Pergamon

Int. J. Biochem. Cell Biol. Vol. 29, No. 2, pp. 303–310, 1997

Printed in Great Britain
PII: S1357-2725(96)00088-X 1357-2725/97 $17.00 + 0.00

Some Properties of Inorganic Pyrophosphatase from *Bacillus subtilis*

TOMOMI SHIMIZU,[1] MIZUHIRO IMAI,[2] SHIN ARAKI,[3] KEI KISHIDA,[4] YASUSHI TERASAWA,[5] AKIRA HACHIMORI[1]*

[1]*Institute of High Polymer Research, Faculty of Textile Science and Technology, Shinshu University, 3-15-1, Tokida, Ueda, Nagano, 386, Japan,* [2]*Aiwa Co., Ltd, 202-1, Uenohara, Okaya, Nagano, 394, Japan,* [3]*Food Research Laboratory, Asahimatsu Foods Co., Ltd, 1008, Dashina, Iida, Nagano, 399-25, Japan,* [4]*Obusedo Corp., 808, Obuse, Obuse-machi, Shimotakai-gun, Nagano, 381-02, Japan and* [5]*Nagano Denpa, 837-2, Okada, Shinonoi, Nagano, 381-22, Japan*

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1; PPase) from *Bacillus subtilis* was purified to a homogeneous state electrophoretically when analysed by SDS–PAGE. The enzyme consists of six identical subunits; the molecular weight of the native enzyme estimated by gel filtration was approx. 120 000, and denaturing polyacrylamide gel electrophoresis gave a single band corresponding to 24 000. The enzyme absolutely required a divalent cation for its activity. Mg^{2+} was most effective, showing two steps of concentration-dependent activation. Mg^{2+} could be partially replaced by Mn^{2+} and Co^{2+}. The enzyme was thermostable in the presence of Mg^{2+}, and no loss of activity was observed on the incubation at 55°C for an hour.

Keywords: ***Bacillus subtilis*** **Inorganic pyrophosphatase Thermostability**

Int. J. Biochem. Cell Biol. (1997) **29**, 303–310

INTRODUCTION

Inorganic pyrophosphatase plays an important role in energy metabolism, providing a thermodynamic pull for biosynthetic reactions such as protein, RNA and DNA syntheses (Kornberg, 1962), and thus is essential for the viability of organisms, as has been demonstrated in bacteria (Chen *et al.*, 1990) and yeast (Lundin *et al.*, 1991). The enzyme was purified from several sources and characterized (Morita and Yasui, 1978; Lahti, 1983; Hachimori *et al.*, 1983; Verhoeven *et al.*, 1986; Smirova and Baykov, 1989; Lin and Le Gall, 1990; Jetten *et al.*, 1992; Richter and Schäfer, 1992; Wakagi *et al.*, 1992), the cytoplasmic *Saccharomyces cerevisiae* (Cooperman, 1982; Kuranova *et al.*, 1983, Cooperman *et al.*, 1992; Terzyan *et al.*, 1984; Welch *et al.*, 1983; Gonzales and Cooperman, 1986; Kolakowski *et al.*, 1988) and *Escherichia coli* enzymes (Josse and Wong, 1971; Lahti *et al.*, 1988; Lahti *et al.*, 1990a; Lahti *et al.*, 1990b; Lahti *et al.*, 1991a; Lahti *et al.*, 1991b; Käpylä *et al.*, 1995; Velichko *et al.*, 1995) having been best studied.

Yeast PPase is a homodimer of 286 amino acids (Kolakowski *et al.*, 1988), whereas *E. coli* PPase is a homohexamer of 175 amino acids (Lahti *et al.*, 1988). Terzyan *et al.* (1984) determined the crystal structure of the yeast apo-enzyme at 3Å resolution and identified the active site cavity, which contains 17 polar residues that could interact with bound metal ions and with a substrate. The alignment of the sequences of several soluble PPases including the yeast and *E. coli* enzymes indicated that the active site residues are very well conserved, even though the overall level of sequence similarity is low (Cooperman *et al.*, 1992). Kankare *et al.*

*To whom all correspondence should be addressed.

Abbreviations: PPase, inorganic pyrophosphatase; PPi, inorganic pyrophosphate; PAGE, polyacrylamide gel electrophoresis.

Received 18 July 1996; accepted 19 July 1996.

(1994) determined the three-dimensional structure of the *E. coli* enzyme at 2.7Å resolution. In spite of little sequence similarity beyond the active center and different oligomeric organization, they found that the overall fold of the *E. coli* enzyme subunit is the same as the *S. cerevisiae* subunit, except that the latter is longer at both the N- and C-termini. Such a resemblance in the three-dimensional structure was also found for the PPases from *Thermus thermophilus* on X-ray diffraction determination at 2.0Å (Teplyakov *et al.*, 1994) and from *Sulforobus acidocaldarius* by computer analysis of homology modeling (Meyer *et al.*, 1995). These facts indicate that the three-dimensional structure as well as the active site residues may be the same among PPases irrespective of their origin.

We previously reported the primary structure of the PPase from thermophilic bacterium PS-3 (Ichiba *et al.*, 1990). The overall identity between the *E. coli* and PS-3 enzymes is 48%, and that between the PS-3 and *T. thermophilus* enzyme is 58% including the well-conserved active site residues. These PPases from *E. coli*, thermophilic bacterium PS-3 and *T. thermophilus* are quite thermostable in the presence of divalent cations (Hachimori *et al.*, 1979; Kuranova *et al.*, 1987; Ichiba *et al.*, 1988); no loss of activity was observed on incubation for 1 hr at 60°C for the *E. coli* enzyme, at 70°C for the thermophilic bacterium PS-3 enzyme, and at 80°C for the *T. thermophilus* enzyme. On the other hand, the enzyme from a thermoacidophilic archaebacterium, *S. acidocaldarius*, was reported to be extremely thermostable even in the absence of divalent cation (Wakagi *et al.*, 1992): no loss of activity was observed after incubation for 10 min at 100°C. However, little information explaining the mechanism underlying the thermostability of these PPases is obtainable from their three-dimensional structures or on comparison of their primary structures. In order to obtain a better insight into the mechanism underlying the thermostability of PPases at the molecular level, more information on the primary structure and thermostability from other bacterial PPases is necessary.

Tono and Kornberg (1967) reported the purification and characterization of inorganic pyrophosphatase from *Bacillus subtilis*. However, their purification was performed by means of preparative polyacrylamide gel electrophoresis twice. In the present paper, we employed column chromatographies for purification of the inorganic pyrophosphatase from *Bacillus subtilis*, and some properties including the thermostability of the enzyme are described.

MATERIALS AND METHODS

Materials

ATP, ADP, AMP, GTP, glucose-6-phosphate, glucose-1-phosphate, and fructose-6-phosphate were purchased from Boehringer-Mannheim. Phenylmethylsulfonyl fluoride and *p*-nitrophenyl phosphate were obtained from Wako Pure Chemical Co., Ltd. Sodium tripolyphosphate was from Sigma. Marker Proteins for gel filtration and SDS–PAGE were obtained from Bio-Rad. Phenyl Sepharose Cl-4B and Sephacryl S-300 were obtained from Pharmacia. Phenyl 5-PW and Shodex IEC-DEAE 825 were purchased from Toso Co., Ltd and Showa Denko Co., Ltd, respectively. All other chemicals used were of analytical grade.

Organism and cell growth

Bacillus subtilis strain AC327 was cultured in LB medium. Cells were harvested just before the stationary growth phase by centrifugation and then stored at -20°C.

Enzyme assay

Inorganic pyrophosphatase activity was assayed at 37°C according to the procedure described previously (Hachimori *et al.*, 1975) in the following reaction mixture; 20 mM Tris-HCl (pH 7.3), 2 mM $MgCl_2$, 1 mM sodium pyrophosphate and sufficient enzyme to hydrolyze about 30% of the substrate in 10 min. Specific activity was expressed in units (U) defined in terms of μmoles of inorganic phosphate liberated in 1 min by 1 mg protein.

Protein concentration measurement

The protein concentration in solution was determined with a Pierce BCA protein assay kit, using bovine serum albumin as a standard.

Molecular weight estimation

The molecular weight of the native enzyme was estimated by gel filtration. The protein solution (2 ml) was applied to a Sephacryl S-300 column (2×90 cm) and eluted with a buffer comprising 20 mM Tris-HCl (pH 7.3), 2 mM $MgCl_2$ and 0.1 M KCl. The molecular weight of

the subunit was measured by poly-acrylamide gel electrophoresis in the presence of 0.1% SDS and 1 mM 2-mecaptoethanol according to the method of Laemmli (1970), using a 10% gel. Standard proteins (5 mg each) and inorganic pyrophosphatase were incubated in boiling water bath for 5 min in the presence of 1% SDS and 1 mM 2-mercaptoethanol prior to electrophoresis.

Purification of inorganic pyrophosphatase from Bacillus subtilis

All the procedures were carried out at 4°C except the high performance liquid chromatographies (HPLC), which were carried out at 20°C.

Frozen *B. subtilis* cell paste (200 g) was stirred in a Bead Beater for 1 min with 100 g of glass beads and 200 ml of Buffer A (20 mM Tris-HCl, pH 7.3, 10 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride) containing a small amount of DNase. The homogenate was then left for the glass beads to settle. The supernatant was decanted and the beads were washed with another 200 ml of Buffer A. The decanted fluid was combined and centrifuged, and the supernatant was collected (crude extract). The protein fraction precipitated from the crude extract on 55–90% saturation with ammonium sulfate was collected, dissolved in a small volume of Buffer A, and then dialyzed against the same buffer ($(NH_4)_2SO_4$ fractionation). The protein solution thus obtained was placed on a column of DEAE-cellulose (DE-52, 2.5 × 27 cm), which had been equilibrated with 20 mM Tris-HCl, pH 7.3, containing 10 mM $MgCl_2$ (Buffer B). The column was washed with Buffer B containing 50 mM NaCl, and then the enzyme was eluted with a linear gradient of 50 mM to 200 mM NaCl in Buffer B, fractions of 200 drops being collected (DE-52). The active fractions were collected, brought to 25% saturation with ammonium sulfate, and then applied to a column of Phenyl Sepharose Cl-4B (2.3 × 18 cm), which had been equilibrated with Buffer B saturated 25% with ammonium sulfate. Linear gradient elution, from 25 to 0% ammonium sulfate saturation, with Buffer B was carried out (total volume, 400 ml), fractions of 80 drops being collected (Phenyl Sepharose). The active fractions were combined, dialyzed against Buffer B, and then concentrated to 1 ml in a collodion bag. The protein pool was loaded on to a Shodex IEC-DEAE 825 column (0.8 × 7.5 cm) which had been equilibrated with Buffer B. Ten milliliters of 120 mM NaCl, followed by a 45 ml gradient of 120–250 mM NaCl, both in Buffer B, were applied to the column at the flow rate of 0.75 ml/min, fractions of 10 drops being collected (DEAE-HPLC). The elution profile is shown in Fig. 1(A). The active fractions were collected and brought to 30% saturation with ammonium sulfate. After concentration in a collodion bag, the protein solution was applied to a HPLC-column of Phenyl-5PW (0.75 × 7.5 cm), which had been equilibrated with Buffer B saturated 30% with ammonium sulfate. The column was developed with a linear gradient, from 30 to 0% ammonium sulfate saturation, of Buffer B at the flow rate of 0.75 ml/min, fractions of five drops being collected (Phenyl-HPLC). The elution pattern is shown in Fig. 1(B). The active fractions were collected and their purity was examined by SDS–PAGE. As shown in Fig. 2, the preparation after the HPLC on Phenyl

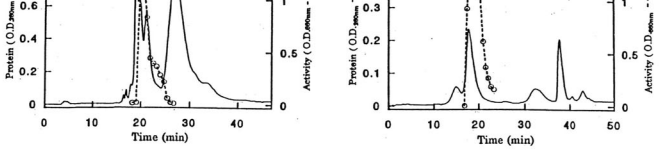

Fig. 1. (A) Anion exchange chromatography on a IEC-DEAE 825 column and (B) hydrophobic chromatography on a Phenyl 5-PW column, of the PPase from *B. subtilis*. The experimental conditions for each chromatography are given in Materials and Methods.

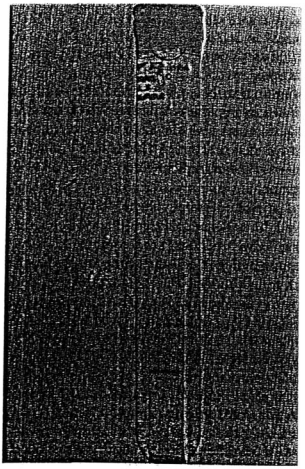

Fig. 2. SDS-PAGE of the purified PPase from *B. subtilis*. A 10% gel was used and 5 μg of protein was applied.

5-PW was homogeneous. The purified PPase was concentrated in a collodion bag and then stored at $-20°C$ in a 50% solution of glycerol in Buffer B. The results of a typical purification of PPase from *Bacillus subtilis* are summarized in Table 1.

RESULTS

Molecular weight

The molecular weight of the purified PPase was estimated to be 120 000 from its elution volume on Sephacryl S-300 gel chromatography [Fig. 3(a)], and that of the subunit to be 24 000 by SDS–PAGE [Fig. 3(B)]. Thus, the PPase from *B. subtilis* appears to consist of six identical subunits.

Effects of divalent cations and pyrophosphate

A divalent cation was absolutely required for the enzyme activity, Mg^{2+} being most effective. As can be seen in Fig. 4, Mg^{2+} activated the enzyme in two steps. When the concentration of sodium pyrophosphate was fixed at 1 mM, the first maximal activity was observed at a Mg^{2+} concentration between 0.5 and 1 mM. With a further increase in the Mg^{2+} concentration, the activity again increased and became constant beyond 3.5 mM Mg^{2+}. Mg^{2+} could be partially replaced by Mn^{2+} and Co^{2+}. However, Mn^{2+} and Co^{2+} did not cause the second activation observed with Mg^{2+}. Other metal ions tested showed no effect on the enzyme activity.

Figure 5 shows the pyrophosphate-concentration dependency of the enzyme activity when the concentration of divalent cations was fixed. With 1 mM Mg^{2+} and Co^{2+}, the maximal activity was observed at the PP_i concentration of 0.5 mM, and the activity decreased beyond that concentration. With 1 mM Mn^{2+}, the activity increased gradually until 1 mM PP_i and then became constant beyond that concentration. When 5 mM Mg^{2+} was employed, the activity increased abruptly with increasing PP_i concentration, and reached a maximum at a PP_i concentration of 1.2–1.5 mM. The activity decreased rapidly beyond this concentration of PP_i.

Substrate specificity

The enzyme was quite specific for the hydrolysis of inorganic pyrophosphate. Some phosphate esters were also examined as substrates by measuring the release of inorganic phosphate. Nucleotide mono-, di- and tri-phosphate, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, *p*-nitrophenylphosphate and polyphosphate did not serve as substrates.

Enzyme stability

The inorganic pyrophosphatase from *B. subtilis* was incubated in 20 mM Tris-HCl buffer, pH 7.3, in the presence and absence of

Table 1. Purification of inorganic pyrophosphatase from *Bacillus subtilis* (200 g of cell paste used)

	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
Crude extract	11 090.0	15 800	1.42	100.0
$(NH_4)_2SO_4$ fractionation	3 260.0	10 460	3.21	66.2
DE-52	406.0	7 026	17.3	44.5
Phenyl sepharose	48.4	6 270	130.0	39.7
DEAE-HPLC	4.11	1 980	482.0	12.5
Phenyl-HPLC	0.20	349	1 750.0	2.2

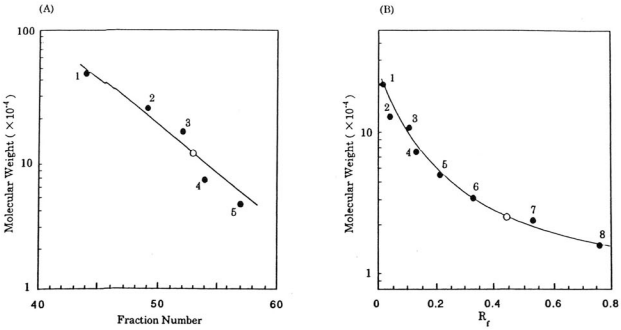

Fig. 3. (A) Molecular weight determination of PPase on Sephacryl S-300 and (B) of its subunit by SDS-PAGE. As molecular size markers, (1) ferritin, (2) catalase, (3) aldolase, (4) bovine surum albumin, and (5) ovalbumin, were all used in (A); and (1) myosin, (2) β-galactosidase, (3) phosphorylase b, (4) bovine serum albumin, (5) ovalbumin, (6) carbonic anhydrase, (7) trypsin inhibitor, and (8) lysozyme, were all used in (B). The open symbols show the positions of PPase and its subunit.

Mg^{2+} at various temperatures, and the remaining activity was measured at various times. The results are shown in Fig. 6. In the absence of Mg^{2+}, the enzyme was quite stable at 30°C, but the enzyme activity decreased gradually on incubation even at 35°C. The rate of inactivation increased with increasing incubation temperature. The enzyme activity was completely lost on incubation for 55 min at 55°C and for 30 min at 60°C [Fig. 6(A)]. However, the enzyme showed remarkable thermostability on the addition of 2 mM Mg^{2+} to the incubation medium. No loss of activity was observed on incubation for 1 hr at 55°C, but the enzyme lost its activity beyond 60°C [Fig. 6(B)].

DISCUSSION

Tono and Kornberg (1967) reported the purification and some properties of the inorganic pyrophosphatase from *Bacillus subtilis*. They purified the enzyme by means of preparative PAGE twice, with a yield of 30%. However, since the preparative PAGE needs the apparatus, we prepared electrophoretically homogeneous PPase from *B. subtilis* by means of several column chromatographies as described in the present paper, although the yield was low. The molecular weight of the purified enzyme was revealed to be 120 000 and that of

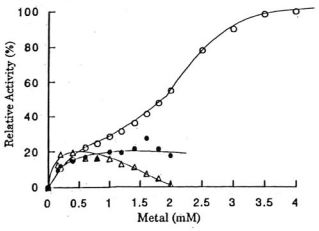

Fig. 4. Effect of divalent cations on the activity of *B. subtilis* PPase. The concentration of sodium pyrophosphate was fixed at 1 mM. ○, Mg^{2+}; △, Mn^{2+}; ●, Co^{2+}.

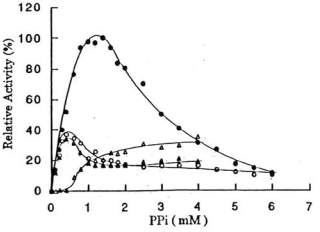

Fig. 5. Effect of the pyrophosphate concentration on the enzyme activity. The activity was measured in the presence of 1 mM Mg^{2+} (○), 5 mM Mg^{2+} (●), 1 mM Mn^{2+} (△) and 1 mM Co^{2+} (▲).

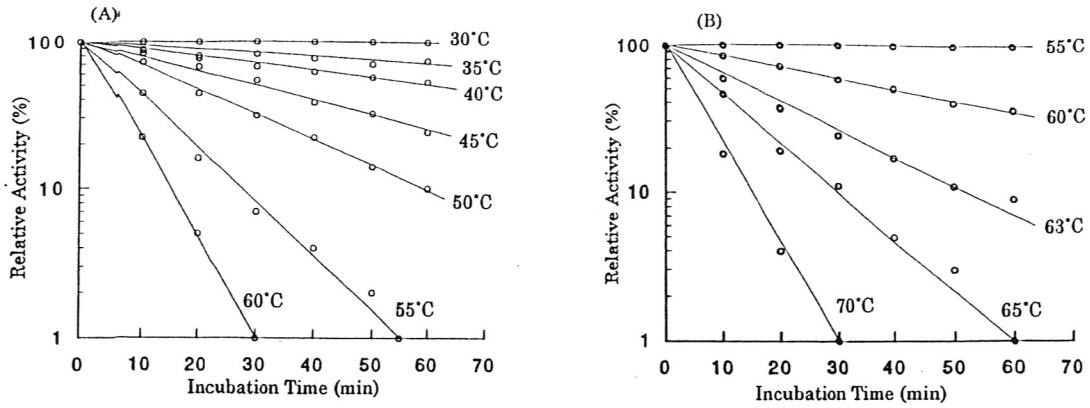

Fig. 6. Thermal inactivation of *B. subtilis* PPase in the (A) absence and (B) presence of 2 mM Mg^{2+}. The enzyme (30 μg/ml) was incubated in 20 mM Tris-HCl, pH 7.3, in the absence and presence of 2 mM Mg^{2+} at various temperatures as indicated. Aliquots were withdrawn at the indicated times and assayed at 37°C.

the subunit to be 24 000 on gel chromatography and SDS–PAGE, respectively, suggesting that the *B. subtilis* PPase consists of six identical subunits. Tono and Kornberg (1967) reported that the molecular weight of their preparation was 68 000, as judged from the results of sucrose gradient sedimentation. Since the amino acid composition and pH-activity profile (data not shown) indicated that our preparation is the same protein with that prepared by Tono and Kornberg (1967), the difference in estimation in molecular weight might be due to the difference in analytical methods. Studies so far report that, most eubacterial and archaebacterial PPases are homohexamers or homotetramers composed of approx. 20 000 subunits (Tominaga and Mori, 1977; Hachimori *et al.*, 1979; Lahti and Niemi, 1981; Verhoeven *et al.*, 1986; Lahti *et al.*, 1988; Richter and Schäfer, 1992; Wakagi *et al.*, 1992; Teplyakov *et al.*, 1994; Van Alebeek *et al.*, 1994; Meyer *et al.*, 1995), except those from *Desulfovibrio desulfuricans*, which is a monomer of molecular weight of 42 000 (Ware and Postgate, 1971) and from *Methanothrix soehngeni* which have an $a_2\beta_2$ oligomeric structure of 33 000 and 35 000 subunits (Jetten *et al.*, 1992). Although we cannot say which estimation is correct at present, our estimation seems to be more likely because PPases from *Bacillus stearothermophilus* and thermophilic bacterium PS-3 which belongs to *Bacillus* are homohexamers composed of about 20 000 subunits (Hachimori *et al.*, 1979).

The *B. subtilis* PPase showed a very characteristic property in terms of cation requirement. Mg^{2+} activated the enzyme in two steps in a concentration-dependent manner, which was not detected by Tono and Kornberg (1967) because they examined the cation requirement with a fixed metal ion concentration: 0.5 mM metal ion at the PPi concentration of 1 mM. They found that Mn^{2+} was most effective at this lower metal ion concentration, which was confirmed in the present study. Beyond 1 mM concentration, the enzyme activity decreased gradually when Mn^{2+} was used, whereas it increased again rapidly with increasing concentration when Mg^{2+} was used, and became constant beyond 3 mM Mg^{2+} when the PP_i concentration was fixed at 1 mM (Fig. 4). However, higher concentration of PP_i inhibited the enzyme activity when the Mg^{2+} concentration was fixed (Fig. 5). Butler and Sperow (1977) suggested three roles of Mg^{2+} in PPase: structural, substrate and activator, in order of effectiveness. Our data suggested that there are more than two binding sites for Mg^{2+} with different affinity constants in the *B. subtilis* enzyme. This is likely because the *E. coli* and yeast enzymes were reported to bind 3–4 Mg^{2+} and 3 Mg^{2+} per subunit molecule, respectively (Cooperman *et al.*, 1992). Since PP_i makes a complex with Mg^{2+} which is the true substrate for PPase, a higher concentration of PP_i probably decreases the effective concentration of free Mg^{2+} which is necessary for letting PPase conformation more active as to the hydrolysis of a substrate. Therefore, *B. subtilis* PPase decreased its activity at higher concentration of PP_i. The conformational change on binding with Mg^{2+} might also make the enzyme molecule thermostable heat denaturation. The Mg^{2+}-induced conformational change and thermostability have been reported for the PPases

from yeast, *E. coli*, *B. stearothermophilus*, thermophilic bacterium PS-3 and *T. thermophilus*, although the maximum temperature for thermostability is different in each case (Hachimori *et al.*, 1979; Kuranova *et al.*, 1987; Ichiba *et al.*, 1988). Thus, Mg^{2+}-induced thermostability might be a common property of PPases irrespective of their origin, except for the enzyme from *Sulfolobus acidocaldarius*, which is extremely thermostable even in the absence of divalent cation (Wakagi *et al.*, 1992). The effect of Mg^{2+} on the conformation of the *B. subtilis* PPase is now being examined in our laboratory.

REFERENCES

Butler L. G. and Sperow J. W. (1977) Multiple roles of metal ions in the reaction catalyzed by yeast inorganic pyrophosphatase. *Bioinorg. Chem.* **7,** 141–150.

Chen J., Brevet A., Froman M., Leveque F., Schmitte J.-M., Blanquet S. and Plateau P. (1990) Pyrophsphatase is essential to the growth of *Escherichia coli*. *J. Bacteriol.* **172,** 5686–5689.

Cooperman B. S. (1982) The mechanism of action of yeast inorganic pyrophosphatase. *Meths Enzymol.* **87,** 526–548.

Cooperman B. S., Baykov A. A. and Lahti R. (1992) Evolutionary conservation of the active site of soluble inorganic pyrophosphatase. *Trends Biochem. Sci.* **17,** 262–266.

Gonzales M. A. and Cooperman B. S. (1986) Glutamic acid-149 is important for enzymatic activity of yeast inorganic pyrophosphatase. *Biochemistry* **25,** 7179–7185.

Hachimori A., Takeda A., Kaibuchi M., Ohkawara N. and Samejima T. (1975) Purification and characterization of inorganic pyrophosphatase from *Bacillus stearothermophilus*. *J. Biochem.* **77,** 1177–1183.

Hachimori A., Shiroya Y., Hirato A., Miyahara T. and Samejima T. (1979) Effect of divalent cations on thermophilic inorganic pyrophosphatase. *J. Biochem.* **86,** 121–130.

Hachimori A., Fujii T., Ohki K. and Iizuka E. (1983) Purification and properties of inorganic pyrophosphatase from porcine brain. *J. Biochem.* **93,** 257–264.

Ichiba T., Shibasaki T., Iizuka E. and Hachimori A. (1988) Cation-induced thermostability of yeast and *Escherichia coli* pyrophosphatase. *Biochem. Cell Biol.* **66,** 25–31.

Ichiba T., Takenaka O., Samejima T. and Hachimori A. (1990) Primary structure of the inorganic pyrophosphatase from thermophilic bacterium PS-3. *J. Biochem.* **108,** 572–578.

Jetten M. S. M., Fluit T. J., Stams A. J. M. and Zehnder A. J. B. (1992) A fluoride-insensitive inorganic pyrophosphatase isolated from *Methanothrix soehngenii*. *Arch. Microbiol.* **157,** 284–289.

Josse J. and Wong S. C. K. (1971) Inorganic pyrophosphatase from *Escherichia coli*. In *The Enzymes* (Edited by Boyer, P. D.) Vol. 4, pp. 499–527, Academic Press, New York.

Kankare J., Genevieve S. N., Salminen T., Glumhoff T., Cooperman B. S., Lahti R. and Goldman A. (1994) The structure of *E. coli* soluble inorganic pyrophosphatase at 2.7Å resolution. *Protein Eng.* **7,** 823–830.

Käpylä J., Hyytiä T., Lahti R., Goldman A., Baykov A. A. and Cooperman B. S. (1995) Effect of D97E substitution on the kinetic and thermodynamic properties of *Escherichia coli* inorganic pyrophosphatase. *Biochemistry* **34,** 792–800.

Kolakowski L. F. Jr., Schlösser M. and Cooperman B. S. (1988) Cloning, molecular characterization and chromosome localization of the inorganic pyrophosphatase (*PPA*) gene from *S. cerevisiae*. *Nucleic Acid Res.* **22,** 10441–10452.

Kornberg A. (1962) On the metabolic significance of phosphorolytic and pyrophosphorolytic reactions. In *Horizons in Biochemistry* (Edited by Kasha H. and Pullman P.), pp. 251–264, Academic Press, New York.

Kuranova I. P., Terzyan S. S., Voronova A. A., Smirnova E. A., Vanistein B. K., Höhne W. and Hansen G. (1983) Active site structure of the inorganic pyrophosphatase from baker's yeast based on the X-ray investigation. *Bioorg. Khim* **9,** 1611–1619.

Kuranova I., Obmolova G. and Konarava N. V. (1987) Inorganic pyrophosphatase from the extremely thermophilic bacterium *Thermus thermophilus*. *Dokl. Acad. Nauk. SSSR* **295,** 1013–1016.

Laemmli U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lahti R. and Niemi T. (1981) Purification and some properties of inorganic pyrophosphatase from *Streptococcus faecalis*. *J. Biochem.* **90,** 79–85.

Lahti R. (1983) Microbial inorganic pyrophosphatase. *Microbiol. Rev.* **47,** 169–179.

Lahti R., Pitkäranta T., Valve E., Ilta I., Kukko-Kalske E. and Heinonen J. (1988) Cloning and characterization of the gene encoding inorganic pyrophosphatase of *Escherichia coli K*-12. *J. Bacteriol.* **170,** 5901–5907.

Lahti R., Kolakowski L. F. Jr., Heinonen J., Vihinen M., Pohjanoksa K. and Cooperman B. S. (1990a) Conservation of functional residues between yeast and *E. coli* inorganic pyrophosphatase. *Biochem. Biophys. Acta* **1038,** 338–345.

Lahti R., Pohjanoksa K. itkär, Pitkäranta T., Heikinheimo P., Salminen T., Meyer P. and Heinonen J. (1990b) A site-directed mutagenesis study on *Escherichia coli* inorganic pyrophosphatase. Glutamic acid-98 and lysine-104 are important for structural integrity, whereas aspartic acids-97 and -102 are essential for catalytic activity. *Biochemistry* **29,** 5761–5766.

Lahti R., Perälä M., Heikinheimo P., Pitkäranta T., Kukko-Kalske E. and Heinonen J. (1991a) Characterization of the 5′ flanking region of the *Escherichia coli ppa* gene encoding inorganic pyrophosphatase: mutations in the ribosome-binding site decrease the level of *ppa* mRNA. *J. Gen. Microbiol.* **137,** 2517–2523.

Lahti R., Salminen T., Latonen S., Heikinheimo P., Pohjaoksa K. and Heinonen J. (1991b) Genetic engineering of *Escherichia coli* inorganic pyrophosphatase. Tyr 55 and Tyr 141 are important for the structural integrity. *Eur. J. Biochem.* **198,** 293–297.

Lin M.-Y. and Le Gall J. (1990) Purification and characterization of two proteins with inorganic pyrophosphatase activity from *Desusfovibrio vulgaris*: rubrerythrin and a new highly active enzyme. *Biochem. Biophys. Res. Commun.* **171,** 313–318.

Lundin M., Baltscheffsky H. and Ronne H. (1991) Yeast *PPA2* gene encodes a mitochondrial inorganic pyrophos-

phatase that is essential for mitochondrial function. *J. Biol. Chem.* **266,** 12168–12172.

Meyer W., Moll R., Kath T. and Schäfer G. (1995) Purification, cloning and sequencing of archabacterial pyrophosphatase from the extreme thermoacidophilic *Sulforobus acidocaldiaus*. *Arch. Biochem. Biophys.* **319,** 149–156.

Morita J. and Yasui T. (1978) Purification and some properties of a neutral muscle pyrophosphatase. *J. Biochem.* **83,** 719–726.

Richter O.-M. H. and Schäfer G. (1992) Purification and enzyme characterization of the cytoplasmic pyrophosphatase from the thermophilic archaebacterium *Thermoplasma acidophilum*. *Eur. J. Biochem.* **209,** 343–349.

Smirova I. N. and Baykov A. A. (1989) Inorganic pyrophosphatase of rat liver cytosol: isolation and properties. *Biokhimiya* **54,** 228–234.

Tominaga N. and Mori T. (1977) Purification and characterization of inorganic pyrophosphatase from *Thiobacillus thiooxidans*. *J. Biochem.* **81,** 477–483.

Teplyakov A., Obmolova G., Willson K. S., Ishii K., Kaji H., Samejima T. and Kuranova I. (1994) Crystal structure of inorganic pyrophosphatase from *Thermus thermophilus*. *Protein Sci.* **3,** 1098–1107.

Terzyan S. S., Voronova A. A., Smirnova E. A., Kuranova I. P., Nekrasov Y. V., Arutyunyan E. G., Vanishtein B. K., Höhne W. and Hansen G. (1984) Structure of yeast inorganic pyrophosphatase at 3.0Å resolution. *Bioorg. Khim.* **10,** 1469–1482.

Tono H. and Kornberg A. (1967) Biochemical studies of bacterial sporulation-III: inorganic pyrophosphatase of vegetative cells and spores of *Bacillus subtilis*. *J. Biol. Chem.* **242,** 2375–2382.

Van Alebeek G.-J. W. M., Keltjens J. T. and Van der Drift C. (1994) Purification and characterization of inorganic pyrophosphatase from *Methanobacterium thermoautotrophicum* (strain ΔH). *Biochim. Biophys. Acta* **1206,** 231–239.

Velichko I., Volk S. E., Dudarenkov V. Y., Magretova N. M., Chernyak V. Y., Goldman A., Cooperman B. S., Lahti R. and Baykov A. A. (1995) Cold lability of the mutant forms of *Escherichia coli* inorganic pyrophosphatase. *FEBS Lett.* **359,** 20–22.

Verhoeven J. A., Schenek K. M., Meyer R. R. and Trela J. M. (1986) Purification and characterization of an inorganic pyrophosphatase from the extreme thermophile *Thermus aquaticus*. *J. Bacteriol.* **168,** 318–321.

Wakagi T., Lee C.-H. and Ohshima T. (1992) An extremely stable inorganic pyrophosphatase from the cytosol of a thermoacidphilic archaebacterium, *Sulfolobus acidocaldarius* strain 7. *Biochim. Biophys. Acta* **1120,** 289–296.

Ware D. A. and Postgate J. R. (1971) Physiological and chemical properties of a reductant-activated inorganic pyrophosphatase from *Desulfovibrio desulfuricans*. *J. Gen. Microbiol.* **67,** 145–160.

Welch K. M., Jacobyansky A., Springs B. and Cooperman B. S. (1983) Catalytic specificity of yeast inorganic pyrophosphatase for magnesium ion as a cofactor. An analysis of divalent metal ion and solvent isotope effects on enzyme function. *Biochemistry* **22,** 2243–2248.