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Expression of *Saccharomyces cerevisiae* inorganic pyrophosphatase in *Escherichia coli*

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A segment of DNA encoding *Saccharomyces cerevisiae* inorganic pyrophosphatase (ppa gene) was amplified by the polymerase chain reaction. The pSCH1 and pSCB6 plasmids containing the ppa gene were obtained. Transformation of the *E. coli* BL21 strain with the resulting recombinant plasmids and selection of clones having extremely high expression of inorganic pyrophosphatase (PPase) were carried out. Superproduction of recombinant *S. cerevisiae* PPase up to 50% of the total bacterial protein was achieved. The enzyme was readily obtained and purified to homogeneity with the use of a simple purification technique. This work is the first description of *S. cerevisiae* PPase superproducer creation.

Saccharomyces cerevisiae; Inorganic pyrophosphatase; Gene; Recombinant vector; Expression; *Escherichia coli*; PCR

1. INTRODUCTION

Inorganic pyrophosphatases catalyze inorganic pyrophosphate hydrolysis in the presence of Mg^{2+}. These enzymes are essential for controlling the level of PP_i in the cell. PP_i is formed principally as a product of many biosynthetic reactions that utilize ATP. PPase from *Saccharomyces cerevisiae* is the best-studied enzyme of this class. It consists of two identical subunits of 32 kDa. The amino acid sequence of this enzyme has been determined and the ppa gene has been recently cloned [1,2]. *S. cerevisiae* PPase is the only enzyme of this class for which the 3 Å structure has been determined [3,4]. The most significant result of this work is the demonstration of an active site cavity within the enzyme structure which contains binding sites for at least three metal ions as well as for two phosphates. Using X-ray crystallographic studies, seventeen polar groups were located at the active site. Eight complete PPase sequences from different organisms have been defined, but only 24 residues are conserved among all the eight sequences [5]. Strikingly, 15 of these residues are in the active site cavity. The importance of some functional amino acid residues has been estimated by chemical modification. Detailed kinetic studies allowed the determination of the minimal kinetic scheme for *S. cerevisiae* PPase catalysis and of all microscopic rate constants. Catalysis has been found to proceed by two parallel pathways, involving 3 or 4 Mg^{2+}/PPI_i or 2 P_i [6].

The results of the sequencing, structural and kinetic studies provide no way of elucididating the detailed mechanism of chemical catalysis and regulation of the enzyme activity. The most informative among currently available methods for solving this problem is site-specific mutagenesis. Thus, there is a need to obtain high expression of the *S. cerevisiae* PPase cloned gene. This paper is concerned with expression of the *S. cerevisiae* PPase gene in *E. coli* and purification of the recombinant enzyme.

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2. MATERIALS AND METHODS

2.1. *DNA manipulation methods*

Isolation and purification of DNA from the *Saccharomyces cerevisiae* SKY594 strain and of plasmid vectors pUC19 and pET3c, digestion of plasmids by restriction endonucleases, isolation of restriction fragments from agarose gels, dephosphorylation of the linearized vectors with calf intestinal phosphatase, ligation and transformation of E. coli strains were carried out as described in [7,8].

2.2. *Polymerase chain reaction (PCR)*

PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments). 25 ml of the reaction mixture contained 2–4 mg of *S. cerevisiae* DNA digested by *Hin*dIII, 4 mg of each 20-mer oligonucleotide, 200 mM dNTPs in PCR buffer (67 mM Tris-HCl (pH 9.1), 100 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 1.8 mg/ml BSA). The samples were heated at 97°C for 10 min. Primer extension was initiated by adding 2–5 units of Taq-polymerase. The subsequent cycles were carried out at 94°C for 1 min, at 44°C for 2 min and at 72°C for 3 min. After 30 cycles of primer extension 5 ml of the samples were electrophoresed in 1.5% agarose gel [8].

2.3. *Plasmid construction*

The recombinant plasmid supporting the ppa gene was constructed by ligating the PCR product into the pUC19 vector linearized with *Sma*I and dephosphorylated. *E. coli* strain XL1 was transformed with the ligase mixture and grown overnight in LB medium. The plasmid from the recombinant clones was isolated and digested by *Eco*RI and *Hin*dIII or *Nde*I and *Hin*dIII (*Bam*HI). The *Nde*I–*Hin*dIII (*Bam*HI) fragments were isolated and purified. Vector pET3c linearized with *Nde*I and *Hin*dIII (*Bam*HI) was ligated with the *Ned*I–*Hin*dIII

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(*Bam*HI) fragments. *E. coli* strain BL21 (DE3) was transformed with the ligase mixture and grown overnight in LB medium. Recombinant plasmids pSCB6, containing the *Nde*I–*Bam*HI fragment, and pSCH1, containing the *Nde*I–*Hin*dIII fragment, were obtained. *E. coli* strain BL21 (DE3) was transformed with pSCB6 or pSCH1.

2.4. *Expression of* S. cerevisiae *inorganic pyrophosphatase*

A single colony was picked from an LB medium plate and cells were grown overnight in 3 ml of the LB medium containing ampicillin (20 mg/ml) under stirring at 37°C. The cells (0.5 ml) were pelleted by centrifugation, resuspended in 100 ml of 50 mM Tris-HCl (pH 7.6) containing lysozyme (1 mg/ml), frozen and melted. Fifteen min later the reaction mixture was centrifuged and the precipitate was discarded. The enzyme activity was detected in the supernatant.

2.5. *Method for PPase production*

For PPase production, transformants were grown under vigorous stirring at 37°C to an A_{550} of 5.0 in 180 ml of the LB medium, containing ampicillin (20 mg/ml). The cells were pelleted by centrifugation (4,000 × *g*, 30 min, 4°C), resuspended in 2 ml of 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 100 mM NaCl, 2 mM phenylmethylsulfonylfluoride and disrupted by lysozyme [8].

Then 3 ml of 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 100 mM NaCl were added to the reaction mixture. The suspension was centrifuged for 20 min at 15,000 × *g* and 4°C, and nucleic acids were precipitated from the supernatant by the slow addition of a one-third volume of 5% streptomycin sulfate. After incubation for 20 min at 0°C, the precipitate was pelleted and discarded. The proteins were precipitated with $(NH_4)_2SO_4$ (90% saturation. After gel filtration (Sephadex G-50 coarse) the protein solution in 30 mM Tris-HCl (pH 7.6) containing 3 mM PP_i was applied to a DEAE-Sepharose column. Elution was carried out with the same buffer containing 1 mM PP_i and 0.02, 0.1 and 0.2 M NaCl, subsequently. The purity of the enzyme preparation was checked by native 12% PAGE-electrophoresis and gel filtration on a Toyopearl column. The PPase activity was assayed according to [9].

3. RESULTS AND DISCUSSION

The aim of this work was to produce with high yield and purify to homogeneity *Saccharomyces cerevisiae* PPase from *E. coli*. The present paper describes the procedure employed.

The cloning and sequencing of the *S. cerevisiae* ppa gene [2] enabled us to apply molecular biology techniques to develop high expression of *S. cerevisiae* PPase. The polymerase chain reaction was used to amplify a DNA segment containing the ppa gene of the known sequence. Two 20-mer specific primers were designed for this purpose:

5′-GCATATGACCTACACTACCA-3′ (primer 1),
5′-GGCCTTGTAGGAGGCAAGAT-3′ (primer 2).

The first primer contained the *Nde*I site at the 5′-end. The melting temperature of the duplex formed by this primer and matrix DNA was equal to 46°C. The second primer was complementary to matrix DNA and the melting temperature of this duplex was equal to 62°C. PCR was carried out as described in section 2. The conditions were so that synthesis of the desired 1.0 kb fragment took place (Fig. 1). The synthesized blunt-ended fragment was ligated into the *Sma*I site of plasmid pUC19. The resulting recombinant plasmid was used for the transformation of the *E. coli* XL1 strain and six recombinant clones were obtained. The 1.0 kb fragment starting from the *Eco*RI site and ending at the *Hin*dIII site was generated from the plasmid and the existence of an insert encoding the total ppa gene was proved. Digestion of the recombinant plasmid by *Nde*I and *Bam*HI or *Nde*I and *Hin*dIII enabled us to obtain an insert of correct orientation. These fragments were ligated with the pET3c plasmid digested by *Nde*I and *Bam*HI or *Nde*I and *Hin*dIII. This plasmid vector is convenient for the production of foreign proteins in *E. coli* because it contains a strong f10 promotor for T7 DNA polymerase which is capable of producing complete transcripts of almost any DNA that is placed under control of the T7 promotor [10].

The recombinant plasmids were transformed into the *E. coli* BL21 (DE3) strain. The transformants were examined and heavy plasmids were selected. Plasmid pSCB6, containing the *Nde*I–*Bam*HI insert, and plasmid pSCH1, containing the *Nde*I–*Hin*dIII insert, were obtained. The pSCH1 plasmid was more stable than pSCB26. The *E. coli* BL21 (DE3) strain was transformed with pSCB6 or pSCH1. Two clones containing pSCB6 and 40 clones containing pSCH1 were obtained and the level of the PPase activity was determined in them. Two clones containing pSCB6 and 30 clones containing pSCH1 had extremely high PPase activity.

The extremely high expression of the plasmid-encoded PPase made possible the *S. cerevisiae* PPase production of up to 5% of the *E. coli* cell weight or up

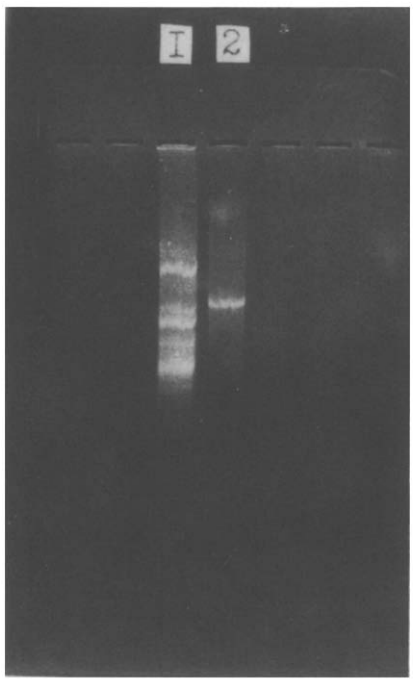

Fig. 1. Agarose gel electrophoresis of the PCR product. Lane 1, M13 mp9 MspI markers; lane 2, *S. cerevisiae* DNA fragment 1.0 kb in length.

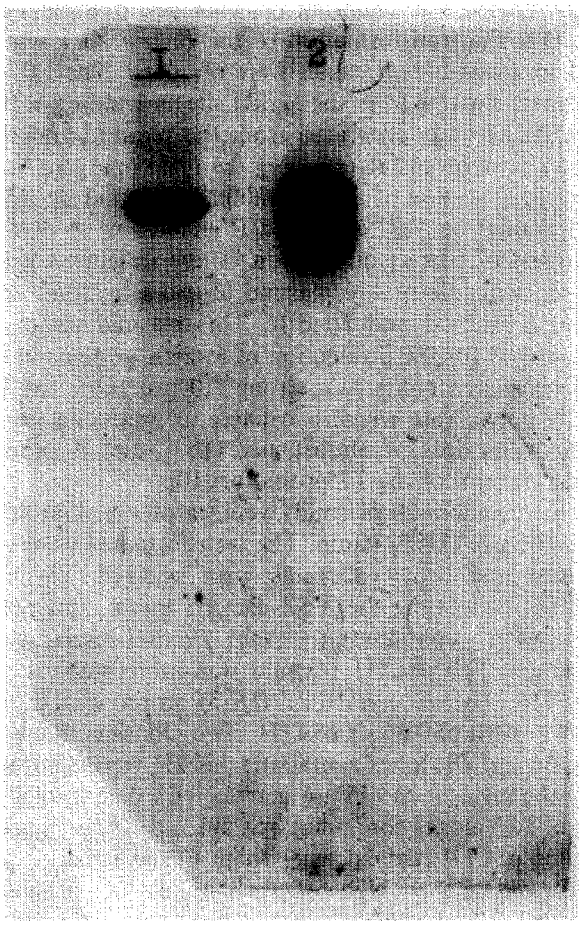

Fig. 2. 12% PAGE of *S. cerevisiae* PPase. Lane 1, the recombinant enzyme; lane 2, the native enzyme.

to 50% of the total *E. coli* protein content. Hence, the enzyme was readily obtained and purified to homogeneity as described in section 2 owing to the high production of recombinant *S. cerevisiae* PPase. So, 20 mg of *S. cerevisiae* PPase can be produced starting from 180 ml of the overnight *E. coli* culture (400 mg of cells). The enzyme specific activity is equal to 1300 EU at 25°C (native enzyme has 1500 EU at 30°C [11]). The recombinant *S. cerevisiae* PPase is homogeneous according to native PAGE-electrophoresis and gel filtration data. Electrophoretic mobilities of the recombinant *S. cerevisiae* PPase and of the enzyme isolated by the classical biochemical procedure were approximately the same (Fig. 2). The retention time of the recombinant PPase corresponded to a molecular mass equal to 66.3 ± 3.7 kDa according to gel filtration data. The native enzyme has 64 kDa.

It is worth noting that only 100 mg of PPase may be obtained from 2 kg of baker's yeast [11]. The modern strategy based on using a recombinant plasmid containing the ppa gene allows one to isolate 20 mg of *S. cerevisiae* PPase from 0.4 g of bacterial culture. The sizeable accumulation of *S. cerevisiae* PPase in a foreign host invites the detailed investigation of structural and kinetic properties of recombinant PPase. This work is now in progress.

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