MARZENNA KOSKOWSKA-DZIADOWICZ, MALGORZATA OCHMAN
and TERESA SZYMCZYK

PURIFICATION OF INORGANIC PYROPHOSPHATASE
FROM RAT SALIVARY GLANDS

Department of Biochemistry, Institute of Biopharmacy, Medical Academy in Warsaw,
Banacha 1, 02-097 Warszawa, Poland

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Inorganic pyrophosphatase [EC 3.6.1.1] isolated from rat sublingual and
submandibular glands was purified 2300-fold and 2600-fold, respectively. The purified
enzymatic preparations separated on electrophoresis into two protein bands, of which
only one showed the pyrophosphatase activity.

Inorganic pyrophosphatase from rat salivary glands is a monomeric anionic
protein, its isoelectric point is 5.42 and 4.90 for the sublingual and submandibular
glands, respectively.

Practically no data on inorganic pyrophosphatase of salivary glands have
been reported in biochemical literature. The present work is a continuation of
our studies on metabolic features of these organs. An earlier communication by
Koskowska-Dziadowicz & Szymczyk [1] reported on subcellular localization
of inorganic pyrophosphatase in subcellular structures of rat sublingual and
submandibular salivary glands. The present work describes purification of the
enzyme as an initial step to further studies on its properties.

MATERIALS AND METHODS

Reagents. Sodium pyrophosphate (acid, pure), bovine serum albumin,
Coomassie brilliant blue R-250 and sucrose were from Serva (Heidelberg,
F.R.G.); MgCl₂ (1 M solution), acrylamide and N′N′-methylenebiscrylamide
were from B.D.H. Chemicals Ltd. (Poole, Dorset, Great Britain); cytochrome c
and Tris were from Koch-Light Lab. Ltd. (Colnbrook, Great Britain); 1,4-
-dithiothreitol (DTT) was purchased from Loba-Chemie (Vienna, Fischamend,
Austria); myoglobin from horse heart, α-chymotrypsinogen A, ovalbumin and
bovine serum γ-globulin were from Sigma Chem. Co. (St. Louis, MO., U.S.A.);
CM-cellulose (DE 11) and DEAE-cellulose (DE 11) were from Whatman
Biochemicals (Maidstone, Kent, Great Britain); Con A Sepharose, Sephadex
G-25 and Sephadex G-100 were from Pharmacia (Uppsala, Sweden). Other reagents, of analytical grade, were obtained from Ciech (Gliwice, Poland). Animals. Wistar male rats weighing 180-200 g were killed by rupture of the spinal cord. The sublingual and submandibular salivary glands encapsulated together in a connective-tissue sac, were immediately isolated and separated by the procedure described by Szymczyk & Jachimowicz [2], whereupon they were washed with an isotonic sucrose solution (4°C).

Homogenates. The separated submandibular and sublingual glands were homogenized in a Potter-Elvehjem glass-Teflon homogenizer with cooling in a water-ice mixture. Homogenates of the sublingual and submandibular glands, 10% and 20% (w/v) respectively, were prepared in a solution containing: 0.32 M sucrose, 10 mM Tris/HCl buffer, pH 7.5, 5 mM MgCl₂ and 1 mM DTT.

The cytosol fraction was obtained by centrifugation of the homogenates of both kinds of salivary glands at 20,000 × g for 30 min, followed by centrifugation of the supernatant at 120,000 × g for 90 min.

Determination of the enzymatic activities. The inorganic pyrophosphatase activity was assayed according to Irie et al. [3]. The activity unit is defined as µmol of inorganic phosphate liberated in the reaction per minute, and specific activity as µmol P_i per minute per 1 mg protein.

Determination of protein. Protein was assayed according to Lowry et al. [4], with bovine serum albumin as a standard.

Determination of inorganic phosphate. Inorganic phosphate was assayed according to Fiske-Subbarow in the modification of Lohmann & Jendrassik [5].

Fractionation with ammonium sulphate. To the obtained cytosol fraction containing inorganic pyrophosphatase, crystalline ammonium sulphate was added up to the required concentration. The mixture was stirred on a magnetic stirrer for 1 h at 0-4°C and centrifuged for 30 min at 20,000 × g. The sediment was dissolved in 10 mM Tris/HCl buffer (pH 7.5 for the sublingual gland and pH 8.0 for the submandibular gland) containing 5 mM MgCl₂ and 1 mM DTT. The reactions obtained at 0.55-0.75 and 0.40-0.65 ammonium sulphate saturation for the sublingual and submandibular glands, respectively, were subjected to chromatography on Sephadex G-25 gel columns (1 × 20 cm), to remove ammonium sulphate. Proteins were eluted from the column with the same buffer in which they had been dissolved after centrifugation.

CM-cellulose chromatography. The enzymatic preparation obtained by ammonium sulphate fractionation (450 and 300 mg protein for the sublingual and submandibular gland, respectively) was applied to a CM-cellulose column (1.5 × 40 cm) equilibrated with 10 mM Tris/acetate buffer, pH 6.8, containing 5 mM MgCl₂ and 1 mM DTT. Protein was eluted from the column with 100 ml of the same buffer and a linear NaCl concentration gradient (0-0.4 M) in 10 mM Tris/acetate buffer, pH 6.8, fractions of 5 ml were collected, and
protein content and inorganic pyrophosphatase activity were determined. Fractions showing enzymatic activity were pooled and concentrated by dialysis against 60% sucrose in 10 mM Tris/HCl buffer, pH 8.6, containing 5 mM MgCl₂ and 1 mM DTT. The concentrated preparation was passed on through a Sephadex G-25 gel column (1 × 20 cm) to remove sucrose. Protein was eluted from the column with the buffer used for dialysis but not containing sucrose.

DEAE-cellulose chromatography. The enzymatic preparation obtained at the previous step (about 100 mg protein), was applied to a DEAE-cellulose column (1.5 × 40 cm) equilibrated with Tris/HCl buffer, pH 8.6, containing 5 mM MgCl₂ and 1 mM DTT. The column was washed with 100 ml of the same buffer and the adsorbed protein was eluted with a linear NaCl concentration gradient (0 - 0.4 M), in 10 mM Tris/HCl buffer, pH 8.6. Fractions of 5 ml were collected and protein content and inorganic pyrophosphatase activity were determined. The fractions showing enzymatic activity were pooled and concentrated by dialysis against 60% sucrose in 10 mM Tris/HCl buffer, pH 7.0, containing 5 mM MgCl₂ and 1 mM DTT. Sucrose was removed from the concentrated preparation in the same way as described above.

Affinity chromatography on a Con A Sepharose column. The enzymatic preparation obtained (10-20 mg protein) was applied to the column (0.5 × 5 cm) equilibrated with 10 mM Tris/HCl buffer, pH 7.0, containing 5 mM MgCl₂, 1 mM DTT and 154 mM NaCl [6]. Protein was eluted from the column with 80 ml of the same buffer, then with 80 ml of 50 mM α-methyl-D-glucose solution in the same buffer. Fractions of 2 ml were collected and protein and activity of inorganic pyrophosphatase were determined. The fractions showing enzymatic activity were pooled and concentrated by dialysis against 60% sucrose solution in 10 mM Tris/HCl buffer, pH 7.5, containing 5 mM MgCl₂ and 1 mM DTT. Sucrose was removed from the concentrated preparation on a Sephadex G-25 gel column.

Molecular sieving on a Sephadex G-100 column. The enzymatic preparation (2-5 mg protein) was applied on the column (1 × 40 cm) equilibrated with 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM MgCl₂, 1 mM DTT and 100 mM NaCl, and protein was eluted with the same buffer. Fractions of 2 ml were collected and protein and inorganic pyrophosphatase activity were determined. Fractions showing enzymatic activity were pooled and concentrated by dialysis against 60% sucrose solution in 10 mM Tris/HCl buffer, pH 7.5, containing 5 mM MgCl₂ and 1 mM DTT. Sucrose was removed as before.

Polyacrylamide gel chromatography. Electrophoretic separation of the enzymatic preparation (30 - 150 μg in a volume of 10 - 50 μl) was performed in glass tubes (0.5 × 6 cm), in 8% polyacrylamide gel of pH 6.0, 8.0 and 9.8 [7], at 2.5 mA per tube, for 2 h. The 3% polyacrylamide concentrating gel formed the upper layer. The separated proteins were stained in the gel with a 0.2% solution of Coomassie blue in the water/methanol/acetic acid (5:5:1, by vol.)
system for 3-4 h. The gel was destained by several cycles of extraction with 7% acetic acid. The gels with separated, stained protein fractions were photographed. Semipreparative electrophoresis at each pH values used was run in eight parallel tubes. Two gel slabs with separated proteins were stained (matrix), and the fragments corresponding to protein bands visible on the matrix were cut out from the remaining six slabs. These fragments were homogenized in 100 mM Tris/HCl buffer, pH 7.5, containing 5 mM MgCl₂ and 1 mM DTT, and protein was eluted during 30 min. Both electrophoresis and elution of protein were carried out at 0-4°C. The activity of inorganic pyrophosphatase was determined in successive fractions of the eluate.

Electrofocusing. Isoelectric focusing of the cytoplasmic fraction (about 30 mg protein) and of the highly purified enzymatic preparation (about 5 mg protein) was performed on a LKB column (110 ml, type 8100). The preparations were separated in a linear sucrose concentration gradient (0-50%, v/w) in 10 mM Tris/HCl buffer, pH 7.5, containing 5 mM MgCl₂, 1 mM DTT and 1% Ampholine (pH 3.5-10.0). After 44-48 h, fractions of 1 ml were collected and their pH value was determined with the use of a Radiometer pH-meter provided with a microelectrode; protein content and inorganic pyrophosphatase activity were also determined. Prior to activity determination, the fractions collected from the column were dialysed against 10 mM Tris/HCl buffer, pH 7.5, containing 5 mM MgCl₂ and 1 mM DTT to remove sucrose and Ampholine.

Molecular mass determination. This was performed by gel filtration on a Sephadex G-100 column [8]. To the column (2.6 x 40 cm) equilibrated with 50 mM Tris/HCl buffer, pH 7.5, containing 5 mM MgCl₂, 1 mM DTT and 100 mM NaCl, were successively applied standard proteins (10 mg each): cytochrome c (Mₘ, 12400), horse heart myoglobin (17800), chymotrypsinogen A (25000), ovalbumin (45000) bovine serum albumin (67000), bovine serum γ-globulin (150000) and 5-10 mg of protein of the inorganic pyrophosphatase preparation. The proteins were eluted with the same buffer. Fractions of 2 ml were collected at a flow rate of 0.2 ml/min. Cytochrome c and horse myoglobin were determined in the fractions in a Unicam type SP 1800 spectrophotometer at 550 nm, and other protein standards at 280 nm.

RESULTS AND DISCUSSION

Fractionation by ammonium sulphate precipitation followed by ion-exchange chromatography on CM-cellulose (Fig. 1) and DEAE-cellulose (Fig. 2), affinity chromatography on Con A Sepharose (Fig. 3) and molecular sieving on Sephadex G-100 (Fig. 4) permitted to obtain for the first time a highly purified inorganic pyrophosphatase preparation from rat tissues. The purification factor for the enzyme from the sublingual salivary gland was 2300, and for the submandibular gland 2600 (Table 1). Following electrophoresis
Fig. 1. CM-cellulose column chromatography of inorganic pyrophosphatase from rat salivary glands. A, Sublingual gland; B, submandibular gland. For conditions see Methods. •, Enzyme activity; ▲, protein content
Fig. 2. DEAE-cellulose column chromatography of inorganic pyrophosphatase from rat salivary glands. A, Sublingual gland; B, submandibular gland. For conditions see Methods. ●, Enzyme activity; ▲, protein content.
Fig. 3. Affinity column chromatography on Con A Sepharose of inorganic pyrophosphatase from rat salivary glands. A, Sublingual gland; B, submandibular gland. For conditions see Methods. ○, Enzyme activity; ▲, protein content
Fig. 4. Sephadex G-100 gel filtration of inorganic pyrophosphatase from rat salivary glands. A. Sublingual gland; B, submandibular gland. For details see Methods. ○, Enzyme activity; ▲, protein content
over the pH range of 6.0-9.8 the purified preparation separated into two protein bands, of which only one showed the ability to hydrolyze inorganic pyrophosphate (Figs. 5 and 6). Inorganic pyrophosphatase from the two salivary glands of the rat shows identical electrophoretic mobility on polyacrylamide gel and, like the cytosolic enzyme from rat liver [3] is an anionic protein with the isoelectric point of 5.4 and 4.9 for the sublingual and submandibular glands, respectively (Figs. 7 and 8). Molecular mass of the enzyme from either kind of salivary gland calculated from gel filtration data (Fig. 9), is about 70,000.

**Table 1**

**Purification of inorganic pyrophosphatase from rat salivary glands**

For conditions of purification at the successive steps, see Methods. The amount of material used for purification was for the sublingual gland 300 g of tissue (about 10,000 glands) and for the submandibular gland 500 g of tissue (about 5000 glands)

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Sublingual gland</th>
<th>Submandibular gland</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Total protein (mg)</td>
<td>Total activity (units)</td>
</tr>
<tr>
<td>Cytosol Ammonium sulphate ppt.</td>
<td>10080.0</td>
<td>29030</td>
</tr>
<tr>
<td>CM-cellulose</td>
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<tr>
<td>DEAE-cellulose</td>
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<td>Con A</td>
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<tr>
<td>Sepharose</td>
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<tr>
<td>Sephadex G-100</td>
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<td>14883</td>
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To check whether inorganic pyrophosphatase from the cytosol of the rat salivary gland cells occurs in a single form or in several heterogeneous molecular forms, both the enzyme present in the total cytosolic fractions and the highly purified preparation were subjected to isoelectric focusing (Figs. 7 and 8, respectively); this was done to exclude the possibility that some enzyme forms were lost during the purification procedure. The results obtained show unequivocally that, in the cells of either kind of salivary glands, inorganic pyrophosphatase occurs in a single molecular form. This is in agreement with the data for the enzyme from the cytosol of rat liver cells, reported by Irie et al. [3]. The existence of a single enzyme form in rat salivary glands is additionally confirmed by the elution profiles, since from all types of columns applied in our work, a single active fraction was consistently obtained. A more diffuse profile
Fig. 5. Polyacrylamide gel electrophoresis of the cytoplasmic fraction from rat sublingual gland, containing inorganic pyrophosphatase. For conditions see Methods. pH Values: a, 6.0; b, 8.0; c, 9.8. The upper narrow band shows no inorganic pyrophosphatase activity; the lower, wider band is enzymatically active.

Fig. 6. Polyacrylamide gel electrophoresis of the cytoplasmic fraction from rat submandibular gland, containing inorganic pyrophosphatase. For conditions see Methods. pH Values: a, 6.0; b, 8.0; c, 9.8. The upper narrow band shows no inorganic pyrophosphatase activity; the lower, wider band is enzymatically active.
of the enzyme present in the total cytosolic fraction (Fig. 7) testifies only that preparations from salivary glands contain a large amount of mucus.

The experiments with gel filtration on a Con A Sepharose column of a partly (100-fold) purified preparation of inorganic pyrophosphatase from either salivary gland was carried to address the question whether the enzyme studied is a glycoprotein. Concanavalin A is able to bind specifically

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Fig. 7. Isoelectric focusing of inorganic pyrophosphatase of the total cytosolic fraction of rat salivary glands: A, Sublingual gland; B, submandibular gland. For conditions see Methods.
Fig. 8. Isoelectric focusing of inorganic pyrophosphatase in the highly purified preparations from rat: A, sublingual and B, submandibular gland (purification factor 2300 and 2600, respectively). For conditions see Methods.
D-mannose and D-glucose through hydroxyl groups at carbons C-3, C-4 and C-6 (Goldstein et al. [9]). The enzyme from either salivary gland showed no affinity to Con A Sepharose (Fig. 3); thus, it can only be concluded that, if inorganic pyrophosphatase from rat salivary glands is a glycoprotein, its sugar moiety contains neither D-mannose nor D-glucose, or both these sugars have no free hydroxyl groups at positions 3, 4 and 6 of the pyran ring.

REFERENCES


