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Purification and Characterization of Purine Nucleoside Phosphorylase from *Proteus vulgaris*

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Purine nucleoside phosphorylase was isolated and purified from cell extracts of *Proteus vulgaris* recovered from spoiling cod fish (*Gadus morhua*). The molecular weight and isoelectric point of the enzyme were 120,000 ± 2,000 and pH 6.8. The Michaelis constant for inosine as substrate was 3.9×10^{-5}. Guanosine also served as a substrate ($K_m = 2.9 \times 10^{-5}$). However, the enzyme was incapable of phosphorylizing adenosine. Adenosine proved to be useful as a competitive inhibitor and was used as a ligand for affinity chromatography of purine nucleoside phosphorylase following initial purification steps of gel filtration and ion-exchange chromatography.

The accumulation of ATP catabolites has been shown to be a reliable indicator of edible fish quality (8, 33). After death, the dephosphorylation and deamination of ATP usually goes to completion within 1 day and results in the accumulation of IMP. The subsequent dephosphorylation of IMP and increase in inosine level is due to autolytic enzymes (8, 12) and is generally the first indication of quality deterioration in fish. The eventual breakdown of inosine to hypoxanthine (Hx) is usually an indication of the last stage of edible fish quality. The accumulation of Hx is thought to result from the action of both autolytic and bacterial enzymes (22, 29). Nucleoside hydrolase (EC 3.2.2.2) and nucleoside phosphorylase (EC 2.4.2.1) were isolated from the muscle tissue of Pacific lingcod (30, 31) and were postulated to be partially responsible for the accumulation of Hx in this tissue. Studies with Atlantic cod (*Gadus morhua*) muscle (29) indicated that, while autolytic enzymes are responsible for the dephosphorylation of IMP, the subsequent conversion of inosine to Hx was largely due to the presence of spoilage bacteria. In fact, attempts were unsuccessful to isolate an enzyme capable of converting inosine to Hx in fresh cod. However, large quantities of the enzyme(s) were present in spoiling fillets (P. J. LeBlanc, M. S. thesis, Technical University of Nova Scotia, Halifax, 1987). Cultures of spoilage bacteria were isolated from cod and screened for their ability to convert inosine into Hx. A culture of *Proteus vulgaris* exhibited the greatest inosine phosphorolyzing activity in spoiling cod, and the objective of the present study was to isolate and characterize the enzyme involved.

MATERIALS AND METHODS

Preparation of cell extracts. *P. vulgaris* cultures isolated from spoiling cod fillets were identified as outlined by Lautrop (17). The cultures were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) and harvested by centrifugation at the peak of the exponential growth phase as measured by A_{660}. The cell pellets were suspended in 0.05 M Tris hydrochloride buffer (pH 8.0), and cell extracts were prepared by ultrasonic disruption for seven 2-min intervals at the maximum setting (Biosonik III; Bronwill Scientific, Rochester, N.Y.). Cell extracts were then clarified by centrifugation at 10,000 × *g* for 45 min. Extracts were stored at −80°C until use. In some cases, the extracts were concentrated with an Amicon XM 100 (100,000-molecular-weight cutoff) membrane from which no enzyme activity was detected in the filtrate.

Enzyme assay conditions. An enzyme assay based on the spectrophotometric method of Kalckar (13) was used for inosine phosphorylase activity measurements. For determination of optimum pH, reaction mixtures containing 1.8 ml of 0.05 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] hydrochloride, pH 6.65 to 7.4; *N*-tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid (TES-NaOH) at pH 7.0 to 7.5; or Tris hydrochloride at pH 7.4 to 8.4 were added to 0.10 ml of 7.3 mM inosine (Sigma Chemical Co., St. Louis, Mo.), 0.01 ml of xanthine oxidase (0.4 U/ml; Boehringer Mannheim, Dorval, Quebec), and 0.10 ml of 0.10 M sodium phosphate, pH 7.3. The reaction was initiated by adding 0.10 ml of the enzyme preparation, and the activity was measured as the increase in A_{293} at 23°C, using a Phillips PU 8800 UV/VIS spectrophotometer. All subsequent assays were performed as above, using 0.05 M TES–NaOH buffer, pH 7.3

For determination of kinetic properties, the assay mixture contained 0.05 ml of phosphate-free xanthine oxidase (0.04 U/ml), 0.05 to 0.20 ml of 2.0 mM inosine, 0.025 to 0.20 ml of 0.10 M sodium phosphate, and 0.025 ml of enzyme preparation and was made up to 2.0 ml with 0.05 M TES, pH 7.3. The activity was expressed in concentration units based on the molar absorbancy change for inosine to uric acid of 12,500 at 293 nm (13). The assay with guanosine as the substrate was determined spectrophotometrically by the decrease in A_{257} due to the depletion of guanosine to guanine with a molar absorbancy decrease of 5,000 (4). The reaction mixture contained 0.025 to 0.10 ml of 2.0 mM guanosine, 0.05 to 0.20 ml of 0.10 M sodium phosphate, and 0.10 ml of the enzyme preparation and was made up to 2.0 ml with 0.05 M TES, pH 7.3. All assays for kinetic evaluations were performed at 25°C. Adenosine was also assayed as a substrate by monitoring the change in A_{250}. The reaction mixture contained 0.25 ml of 2 mM adenosine, 0.2 ml of 0.1 M sodium phosphate, and 0.2 ml of enzyme preparation and was made up to 2.0 ml with 0.05 M TES, pH 7.3.

Purification of PNP. All procedures in the purification of purine nucleoside phosphorylase (PNP) were performed at 3°C. Cell extract prepared from *P. vulgaris* was applied to a Sephacryl S-300 (Pharmacia) column (2.5 by 80 cm). Elution

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was accomplished with 0.05 M Tris hydrochloride buffer (pH 8.0) at a flow rate of 0.25 ml/min. Active fractions were pooled and dialyzed against 0.05 M sodium citrate (Baker Chemicals), pH 6.0, for 12 h. The dialyzed samples were then applied to a DEAE-Sephacel (Pharmacia) ion-exchange column (1.8 by 8.0 cm) which was equilibrated with 0.05 M sodium citrate, pH 6.0. The column was then washed with 2 volumes of the starting buffer followed by an 80-ml continuous gradient elution of 0.05 to 0.12 M citrate at a flow rate of 0.4 ml/min. Fractions containing activity were pooled and dialyzed against 0.01 M disodium-PIPES hydrochloride (Sigma), pH 7.0, for 12 h. The dialyzed sample was then applied to an Agadenosine (Pharmacia) affinity column (1.1 by 7.5 cm). The column was washed with 20 ml of 0.01 M PIPES, pH 7.0, followed by 30.0 ml of the same buffer with a continuous gradient of 0.0 to 1.5 M KCl at a flow rate of 6.0 ml/h. The active fractions were pooled and dialyzed against 0.02 M TES and stored at −80°C.

Isoelectric focusing of PNP. Samples of purified enzyme containing 2% Pharmalyte 3-10 (Pharmacia) were applied to 10% T–3% C [where T denotes total concentration of acrylamide monomer plus bis-(*N,N'*-methylene-bis-acrylamide) cross-linker, and C denotes the percentage of T represented by bis cross-linker] polyacrylamide tube gels. Enzyme identification was performed with activity staining of the gel by immersing it in a solution of 0.05 M TES–NaOH (pH 7.3), 0.02% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma), 8 mM inosine, and 0.0008 U of xanthine oxidase per ml. Gels were incubated at 35°C until a dark blue-black band appeared (within 30 min). The gels were fixed in a solution consisting of 150 ml of methanol, 350 ml of water, 17.25 g of sulfosalicylic acid, and 57.5 g of trichloroacetic acid and then stained with Coomassie blue G-250 for determination of pI.

Polyacrylamide gel electrophoresis. The native molecular weight of PNP was determined by gradient slab polyacrylamide gel electrophoresis. The enzyme preparation was applied to a 4 to 30% polyacrylamide gradient slab gel with 4% bisacrylamide cross-linker. The electrophoresis buffer was composed of 0.09 M Tris, 0.08 M boric acid, and 0.0025 M disodium EDTA, pH 8.4. Electrophoresis was carried out at 150 V for 15 h in a Pharmacia GE-4 electrophoresis apparatus. The enzyme was stained for activity with 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide as above, and the apparent molecular weight was determined with molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.).

Protein determinations. Protein content was determined by the method of Lowry et al. (20), using bovine serum albumin (fatty acid-free, grade V; Sigma) as the standard.

RESULTS AND DISCUSSION

Initial attempts to recover either inosine nucleosidase or nucleoside phosphorylase from very fresh fish were fraught with difficulty. Only by using partially decomposed muscle tissue were we able to recover measurable amounts of activity. Seven bacterial cultures obtained from spoiling fillets were screened for their ability to produce Hx from inosine.

Only two cultures (*P. vulgaris* and *Pseudomonas fluorescens*) contained any measurable activity, with the specific activity of *P. vulgaris* cultures being approximately twice that of *Pseudomonas fluorescens*. *P. vulgaris* was used as a source of PNP for all subsequent experiments. Little activity was observed prior to ultrasonic disruption of the cells,

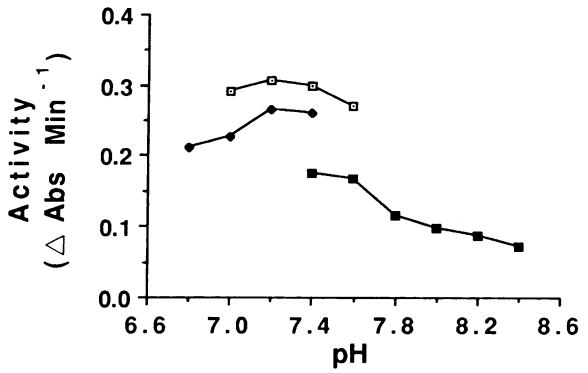

FIG. 1. Activity assay performed at various pH values, using 0.05 M PIPES hydrochloride (■), TES-NaOH (⊡), or Tris hydrochloride (◆) buffers.

suggesting that the enzyme was primarily intracellular in nature. Upon extensive dialysis against phosphate-free buffers, little activity was observed, suggesting that nucleoside phosphorylase was far more important than inosine nucleosidase in the bacterial production of Hx.

PNPs have been purified from a variety of sources (2, 5, 9, 10, 14, 21, 23–25). Kinetics, physical properties, and catalytic specificity may differ depending on the source. In general, most have been found to exhibit maximum activity between pH 6.5 and 8.0 (25). The PNP in the present study exhibited maximum activity with the TES buffer in the pH range of 7.0 to 7.5 (Fig. 1). This is perhaps not surprising since this is generally the pH range for spoiling fish tissue. All subsequent activity measurements were performed at pH 7.3 in TES buffer.

A three-step purification of the cell extracts was performed by gel filtration, ion-exchange chromatography, and affinity chromatography, using adenosine as the ligand. Gel filtration on Sephacryl S-300 (Fig. 2) resulted in the recovery of a single peak of nucleoside phosphorylase activity which, when applied to a DEAE-Sephacel column (Fig. 3), resulted in a 5-fold increase in specific activity from the Sephacryl column and an overall purification of 23-fold from the crude

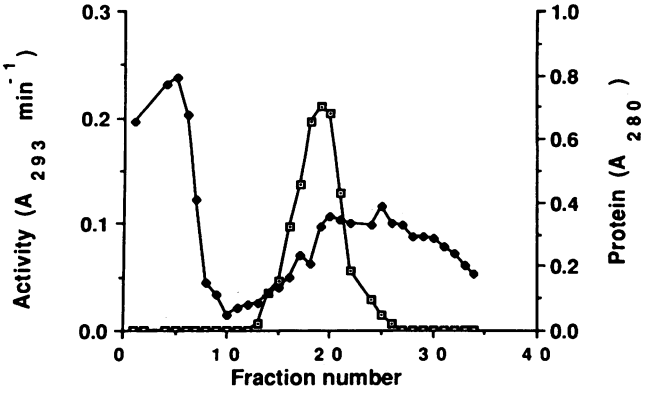

FIG. 2. Gel filtration elution of PNP on a Sephacryl S-300 column (2.5 by 80 cm). Elution was performed with 0.05 M Tris hydrochloride, pH 8.0, at a flow rate of 0.25 ml/min. The void volume was 145 ml, and each fraction volume was 4.0 ml. Symbols: ⊡, activity; ◆, protein. Fractions 13 to 26 were pooled for subsequent application to the DEAE-Sephacel column.

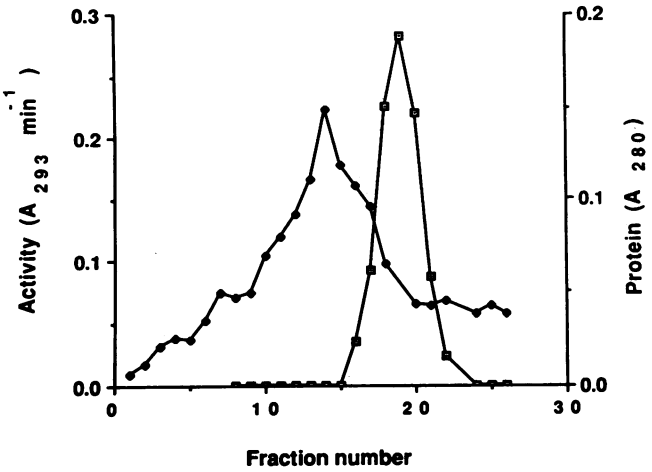

FIG. 3. DEAE-Sephacel ion-exchange chromatography elution of PNP on a 1.8- by 8.0-cm column. Elution was obtained with an 80-ml concentration gradient from 0.05 to 0.12 M sodium citrate, pH 6.0, at a flow rate of 0.4 ml/min. Fraction volume was 3.1 ml. Symbols: ⊡, activity; ◆, protein. Fractions 16 to 22 were pooled for subsequent application to the Agadenosine column.

cell extract. Affinity chromatography (Fig. 4) resulted in a further 23-fold purification from the ion-exchange step (500-fold from the crude extract), yielding an overall recovery of 69% for all three steps. Table 1 summarizes the results of the purification procedure.

The isoelectric point of PNP was determined on polyacrylamide gels by comparison with standards. The PNP active band, stained with the redox dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, coincided with a single protein with a pI of 6.8. No apparent loss of resolution through diffusion was observed when fixation and Coomassie blue staining of the gels was performed following the activity staining. PNPs from other sources have pIs ranging from 4.3 for *Enterobacter cloacae* (21) to 6.25 from human erythrocytes (1).

TABLE 1. Summary of purification of PNP from *P. vulgaris*

Step	Activity (μmol min^{-1})	Protein (mg)	Sp act (U mg^{-1})	Recovery (%)	Purification (fold)
Crude extract	6.86	165.6	0.04	100	1
Sephacryl	6.90	37.7	0.18	100	4.2
Sephacel	5.88	6.4	0.92	85.7	23
Agadenosine	4.75	0.22	21.6	69.2	540

Molecular weight determination of PNP was performed under nondenaturing conditions by subjecting the enzyme preparation to gradient slab polyacrylamide gel electrophoresis. The apparent molecular weight of PNP was 120,000 ± 2,000 when compared with standards. PNPs isolated from mammalian tissues have molecular weights ranging from 48,000 to 92,000 (14, 18, 19, 23, 28). Bacterial PNPs studied to date may be grouped into two categories: high-molecular-weight (130,000 to 140,000) types isolated from *Salmonella typhimurium* and *Escherichia coli* (11), and low-molecular-weight (80,000 to 95,000) types isolated from *Bacillus cereus* (5, 6) and *Enterobacter cloacae* (21). Also, the PNPs isolated from human erythrocytes (15) and calf spleen (1) fall into the low-molecular-weight category.

Thus, the PNP prepared from cell extracts of *P. vulgaris* involved in seafood spoilage is similar in size to those of *E. coli* (138,000 ± 10%) (11) and *S. typhimurium* (130,000 ± 10%) (26).

Double-reciprocal kinetic plots with inosine as the variable substrate and phosphate as the changing fixed substrate were constructed. Both slopes and intercepts changed for different concentrations of inorganic phosphate (Fig. 5). This nonparallel pattern rules out a ping-pong reaction mechanism (3) in which a ribosylated or phosphorylated enzyme

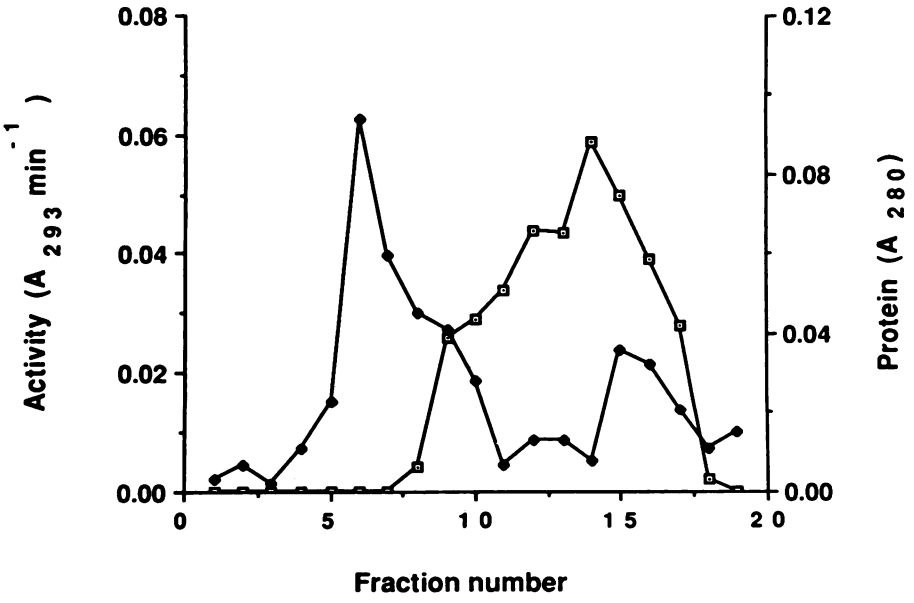

FIG. 4. Affinity chromatography elution of PNP from Agadenosine on a 1.1- by 8.0-cm column. Elution was obtained with a continuous KCl concentration gradient from 0 to 1.5 M in 0.01 M PIPES hydrochloride, pH 7.0, at a flow rate of 6.0 ml/h. Fraction volume was 1.5 ml. Symbols: ⊡, activity; ◆, protein. Fractions 8 to 18 were pooled for electrophoresis.

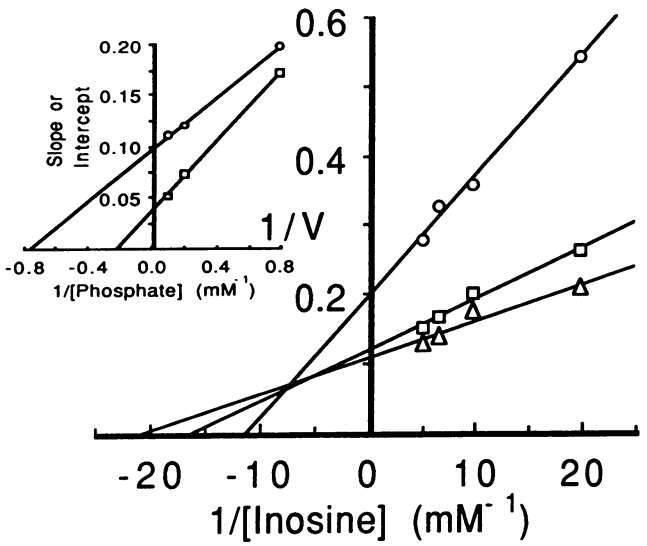

FIG. 5. Initial velocity with inosine as the variable substrate at phosphate concentrations of 1.3 (○), 5.1 (□), and 10.0 (△) mM. The reciprocal reaction velocity (micromoles per milligram per minute) is plotted against the reciprocal inosine concentration. (Inset) Replots of the slopes (□) and intercepts (○) with respect to the reciprocal phosphate concentration. All data points are the average of two values. All activity assays were performed at 25°C.

intermediate would exist and is in agreement with other kinetic studies with PNP which indicate that the reaction takes place on a ternary complex when both substrates are simultaneously bound to the enzyme (10, 15, 16, 19). Kinetic parameters obtained by plotting the slopes and intercepts of the reciprocal plots against 1/[phosphate] (inset, Fig. 5) are summarized in Table 2. The K_m for inosine, 3.9×10^{-5} M, compares favorably with those reported from human erythrocytes (5.8×10^{-5} M [15]) and ascites cells (3×10^{-5} M [7]), fish muscle (3.2×10^{-5} M [32]), and other members of the family *Enterobacteriaceae* such as *Enterobacter cloacae* (21) and *E. coli* (11) (9×10^{-5} M) and *S. typhimurium* (5×10^{-5} to 7×10^{-5} M [11, 26]). The K_m we reported was determined at low inosine concentrations (<0.2 mM), which should be noted since substrate activation has been observed with higher inosine levels (2, 15). The K_{ia} values shown in Table 2 are the dissociation constants of the substrates from the enzyme-substrate complexes, for each substrate.

Although inosine was used as the assay substrate during the purification of PNP, the enzyme also catalyzes the phosphorolysis of guanosine, indicating broad specificity. Double-reciprocal plots with guanosine as the variable substrate and phosphate as the fixed substrate showed a nonparallel pattern similar to that obtained with inosine as substrate. The K_m for guanosine was similar to that of inosine (Table 2). However, the specific activity with inosine as substrate was double that obtained with guanosine. This is similar to the enzyme isolated from *B. cereus* (5) and *Enterobacter cloacae* (21), with which the phosphorolysis of inosine doubled that of guanosine. However, the PNP isolated from *E. coli* and *S. typhimurium* yielded similar phosphorolysis rates with inosine and guanosine as substrates (11).

TABLE 2. Kinetic parameters estimated from plots and replots of kinetic data for PNP from *P. vulgaris*

Substrate	K_{ia} (M)	K_m (M)	V_{max} (μmol mg^{-1} min^{-1})
Inosine	1.4×10^{-4}	3.9×10^{-5}	10.2
Guanosine	2.5×10^{-5}	2.9×10^{-5}	5.8
Phosphate			
With inosine		1.3×10^{-5}	10.3
With guanosine		1.5×10^{-5}	5.8

While most PNP preparations have specificity for inosine and guanosine as well as their respective bases, little or no activity with adenine or adenosine as substrates has been reported (16, 21, 34) except for the preparations obtained from *E. coli* and *S. typhimurium*, in which adenosine was phosphorylized as efficiently as inosine and guanosine (11, 26). Adenine is a competitive inhibitor of PNP from sarcoma 180 cells (34), indicating that it may bind some mammalian PNPs. The PNP described here from *P. vulgaris* showed no measurable phosphorolizing activity with adenosine. Although adenosine is not a substrate for PNP from *P. vulgaris*, its ability to bind the enzyme as a ligand in affinity chromatography has proven valuable in enzyme purification. Kinetic analysis with adenosine as the inhibitor of inosine phosphorolysis was undertaken to determine the K_i value and whether the site of attachment on the affinity column is indeed the active site. The inhibition pattern obtained is indicative of a competitive inhibition mechanism (3), therefore confirming active site binding. The K_i of adenosine was 4.9×10^{-5} from the replot of the slopes from the double-reciprocal activity plots.

The breakdown of inosine in Atlantic cod is at least in part due to bacterial PNP. In a previous study (29), we showed that spoilage bacteria could accelerate Hx production in cod fillets by at least 2 days. The PNP recovered from spoilage bacteria was examined in the present study as an attempt to gain a more complete understanding of the events taking place during spoilage of seafood.

It may be that PNP and other autolytic or bacterial enzymes could be used in the rapid analysis of seafood quality since it has been shown that the speed of ATP breakdown may be used as an indicator of edibility (27).

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