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ORIGINAL PAPER

Efficient solubilization, purification of recombinant extracellular α-amylase from *pyrococcus furiosus* expressed as inclusion bodies in *Escherichia coli*

Lisa Wang · Qinhua Zhou · Huayou Chen · Zhongmei Chu · Jianfeng Lu · Yi Zhang · Shengli Yang

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Abstract The gene encoding the *Pyrococcus furiosus* extracellular α-amylase (PFA) was amplified by PCR from *P. furiosus* genomic DNA and was highly expressed in *Escherichia coli* BL21-Codon Plus (DE3)-RIL. The recombinant α-amylase was mainly expressed in the form of insoluble inclusion bodies. An improved purification method was established in this paper. The solubilization of the inclusion bodies was achieved by 90°C treatment for 3 min in Britton–Robinson buffer at pH 10.5. The solubilized PFA was then diluted and subsequently purified by Phenyl Sepharose chromatography. The overall yield of the new purification method was about 58,000 U/g wet cells, which is higher than previously reported.

Keywords *Pyrococcus furiosus* · *Escherichia coli* · α-Amylase · Inclusion bodies · Purification · Thermal stability

L. Wang · Q. Zhou · H. Chen · Z. Chu · J. Lu · Y. Zhang (✉) · S. Yang
Shanghai Institutes for Biological Sciences,
Chinese Academy of Science,
500 Caobao Road, 200233 Shanghai, China
e-mail: yzhang@sibs.ac.cn

L. Wang · H. Chen · Y. Zhang · S. Yang
Graduate School of Chinese Academy of Science,
100049 Beijing, China

Q. Zhou
College of Medicine, Jiaxing University,
314001 Jiaxing, China

Introduction

α-Amylases are among the most important commercial enzymes, having wide applications in starch-processing, brewing, alcohol production, textile, and other industries [3, 5]. Therefore, any improvement in the enzyme production yield, thermostability or activity will have a direct impact in the process performance, economics, and feasibility [14].

α-Amylases (EC 3.2.1.1) are endo-type enzymes that hydrolyses α-1,4-glucosidic linkages in starch substrate backbone. They make up the first step in starch liquefaction process and play a key role in starch conversion technology. Thermostable and acid-stable amylases are preferred since gelatinization of starch granules is completed above 100°C, and the natural pH of starch slurry is ~4.5. Moreover, the commonly used thermostable α-amylase usually required additional Ca^{2+}, which is a potent inhibitor for xylose isomerase subsequently used in processing starch hydrolysate to fructose syrup. The extracellular α-amylase from *Pyrococcus furiosus* (PFA) displays a temperature optimum of 100°C and retains above 80% of maximal activity at pH 4.5 without calcium salt addition [8]. These predominant properties of PFA make this enzyme a potential candidate for industrial application.

Due to the inherent difficulty of cultivation of *P. furiosus*, it is difficult to obtain a sufficient amount of cells for large-scale enzyme production. Several studies have focused on the production of recombinant PFA. The PFA gene has been cloned and expressed in active form in *Bacillus subtilis* and *Escherichia coli* successively [5, 8]. However, when higher level expression was achieved, the recombinant PFA always accumulated in the insoluble form as the inclusion bodies.

Several studies have tried to express the PFA as the soluble form. Results have shown that coexpression of PFA with thioredoxin in *E. coli* at 18°C is able to diminish inclusion body formation [5], and fusion expression of PFA with intein in *E. coli* also lead to a lower level of inclusion body formation [7]. However, all these soluble expressions resulted in the lower levels of total recombinant protein production. Previously, a method to extract the recombinant PFA from the inclusion bodies with glycerol has been reported [12]. We have tried the glycerol extraction method in our experiment and found that glycerol could only extract a portion of the recombinant proteins. Large amounts of protein remained insoluble after glycerol extraction thrice. We reported here an efficient method to purify the recombinant PFA from the inclusion bodies. By this method, a higher yield of active enzyme was achieved.

Materials and methods

Cloning and expression of PFA

The PFA gene without its signal peptide sequence was amplified by PCR from genomic DNA of *P. furiosus*. Forward primer 5′-ATGAAATACTTGGAGCTTGAAGAG-3′ and reverse primer 5′-AAGAAGCTTATCACCCAACACCACAATAACTC- 3′ were deduced from the open reading frame of the *P. furiosus* extracellular α-amylase gene (GenBank accession no. AF001268) [5]. A Hind III recognition site (underlined) was fused to the reverse primer. The PCR product amplified by *Pyrobest*™ DNA Ploymerase (Takara Bio, Shiya, Japan) was digested with Hind III and ligated into the NdeI (blunted with T4 DNA polymerase) and Hindβ site of the **pT7473** vector to get **pT7473-amyl**, then transformed into *E. coli* DH5α. The **pT7473-amyl** was isolated and the cloned gene was confirmed by DNA sequencing. The plasmid was then transformed into *E. coli* strain BL21-Codon Plus (DE3)-RIL for expression.

Isolation and Purification of inclusion bodies of PFA

Escherichia coli strain BL21-Codon Plus (DE3)-RIL cells transferred with the **pT7473-amyl** plasmid were cultured in LB-ampicillin (100 μg/ml)-kanamycin (50 μg/ml) medium at 37°C to an optical density at 600 nm of 0.7–0.9, then induced at 37°C with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Cells were harvested by centrifugation after 4 h of induction and suspended in 50 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl and 1 mM EDTA (buffer A). The cell suspension was then subjected to sonication for disruption and centrifuged at 12,000 g for 30 min to isolate inclusion bodies from cell debris. The inclusion bodies thus obtained were washed with buffer A containing 1% Triton X-100. Finally, the pellet was washed twice in distilled water to remove contaminating salts and detergent and stored at 20°C until used.

Solubilization of PFA from inclusion bodies

Solubilization of PFA was carried out in 120 mM Britton–Robinson buffer (40 mM boric acid; 40 mM acetic acid; 40 mM phosphoric acid adjusted to appropriate pH with NaOH) at different pH and temperature. Typically, 1 ml purified inclusion bodies (5 mg/ml) in 50 mM Tris buffer (pH 8.0) were added in each microcentrifuge tubes and centrifuged. The supernatant was discarded and 1 ml of Britton–Robinson buffer at different pH (2.5–12) was added, respectively, to the pellets. The suspension was vortexed and heated at 90°C for 3 min, then quickly chilled on ice. The samples were again centrifuged at 10,000 rpm for 30 min. Protein concentrations in the supernatant fractions were determined by the method of Bradford [2] and the enzyme activities were assayed by the DNS method [1] after appropriately diluting. To determine the effect of temperature on solubility of the recombinant protein, a fixed amount of inclusion bodies was solubilized at pH 10.5 and heated at different temperatures (from 50°C to 100°C).

Purification of PFA

The PFA inclusion bodies from 5.8 g of wet cells were solubilized in 60 ml of 120 mM Britton–Robinson buffer (pH 10.5), and heated at 90°C for 3 min, then centrifuged at 17,000 g for 40 min. The supernatant was diluted five times with 120 mM Britton–Robinson buffer (pH 8.5) (pH was adjusted to about 9.0). After another centrifugation step, PFA in the supernatant was further purified by hydrophobic interaction chromatography. The protein solution was loaded onto a phenyl Sepharose 6 Fast Flow column (Amersham Pharmacia Biotech, Freiburg, Germany), which was pre-equilibrated with 120 mM Britton–Robinson (pH 8.5) at a flow rate of 0.8 ml/min. Then the column was washed with five column volumes of equilibration buffer followed by a ten-column-volume increasing linear gradient from 50 mM sodium acetate (pH 6.0) to 50 mM sodium acetate (pH 6.0) containing 50% (v/v) ethylene

glycol. The PFA was eluted from the column with 50 mM sodium acetate (pH 6.0) containing 50% (v/v) ethylene glycol.

The glycerol extraction method reported by Linden was also performed according to the same procedures presented in the reference [12] and the purified proteins were used for enzyme assays as standards.

Enzyme assays

α-amylase activity was assayed according to Bernfeld [1]. In brief, 0.02 ml of suitably diluted enzyme solution was incubated with a reaction mixture containing 0.48 ml of 1% (w/v) soluble starch in 50 mM of acetate buffer, pH 5.5 at 98°C for 15 min. A control without enzyme was used. The hydrolysis was stopped by cooling the mixture on ice. The reducing sugars formed were measured by the addition of DNS (3, 5- dinitrosalicylic acid) reagent, using glucose as the standard. One unit of enzymatic activity is defined as the amount of enzyme required to liberate 1 μmol of reducing sugar as glucose per minute under the assay conditions. Protein concentration was determined by the Bradford method [2] using bovine serum albumin as the standard.

The thermal stability of recombinant α-amylase was investigated by determination of residual activities of enzyme solution after incubation for different periods at high temperature. The enzyme samples were incubated for various times at 90–128°C. Samples were removed at regular intervals and cooled on ice and the remaining activities were assayed.

Gel electrophoresis

Protein samples were analyzed by polyacrylamide denaturing gel electrophoresis (SDS-PAGE) using 10% gels essentially was performed according to Laemmli [11]. Protein samples were treated in denaturing buffer [2% SDS and 100 mM DDT (final concentrations)] at different temperatures for 10 min before loading. Low-MW protein markers were used as standards. Native PAGE was performed under the same conditions as described above except for the absence of SDS in the gel and buffer system. Proteins were stained with Coomassie brilliant blue R-250.

For the detection of protein band exhibiting amylase activities the gels were immersed in 50 mM acetate buffer (pH 5.5) at 40°C for 15 min, then incubated in the same buffer containing 1% (w/v) soluble starch for 15 min at 100°C, and finally incubated in a solution containing 10 mM iodine and 100 mM potassium iodide until a clear zone became visible.

Results

Cloning and expression of PFA

The PFA gene was amplified from *P. furiosus* genome DNA by PCR. The expression plasmid was constructed as described in materials and methods. The expression level of PFA was around 20% of the total cellular protein and most of the recombinant PFA was expressed as inclusion bodies. The molecular weight of the expressed protein was around 43 kDa (Fig. 1), which was very close to the calculated molecular mass of the amylase.

Solubilization of PFA from Inclusion bodies in different pH and temperature

It has been reported that heating for a while facilitated solublilization of recombinant proteins from *P. furiosus* expressed in *E. coli* in some cases [5, 6, 15], and PFA was stable at 90°C. Thus we tried to solubilize the PFA inclusion bodies at different pH at 90°C. Protein concentrations and enzyme activities of PFA solubilized in Britton–Robinson buffer at different pH after heating at 90°C for 3 min were shown in Fig. 2a. Inclusion bodies were difficult to solubilize at pH among 4.0–6.0 (Fig. 2a). Both of the protein concentration and the enzyme activity tended to increase with the increase of pH (Fig. 2a). At pH 10.5, the enzyme activity was about eight times higher than that at neutral pH. Higher alkaline pH (>10.5), even though helped to solubilize more inclusion bodies, resulted in lower

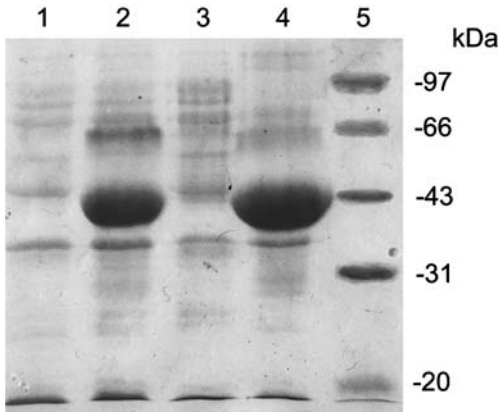

Fig. 1 SDS-PAGE analysis of recombinant PFA expression in *E. coli* after induction with IPTG. *Lane 1 E. coli* BL21 (DE3) RIL with plasmid **pT7473**; *lane 2 E. coli* BL21(DE3) RIL containing plasmid **pT7473-amyl** after induction with IPTG; *lane 3* supernatant of total cell lysate after induction, containing **pT7473-amyl**; *lane 4* pellet of total cell lysate after induction, containing **pT7473-amyl**; *lane 5* protein molecular mass standards

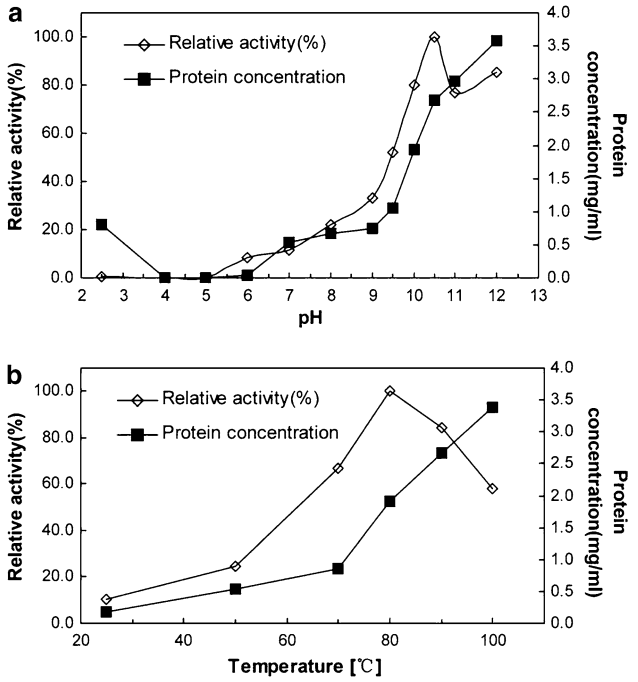

Fig. 2 Influences of pH (**a**) or temperature (**b**) on the solubilization of PFA inclusion bodies. Protein concentration was measured by Bradford [2] and the enzyme activity was assayed by DNS

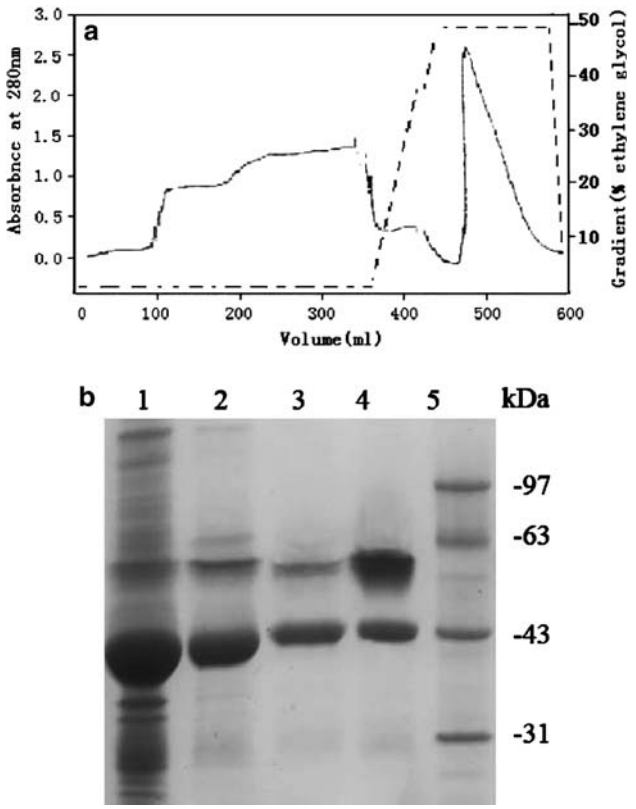

Fig. 3 **a** Elution curve of hydrophobic interaction chromatography of PFA. PFA was solubilizated in Britton–Robinson buffer at pH 10.5 after heating and diluted into Britton–Robinson buffer at pH 8.5 before loading onto Phenyl Sepharose. Stationary phase: Phenyl Sepharose 6 Fast Flow; linear gradient elution: 100% solution A, 50 mM sodium acetate (pH 6.0) to 100% solution B, 50 mM sodium acetate (pH 6.0) containing 50% (V/V) ethylene glycol. Flow rate: 0.8 ml/min. **b** SDS-PAGE of samples taken from various steps of PFA purification. Lane 1, pellet of bacterial lysate; lane 2, solubilized sample by Britton–Robinson buffer (pH 10.5) after heating; lane 3, sample after dilution; lane 4, purified PFA by phenyl sepharose; lane 5, protein molecular mass standards

enzyme activity (Fig. 2a), pH 10.5 was a preferable choice for both protein solubility and enzyme activity. The effect of different temperatures on the solubility and enzyme activity of PFA was presented in Fig. 2b. Although the highest activity was observed at 80°C, solubility was much lower (less than 2.0 mg/ml). Considered both of the enzyme activity and protein solubility, the preferred condition for solubilization of the inclusion bodies was selected as pH 10.5, at 90°C for 3 min.

Purification of PFA

The PFA was so hydrophobic that it could be directly absorbed onto the phenyl-sepharose column in the absence of any salt [5]. PFA inclusion bodies were solubilized in Britton–Robinson buffer (pH 10.5) after heating at 90°C for 3 min. The protein solution was then diluted fivefold and pH was adjusted to 9.0. After centrifuging the supernatant was loaded onto a phenyl Sepharose 6 Fast Flow column for purification. The protein was eluted by 50% (v/v) ethylene glycol (Fig. 3a). SDS-PAGE of the purified protein showed two bands (Fig. 3b). Previous reports showed similar results that the PFA tends to form a homodimer even on SDS-polyacrylamide gels [5]. Analysis of the purified protein by native gel showed one single protein band with the same size as determined by activity staining (Fig. 4a). The behavior of the purified protein treated at different temperatures in denaturing buffer was shown in Fig. 4b. The 66 KD band showed enzyme activity and disappeared after heating at 121°C (Fig. 4b, lane 2) indicating as a dimeric band. By this method we finally acquired 91.9 mg purified enzyme from 5.8 g of wet cells and the specific activity was similar to previous reports [5, 12] (Table 1).

The glycerol method for purification of PFA [12] was also performed and the purified protein was used as a standard for thermal stability analysis.

Thermal stability

It was reported that the purified enzyme by glycerol method exhibits similar characteristic properties as the recombinant enzyme that was expressed in soluble

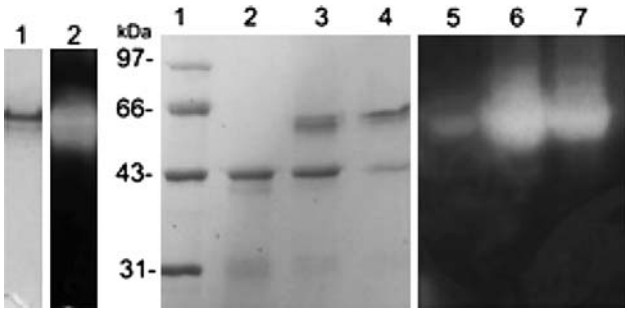

Fig. 4 **a** Native PAGE of the purified recombinant PFA. *Lane 1* was stained by Coomassie blue; *Lane 2* zymogram staining for α-amylase activity. **b** Behavior of PFA in SDS-PAGE. Protein bands were visualized by staining with Coomassie blue (*lanes 1–4*) and α-amylase activity band by zymogram staining (*lanes 5–7*). The protein samples were treated in denaturing buffer at 121°C (*lanes 2, 5*), 100°C (*lanes 3, 6*) or room temperature (*lanes 4, 7*) for 10 min before loading. *Lane 1* protein molecular mass standards

Table 1 Purification of PFA from 5.8 g of wet cells

Purification step	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Total cell protein	1,056.3	138.2	145,950	1.00	100
Centrifugation	834.8	142.1	118,650	1.03	81.3
Solubilization	452.4	1,165.1	527,100	8.43	361
Dilution	347.4	1,489.6	517,500	10.78	355
Phenyl Sepharose	91.9	3,644.2	334,900	26.37	229

form [12]. We have purified the recombinant PFA by the glycerol method according to the reference [12] at the same time and compared it with the PFA purified by the alkaline method presented in this paper. The residual amylase activities were measured according to materials and methods. As shown in Fig. 5a, the purified PFA by these two different methods presented the same thermostable properties after incubating at 100 and 120°C. The thermal stabilities of the recombinant PFA purified by alkaline method at different temperatures were shown in Fig. 5b. The PFA purified by alkaline method showed a half-life of 3 h at 120°C and retained over 10% of its maximal activity even after 40 min of incubation at 128°C (Fig. 5b), which were similar to the results reported before [7, 8, 10].

Discussion

Large-scale and simple purification process with low cost is essential for PFA to meet the requirement in starch industry. This study presents an effective method for purification of PFA from insoluble inclusion bodies. In the present study, PFA gene was cloned and expressed at high levels in *E. coli* as inclusion

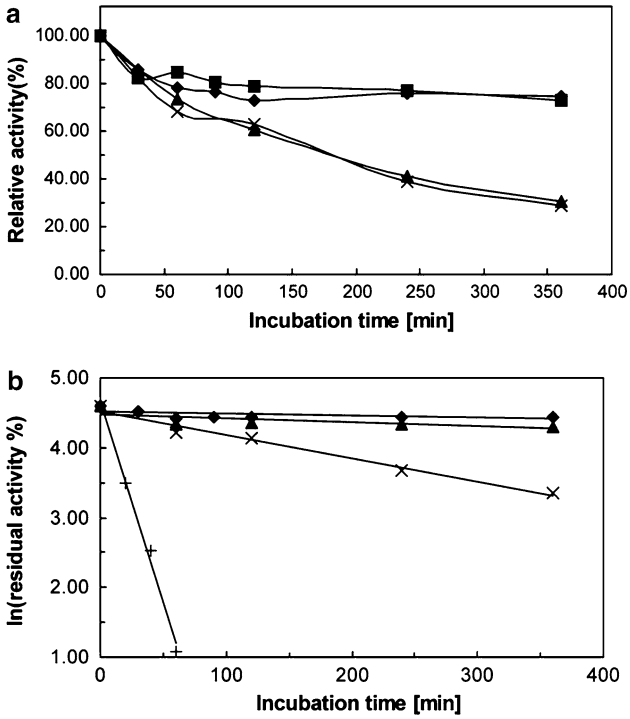

Fig. 5 **a** Thermal stabilities of the recombinant PFA purified by alkaline method (*filled square* and *multi sign*) and by glycerol method (*filled diamond* and *filled triangle*) at 100°C (*filled square* and *filled diamond*) or 120°C (*multi sign* and *filled triangle*). **b** Thermal stabilities of the recombinant PFA purified by alkaline method in 50 mM acetate buffer (pH 6.0) at: 90 (*filled diamond*), 110 (*filled triangle*), 120 (*multi sign*) and 128°C (+)

bodies. The inclusion bodies were solubilized efficiently at alkaline pH along with heat treatment and subsequently purified after passing through a phenyl Sepharose column. The purified enzymes by this alkaline method showed similar characteristic properties with that purified by glycerol method and other methods reported before [5, 8, 12].

One of the major problems associated with high-level expression of recombinant protein in *E. coli* is the formation of inclusion body. A variety of methods can be used to solubilize inclusion bodies; however, the choice of solubilizing agent can greatly impact the subsequent refolding step and the cost of the overall process [4]. Although inclusion body is generally considered to be devoid of biological activity [16, 18], a few reports indicated previously that some recombinant proteins expressed in *E. coli* may form insoluble particles which containing native conformation. Utilization of milder solubilization conditions may help to recover the proteins in the native form from such insoluble particles [17, 18]. On the other hand, proteins inside inclusion body aggregates have been reported to have native-like secondary structures [9, 13, 16]. It is assumed that restoration of this native-like secondary

structure using mild solubilization conditions will help to improve the recovery of bioactive protein in comparison with solubilization using high concentrations of chaotropic agents [13, 16, 18]. In our present study, the recombinant PFA aggregates exhibit high enzymatic activity of 142 U/mg, thus a milder condition is preferred for the solubilization of the inclusion bodies.

Alkaline pH has been successful used to purify recombinant proteins from inclusion bodies [4], especially in the large-scale production of bovine growth hormone [9, 13, 19]. On account of its mildness, low cost and easily scale-up, extreme pH was selected to solubilze PFA inclusion bodies in this study. We found that PFA inclusion bodies could be efficiently solubilized at alkaline pH following a short time of heat treatment. By this simple, economical and efficient method, the yield of active enzyme was greatly increased compared with the works previous reported [7]. This study might be beneficial for the future works on the large-scale productions of thermostable (-amylases with industrial potentials.

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References

1. Bernfeld P (1955) Amylases α- and β-. Methods Enzymol 1:149–158
2. Bradford MM (1976) A rapid and sensitive method for quantication of microgram quantities of protein utilizeing the principle of protein-dye binding. Anal Biochem 72:248–254
3. Crabb WD, Mitchinson C (1997) Enzymes involved in the processing of starches to sugars. Trends Biotechnol 15:349–352
4. Clark ED (2001) Protein refolding for industrial processes. Curr Opin Biotechnol 12:202–207
5. Dong G, Vieille C, Savchenko A, Zeikus JG (1997a) Cloning, sequencing, and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. Appl Environ Microbiol 63:3569–3576
6. Dong G, Vieille C, Savchenko A, Zeikus JG (1997b) Cloning, sequencing, and expression of the gene encoding amylopullulanase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. Appl Environ Microbiol 63:3577–3584
7. Grzybowska B, Szweda P, Synowiecki J (2004) Cloning of the thermostable α-amylase gene from *Pyrococcus woesei* in *Escherichia coli*. Mol Biotechnol 26:101–109
8. Jørgensen S, Vorgias CE, Antranikian G (1997) Cloning, sequencing, characterization, and expression of an extracellular a-amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* in *Escherichia coli* and *Bacillus subtilis*. J Biol Chem 268:16335–16342
9. Khan RH, AppaRao KBC, Eshwari ANS, Totey SM, Panda AK (1998) Solubilization of recombinant ovine growth hormone with retention of native-like secondary structure and its refolding from the inclusion bodies of *Escherichia coli*. Biotechnol Prog 14:722–728
10. Koch R, Spreinat A, Lemke K, Antranikian G (1991) Purification and properties of a hyperthermoactive α-amylase from archaebacterium pyrococcus woesei. Arch Microbiol 155:572–578
11. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
12. Linden A, Niehaus F, Antranikian G (2000) Single-step purification of a recombinant thermostable a-amylase after solubilization of the enzyme from insoluble aggregates. J Chromatogr B 737:253–259
13. Patra AK, Mukhopadhyay R, Mukhija R, Krishnan A, Garg LC, Panda AK (2000) Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from escherichia coli. Protein Expr Purif 18:182–192
14. Rivera MH, Lopez-Munguia, Soberon X, Saab-Rincon G (2003) alpha-Amylase from bacillus licheniformis mutants near to the catalytic site: effects on hydrolytic and transglycosylation activity. Protein Eng 16:505–514
15. Schofield LR, Patchett ML, Parker EJ (2004) Expression, purification, and characterization of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase from *Pyrococcus furiosus*. Protein Expr Purif 34:17–27
16. Singh SM, Panda AK (2005) Solubilization and refolding of bacteiral inclusion body protein. J Biosci Bioeng 99:303–310
17. Tsumoto K, Umetsu M, Kumagai I, Ejima D, Arakawa T (2003) Solubilization of active green fluorescent protein from insoluble particles by guanidine and arginine. Biochem Biophys Res Commun 312:1383–1386
18. Vallejo LF, Rinas U (2004) Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins. Microb Cell Fac 3:11
19. Zeevi M, Artzi HB, Shaked H, Hadary D, Pinhassi D, Salik H, Bartfeld D (1991) Recovery of biologically active recombinant bovine growth hormone from inclusion bodies in *Escherichia coli*. In: White MD (ed) Biologicals from recombinant microorganisms and animal cells. Production and Recovery. VCH, Weinheim, Germany, pp 331–348