Similarity between Alkaline Phosphatase and Nucleotide Pyrophosphatase from Skipjack Liver

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Skipjack liver alkaline phosphatase (EC 3.1.3.1) and nucleotide pyrophosphatase (EC 3.6.1.9) were compared in respect to some physico-chemical and enzymatic properties. Neuraminidase strongly inactivated both enzymes in a similar fashion, whereas muramidase inactivated alkaline phosphatase more slowly than the other enzyme. Nucleotide pyrophosphatase was also inactivated by several proteolytic enzymes more easily than alkaline phosphatase. Sodium lauryl sulfate, urea, and guanidine hydrochloride inactivated both enzymes in a similar manner, and the protective effect of MgCl₂ was also similar. In the presence of MgCl₂, alkaline phosphatase showed the optimum temperature at 35°C and nucleotide pyrophosphatase at 45°C. In the absence of MgCl₂, however, both enzymes showed the identical optimal temperature at 45°C. All the substrates common to both enzymes inhibited them competitively. Nucleotide pyrophosphatase was inhibited by all the substrates specific to alkaline phosphatase but the inhibition types were different for both enzymes with some substrates. All the substrates for nucleotide pyrophosphatase also inhibited alkaline phosphatase by the competitive or the mixed type manner. These results suggested that the two skipjack enzymes are closely similar to each other in their primary or higher-dimensional structure.

As we reported previously,¹,² it is quite difficult to separate alkaline phosphatase (EC 3.1.3.1) and nucleotide pyrophosphatase (EC 3.6.1.9) from skipjack liver, since their behaviors resemble each other. In addition, they were quite similar in some enzymatic and physico-chemical properties. For example, both skipjack enzymes hydrolyzed NADP⁺, NADPH, ATP, ADP, and inorganic pyrophosphate. All these facts strongly suggest that both enzymes have similar molecular structures.

This paper communicates a further enzymatic and physico-chemical comparison of the two skipjack enzymes.

Material and Methods

Material
Livers were obtained from frozen specimens of the skipjack, Katsuwonus pelamis, and kept frozen at -20°C until used.

Purification of Enzymes
Both enzymes were partially purified by the method previously reported.¹,²,³ A 360-fold purified preparation in respect of either enzyme activity was mainly used through the present study. The purified (1,600- and 2,900-fold) nucleotide pyrophosphatase preparations which showed an alkaline phosphatase activity as well, were also used.

Enzyme Assay
Alkaline phosphatase activity was assayed using p-nitrophenyl phosphate as substrate, as described in the preceding paper.⁴ Nucleotide pyrophosphatase activity was determined using 2'-deoxythymidine 5'-p-nitrophenyl phosphate as substrate. The standard incubation mixture contained 0.33mM substrate, 10 mM MgCl₂, 0.15 M Tris-HCl pH 9.0, and 0.02-0.05 ml of enzyme solution with a total volume of 3.0 ml. Liberation of p-nitrophenol by both enzymes was monitored spectrophotometrically at 400 nm. The enzyme activity was calculated using 18 × 10³ as a molar extinction coefficient of p-nitrophenol.

Mixed Substrate Experiment
Mixed substrate experiments were carried out according to Webb et al.⁵ Michaelis constants (Kₘ) for each substrate were determined for both enzymes. The mixed substrate velocity (vₐ) was determined using a solution containing both

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substrates at an equal specific concentration \((\sigma = S/K_m)\). Initial velocities for respective substrates \((v_1 \text{ and } v_2)\) at that concentration were also determined.

**Digestion with Neuraminidase, Muramidase and Some Proteolytic Enzymes**

One milliliter of the enzyme solution was diluted with the same volume of a given buffer and to the mixture was added 0.34 to 12 mg of the following crystalline enzymes; neuraminidase, muramidase, trypsin, \(\alpha\)-chymotrypsin, Nagase, Pronase and papain. The mixtures were then incubated at 37\(^\circ\)C, and at due intervals aliquots were taken and determined for both enzymatic activities.

**Results and Discussion**

**Mixed Substrate Experiment**

To examine whether a single catalytic center of a single protein is responsible for alkaline phosphatase and nucleotide pyrophosphatase activities, some mixed substrate experiments were performed, using the three enzyme preparations of 360-, 1,600- and 2,900-fold purifications. The results are shown in Fig. 1. If each enzyme activity comes from two different proteins, the theoretical value of \(v_1/(v_1+v_2)\) must be 1.0 in spite of \(\sigma\) value (refer to theoretical curve I in Fig. 1). A theoretical value of 0.5 may be obtained at the infinite \(\sigma\) value if both activities come from the same catalytic center of a single protein (refer to theoretical curve II in Fig. 1). As seen in Fig. 1, the curve of 360-fold purified preparation roughly coincided with the theoretical curve II. The two more highly purified preparations, however, presented the curves between both theoretical curves, rather nearer to \(\sigma\). These data suggest that both skipjack enzymes are actually different ones.

**Inactivation by Neuraminidase and Muramidase**

Both enzymes of mammalian origins have been reported to be glycoproteins.\(^6\)\(^7\) For further comparison of skipjack enzymes, the 360-fold purified enzyme was treated by neuraminidase and muramidase. As shown in Fig. 2, neuraminidase drastically inactivated both enzymes in a similar manner. On the other hand, muramidase inactivated alkaline phosphatase very slowly, whereas it inactivated nucleotide pyrophosphatase sharply. These data indicate that both skipjack enzymes are also glycoproteins and their carbohydrate moieties differ from each other.

**Inactivation by Several Proteolytic Enzymes**

The 360-fold purified enzyme solution was digested by trypsin, \(\alpha\)-chymotrypsin, Nagase, Pronase and papain. As shown in Fig. 3, the alkaline phosphatase was generally more resistant to these proteolytic enzymes than the pyrophosphatase. The nucleotide pyrophosphatase was not affected by papain at all. The alkaline phosphatase activity was enhanced to some extent when treated with papain or Pronase. These data indicate that the two enzymes differ from each other in the primary or higher-dimentional protein structure.

**Effect of Denaturing Agents**

Several denaturing agents, sodium lauryl sulfate, urea and guanidine hydrochloride, were tested to see the inactivation effect on both enzymes in the presence or absence of 20 mM MgCl\(_2\). As shown in Fig. 4, these denaturing agents inactivated both enzymes and the presence of MgCl\(_2\) retarded the inactivation of both enzymes. These data suggest that both skipjack enzymes possess quite similar molecular conformations.

**Effect of Temperature**

The temperature-activity profiles of both enzymes were determined between 25 and 50\(^\circ\)C in the presence or absence of 20 mM MgCl\(_2\). As shown
Fig. 2. Digestion of skipjack alkaline phosphatase and nucleotide pyrophosphatase with neuraminidase and muramidase.

- Alkaline phosphatase activity, - Nucleotide pyrophosphatase activity.

(A); Digested with neuraminidase (0.5 mg/ml), (B); Digested with muramidase (0.5 mg/ml).

Fig. 3. Digestion of skipjack alkaline phosphatase and nucleotide pyrophosphatase by several proteolytic enzymes.

- Alkaline phosphatase activity, - Nucleotide pyrophosphatase activity.

(A); Trypsin (5 mg/ml), (B); α-Chymotrypsin (5 mg/ml), (C); Nagase (6 mg/ml), (D); Pronase (0.17 mg/ml), (E); Papain (5 mg/ml).

In Fig. 5, temperature-activity curves in the presence of MgCl₂ significantly differed between both enzymes, the optimal temperature being found at 35°C for alkaline phosphatase and at 45°C for nucleotide pyrophosphatase. In the absence of MgCl₂, however, the curves were very similar, showing the optimum at 45°C and a shoulder at 35°C. These data show that the whole protein conformations or the conformations around active site of both enzymes may be quite similar.

Inhibition by Several Substrates

The substrate specificity of both skipjack enzymes is significantly low as described previously.¹,²
Fig. 4. Inactivation of skipjack alkaline phosphatase and nucleotide pyrophosphatase by some
denaturing agents.

\(\text{O-O}:\) Alkaline phosphatase activity with 20 mM MgCl\(_2\), \(\triangle-\triangle\): Alkaline phosphatase
activity without MgCl\(_2\), •-•: Nucleotide pyrophosphatase activity with 20 mM MgCl\(_2\),
\(\Delta-\Delta\): Nucleotide pyrophosphatase activity without MgCl\(_2\).
(A): Sodium lauryl sulfate (100 mM), (B): Urea (4 M), (C): Guanidine hydrochloride (2 M).

Fig. 5. Temperature-activity profiles of skipjack
alkaline phosphatase and nucleotide pyrophosphatase with and without 20 mM MgCl\(_2\).

Some substrates such as NADP\(^+\), NADPH, ATP, ADP, and inorganic pyrophosphate were similarly
hydrolyzed by both enzymes.

Inhibitory effects on both enzymes of these common substrates and several other substrates were
examined. As shown in Table 1, all of the common substrates inhibited both enzymes competitively.
The inhibition constants for ATP and ADP were rather high against alkaline phosphatase.

The inhibition by several alkaline phosphatase-specific substrates was then examined. As shown
in Table 2, these substrates inhibited alkaline phosphatase competitively except nicotinamide mono-
nucleotide which showed the mixed type inhibition. Nucleotide pyrophosphatase, on the other hand,
was inhibited non-competitively by 2'-AMP, \(\alpha\)-naphthylphosphate and \(\beta\)-glycerophosphate. And
the inhibition constants were lower against alkaline phosphatase except for 5'-AMP.

Table 3 shows the inhibition by some substrates of nucleotide pyrophosphatase and by other inhibitors
such as adenosine, adenine, etc. FAD, NAD\(^+\), and NADH inhibited nucleotide pyrophosphatase competitively, but alkaline phosphatase in the mixed type except for FAD.

### Table 1. Inhibition on skipjack alkaline phosphatase and nucleotide pyrophosphatase activities by
some substrates common to both enzymes

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Alkaline phosphatase</th>
<th>Nucleotide pyrophosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type (K_i (\text{m}))</td>
<td>Type (K_i (\text{m}))</td>
</tr>
<tr>
<td>NADP(^+)</td>
<td>c(^*) 4.2 \times 10^{-5}</td>
<td>c 5.8 \times 10^{-5}</td>
</tr>
<tr>
<td>NADPH</td>
<td>c          1.7 \times 10^{-5}</td>
<td>c 3.3 \times 10^{-5}</td>
</tr>
<tr>
<td>ATP</td>
<td>c          9.0 \times 10^{-4}</td>
<td>c 9.0 \times 10^{-5}</td>
</tr>
<tr>
<td>ADP</td>
<td>c          2.0 \times 10^{-4}</td>
<td>c 4.7 \times 10^{-5}</td>
</tr>
<tr>
<td>Inorganic pyrophosphate</td>
<td>c          —</td>
<td>c —</td>
</tr>
</tbody>
</table>

\(\text{c}\): Competitive inhibition.

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Table 2. Inhibition of skipjack alkaline phosphatase and nucleotide pyrophosphatase by some substrates for the former enzyme

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Alkaline phosphatase</th>
<th>Nucleotide pyrophosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
<td></td>
</tr>
<tr>
<td>$5'$-AMP</td>
<td>c$^1$</td>
<td>4.6 x 10$^{-4}$</td>
</tr>
<tr>
<td>2'-AMP</td>
<td>c</td>
<td>1.0 x 10$^{-4}$</td>
</tr>
<tr>
<td>α-Naphthylphosphate</td>
<td>c</td>
<td>2.6 x 10$^{-6}$</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>c</td>
<td>3.9 x 10$^{-4}$</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>c</td>
<td>5.7 x 10$^{-4}$</td>
</tr>
<tr>
<td>Nicotinamide mononucleotide</td>
<td>m$^3$</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Competitive inhibition, $^2$ Non-competitive inhibition, $^3$ Mixed type inhibition.

Table 3. Inhibition of skipjack alkaline phosphatase and nucleotide pyrophosphatase by some substrates for the latter enzyme and by other inhibitors

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Alkaline phosphatase</th>
<th>Nucleotide pyrophosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type</td>
<td>$K_i$ (m)</td>
</tr>
<tr>
<td>FAD</td>
<td>c$^1$</td>
<td>1.2 x 10$^{-4}$</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>m$^2$</td>
<td>6.4 x 10$^{-4}$</td>
</tr>
<tr>
<td>NADH</td>
<td>m</td>
<td>7.8 x 10$^{-4}$</td>
</tr>
<tr>
<td>Adenosine</td>
<td>No inhibition</td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>n$^3$</td>
<td>2.1 x 10$^{-5}$</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>m</td>
<td>3.2 x 10$^{-5}$</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>n</td>
<td>5.3 x 10$^{-5}$</td>
</tr>
<tr>
<td>Arsenate</td>
<td>c</td>
<td>5.0 x 10$^{-7}$</td>
</tr>
<tr>
<td>Inorganic phosphate</td>
<td>c</td>
<td>1.0 x 10$^{-4}$</td>
</tr>
</tbody>
</table>

$^1$ Competitive inhibition, $^2$ Mixed type inhibition, $^3$ Non-competitive inhibition.

Though a nucleoside, adenosine, did not inhibit both enzymes, adenine inhibited nucleotide pyrophosphatase competitively and alkaline phosphatase non-competitively. Nicotinamide, taurocholate, arsenate, and inorganic phosphate were all inhibitory to both enzymes. And the inhibition constants were also similar to each other, though the types of inhibition were all different between enzymes.

These data suggest again that the active sites of skipjack alkaline phosphatase and nucleotide pyrophosphatase are quite similar.

As reported previously, both skipjack enzymes are completely or almost indistinguishable from each other, in various properties such as molecular weight, isoelectric point, heat stability, optimal pH, the effect by metallic ions, etc. By the present studies, it was disclosed that both enzymes are two distinct proteins, though their structures including active sites are very similar to each other. In this connection, we observed the occurrence of many isoenzymes in skipjack alkaline phosphatase. Hence, the possibility that some of such alkaline phosphatase isoenzymes show also a nucleotide pyrophosphatase activity may not be excluded fully.

ABNEY et al. reported that nucleotide pyrophosphatase and alkaline phosphodiesterase activities in mouse spleen lymphocytes are exhibited by the same or very similar enzymes at the outer aspect of the lymphocyte plasma membrane. TAKEI et al. also demonstrated that nucleotide pyrophosphatase and 5'-nucleotidase in yeast is a single enzyme. It was also reported by EATON and Moss that alkaline phosphatase and inorganic pyrophosphatase activities in human liver and small intestine are shown by the same enzyme with a single active center. Many authors also suggested that rat small intestinal Ca$^{2+}$-activated ATPase is identical with alkaline phosphatase.

In recent years, homology of different enzymes has been elucidated on the basis of their primary or higher structures, and has been discussed in relation to molecular evolution: e.g., serine proteases, phosphorylase and glycogen synthase, lysozyme and α-lactalbumin, phosphagen kinases, etc. Skipjack alkaline phosphatase and nucleotide pyrophosphatase may provide another pair of homologous proteins.
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References