms such as *Bacillus subtilis* and *Saccharomyces cerevisiae* [17,18]. The nature of inclusion bodies and the mechanism of their formation is still under investigation, but a number of observations indicate that recombinant protein aggregation results from the association of misfolded folding intermediates [4,5,19,20]. Hence, inclusion bodies are generally devoid of enzyme activity. In contrast, the recombinant *P. woesei* α-amylase aggregates exhibit high enzymatic activity with a specific activity of 240 U/mg. It is, therefore, more likely that in this case aggregation occurred by specific interaction of mature recombinant protein molecules rather than by misfolded folding intermediates. Furthermore, α-amylase aggregates appear not to be 'real' inclusion bodies. Under these circumstances efforts were made to avoid the use of denaturing agents for the solubilization of protein aggregates. Alternatively, a nondenaturing and protein stabilizing method (glycerol extraction) was employed. After separation of the recombinant protein aggregates and cell debris from the bulk of soluble intracellular *E. coli* proteins the thermostable α-amylase could be solubilized successfully from the pellet fraction by extraction in 0.05 *M* sodium acetate (pH 6.0) containing 3 m*M* EDTA and 20% (v/v) glycerol (buffer A). The analysis of the proteins before and after pellet treatment with buffer A is shown in Fig. 2 (lanes 3–5). After extraction most of the contaminating proteins remained in the pellet fraction resulting in a relatively pure target protein in the soluble fraction.

The sample was further purified by hydrophobic interaction chromatography (HIC). Fig. 3 shows the FPLC chromatogram of 3 ml glycerol–amylase extract. Adsorption of the amylase to the phenyl

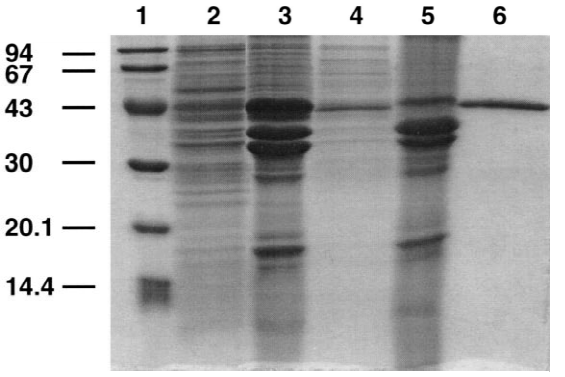

Fig. 2. SDS–PAGE (T=12%, C=2.7%) of samples taken from various steps of α-amylase purification using the recombinant *E. coli* BL21(DE3) cells. Protein bands were visualized by staining with Coomassie blue. Lanes: 1, molecular mass standard proteins (10^{-3} rel. mol. mass units); 2, total cell protein (20 μg); 3, pellet fraction (30 μg); 4, glycerol–amylase extract (7 μg); 5, pellet fraction after amylase solubilization and extraction (20 μg); 6, phenyl superose activity pool (6 μg).

superose matrix occurred in the absence of any additional salt either to the sample or to the equilibration buffer; no amylolytic activity was detectable in the void volume. Under these conditions, however, other proteins did not bind to the column and were eluted mainly in the void peak. The α-amylase was finally eluted with 100% buffer B (50% ethylene glycol in 0.05 *M* sodium acetate, pH 6.0). Analysis of the pooled active fractions by SDS–PAGE (Fig. 2, lane 6) and native gradient-PAGE (Fig. 4, lane 2) showed one single protein band with the same size as determined by activity staining (Fig. 4, lane 3 and Fig. 5, lane 6). The enzyme was purified 258-fold with a final yield of 54% (Table 1). The recombinant amylase expressed in *H. elongata* could also be purified by the same procedure (Fig. 5).

During hydrophobic interaction chromatography, the solvent-accessible hydrophobic regions on the surface of biomolecules interact with the hydrophobic ligands on the adsorbent. This adsorption is salt-promoted so that the binding of proteins is usually carried out at high salt concentrations. The principle for the strengthening of the hydrophobic interaction at high salt concentrations is the same as in salting out precipitation [21]. The strong binding of the α-amylase on phenyl superose indicates that

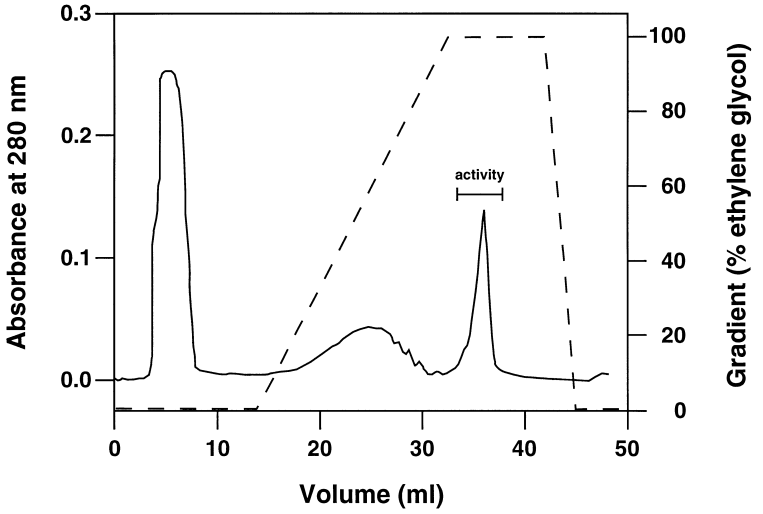

Fig. 3. Hydrophobic interaction chromatography of the glycerol–amylase extract from recombinant *E. coli* BL21(DE3) on phenyl superose HR 5/5 column. 0.7 mg protein was applied to the column. Eluent A: 0.05 *M* sodium acetate (pH 6.0) containing 3 m*M* EDTA and 20% (v/v) glycerol. Eluent B: 0.05 *M* sodium acetate (pH 6.0) containing 50% (v/v) ethylene glycol. One-ml fractions were collected at a flow-rate of 0.1 ml/min. The dashed line shows the gradient profile.

this protein is very hydrophobic. Proteins that adsorb strongly even at low salt concentrations exhibit generally a low water solubility and tend to aggregate readily in aqueous solutions [21]. The formation of α-amylase aggregates in *E. coli* and *H. elongata*, hence, seems to be derived from the mature enzyme. This assumption is supported by the observation that the solubilized α-amylase exhibits similar characteristic properties as the recombinant enzyme that was expressed in soluble form (data not shown). Aggregation as the result of incorrect disulfide bond

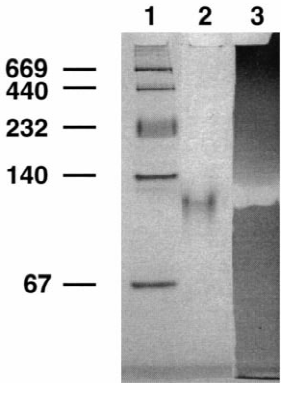

Fig. 4. Native gradient-PAGE (4–20%) of the purified recombinant *P. woesei* α-amylase. Protein bands were visualized by staining with Coomassie blue (lanes 1 and 2) and the α-amylase band by zymogram staining (lane 3). Lanes: 1, molecular mass standard proteins (10^{-3} rel.mol. mass units); 2, purified α-amylase (4 μg); 3, purified α-amylase (0.2 μg).

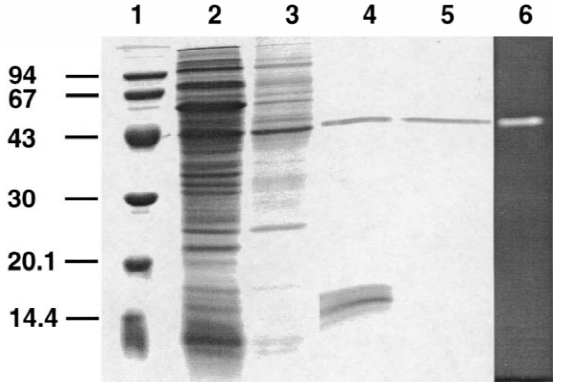

Fig. 5. SDS–PAGE (T=12%, C=2.7%) of samples taken from various steps of α-amylase purification using the recombinant *H. elongata* P1 cells. Protein bands were visualized by staining with Coomassie blue (lanes 1–5) and the α-amylase band by zymogram staining (lane 6). Lanes: 1, molecular mass standard proteins (10^{-3} rel. mol. mass units); 2, total cell protein (25 μg); 3, pellet fraction (10 μg); 4, glycerol–amylase extract (6 μg); 5, phenyl superose activity pool (4 μg).

Table 1
Purification of the pyrococcal α-amylase from 6.5 g of recombinant *E. coli* BL21(DE3) cells

Purification step	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Total cell protein[a]	1026	13.5	13851	1.0	100
Centrifugation[b]	64.8	240.5	15552	17.8	112
Solubilization[c]	18.4	797.8	14680	59.1	106
Phenyl superose[d]	2.2	3483.3	7469	258.0	54

[a] After cell disruption by sonication.
[b] Pellet fraction containing α-amylase aggregates and cell debris was prepared after centrifugation at 54 000 *g* for 30 min.
[c] Solubilization and extraction of α-amylase aggregates in 0.05 *M* sodium acetate (pH 6.0) containing 3 m*M* EDTA and 20% (v/v) glycerol.
[d] Hydrophobic interaction chromatography of the solubilized amylase on a phenyl superose HR 5/5 column.

formation can be ruled out, since the protein precipitates during ultrafiltration (Amicon centricon — 30 units, 30 000 Da membrane molecular weight cut-off, Millipore, Eschborn, Germany) at relatively low protein concentrations even in the presence of 10 m*M* DTT (data not shown).

Recently the α-amylase from *Pyrococcus furiosus* was partly expressed as soluble enzyme and purified by Dong et al. [9]. The purification method consists of a heat treatment step followed by precipitation with 60% $(NH_4)_2SO_4$, two HIC steps on phenyl superose and a final separation by gel permeation chromatography on Sephacryl S200. After each chromatography step the active fractions were pooled and concentrated by ultrafiltration. Due to the hydrophobicity of the amylase this can cause a significant loss of the target protein. Unfortunately, a comparison between the method described by Dong et al. and the one presented in this contribution is not possible since data regarding the yield and purification factors are not available. Another drawback in the purification method described in Ref. [9] is the use of 6 *M* urea at alkaline pH (9.4). This step can cause the formation of isocyanate which may lead to carbamylation of free amino groups of the polypeptide [22].

The simple method described in this presentation will now allow the fast preparation of sufficient amounts of native archaeal α-amylase that will be needed to study structure–function relationships of a heat-stable enzyme and to perform application tests for the starch industry.

4. Conclusions

A simple and effective method for the solubilization of a recombinant thermostable *P. woesei* α-amylase from aggregates has been described in this report. The presented method has several advantages over the purification of the α-amylase expressed in soluble form. The separation of the α-amylase aggregates from the bulk of soluble cell proteins by centrifugation is a fast and effective first purification step. By solubilization of the aggregates under mild conditions the enzyme activity could be retained, making a following renaturation step unnecessary. Further loss of enzyme was minimized by employing a single hydrophobic interaction chromatography step.

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