Substrate Specificity and Salt Inhibition of Five Proteinases Isolated from the Pyloric Caeca and Stomach of Sardine

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To elucidate the mechanism of hydrolysis of fish muscle proteins by fish proteinases in fish sauce production, each pure preparation of three alkaline proteinases and two acid proteinases from sardine was tested for its ability to hydrolyze various proteins and its stability in the presence of 0 to 25% of NaCl. Each of the alkaline proteinases hydrolyzed casein more rapidly than other proteins. A major alkaline proteinase (III) hydrolyzed sarcoplasmic protein from sardine 5-times faster than other alkaline proteinases. Each of two acid proteinases hydrolyzed hemoglobin and myoglobin more rapidly than the other proteins. After preincubation with 25% NaCl, an alkaline proteinase (III) and an acid proteinase (II) were stable although the other proteinases became unstable. The two proteinases, alkaline proteinase III and acid proteinase II, were also stable for three months after the beginning of fish sauce production. The proteolytic activity of each of alkaline and the acid proteinases was strongly inhibited by more than 15% NaCl; however, minimum inhibition was observed when sardine muscle proteins were used as the substrate.

Fish sauce is mainly produced by the digestion of fish muscle proteins by fish proteinases in the presence of a high concentration of NaCl.1-3) The effect of the presence of a high concentration of NaCl on the liberation of proteins and amino acids in fish sauce production has been investigated, using whole fish bodies.4-7) However, no studies have been carried out on the precise mechanism of proteolysis of fish proteins by fish enzymes in the presence or the absence of NaCl, mainly due to the lack of a pure enzyme preparation. Recently we obtained pure preparations of three alkaline proteinases (I, II and III) from the pyloric caeca of sardine8) and two acid proteinases (I and II) from its stomach.9) Alkaline proteinase I from sardine is of the unique type, alkaline proteinase II and III are an anionic α-chymotrypsin-like enzyme and an anionic trypsin-like enzyme, respectively. Acid proteinase I and II from sardine are both similar to mammalian cathepsin D and pepsin except that I and II can hardly hydrolyze a synthetic pepsin substrate, N-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine. These results suggest that sardine proteinases are unique in hydrolyzing various proteins including fish muscle proteins.

The purpose of this paper is to examine the hydrolysis of various proteins by these pure proteinases from sardine and to elucidate the effect of high concentrations of NaCl on the stability and activity of the sardine proteinases.

MATERIALS AND METHODS

Pure proteinases from sardine. Three alkaline proteinases (I, II and III) were isolated by (NH₄)₂SO₄ fractionation, DEAE-cellulose chromatography and gel filtration on Sephadex G-100 from the pyloric caeca of sardine as described in our previous paper.8) Likewise, two acid proteinases I and II were isolated from the stomach of sardine by a similar method.9) The final enzyme preparations were judged homogeneous by multiple criteria and used in all experiments in this paper.

Sardine protein substrates. Three sardine proteins i.e., sarcoplasmic, myofibril and stroma proteins, were fractionated from sardine muscle according to the method of Connell.10-12) and used as a fish protein substrate as described in our previous papers.8,9) Milk casein, egg...
albumin, bovine serum albumin, fibrinogen, fibrin and collagen were obtained from Wako Pure Chemical Co. Bovine pancreatic trypsin, α-chymotrypsin and porcine pepsin from Sigma Chemical Co.

Proteinase activity. Proteinase activity of the enzymes was determined according to a modification of Anson's method\(^\text{13}\) as described in the previous papers.\(^\text{8,9}\) Proteinase activity, after preincubation with a high concentration of NaCl, was determined according to the modification of Anson's method\(^\text{13}\) described by Hayashi et al.\(^\text{14}\)

RESULTS AND DISCUSSION

Hydrolysis of proteins by sardine proteinases

Various proteins including sardine protein were hydrolyzed at their optimal pH by each pure preparation of the alkaline and acid proteinases isolated from sardine.

Alkaline proteinase. Table I shows the relative rate of hydrolysis of various proteins by alkaline proteinase I, II and III. In general, each alkaline proteinase hydrolyzed milk casein more rapidly than all other proteins. However, alkaline proteinase III hydrolyzed sarcoplasmic proteins from sardine muscle more easily than alkaline proteinases I and II or bovine pancreatic trypsin and α-chymotrypsin. As the specific activity toward milk casein for alkaline proteinase III is about double that for I and II (the legend in Table I), it is calculated that III can hydrolyze the sardine muscle protein approximately 5-times faster than I and II.

Acid proteinase. Table II shows the relative rate of hydrolysis of various proteins by acid proteinase I and II. Both enzymes hydrolyzed hemoglobin and myoglobin more rapidly than the other proteins. Acid proteinase I was similar to II in the relative rate of hydrolysis of various proteins shown in Table II. Both acid proteinases could not hydrolyze the sarcoplasmic protein well compared with hemoglobin.

The acid proteinases were different from porcine pepsin in that pepsin hydrolyzed bovine serum albumin more easily than the acid proteinases.

Effect of NaCl on proteinase stability

To know the role of fish enzymes in fish sauce production, the stability and proteolytic activity of each pure preparation of the alkaline and acid proteinases from sardine were examined in the presence of high concentrations of NaCl.

Alkaline proteinase. After each of the alkaline proteinases I, II and III was prein-

<table>
<thead>
<tr>
<th>Protein substrate</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>Trypsin</th>
<th>α-Chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk casein</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>50</td>
<td>73</td>
<td>75</td>
<td>31.1</td>
<td>28.1</td>
</tr>
<tr>
<td>Fish protein*</td>
<td>27.3</td>
<td>32</td>
<td>72.5</td>
<td>11</td>
<td>16.5</td>
</tr>
<tr>
<td>Collagen</td>
<td>27.3</td>
<td>28</td>
<td>37.7</td>
<td>11</td>
<td>6.2</td>
</tr>
<tr>
<td>Bovine albumin</td>
<td>37</td>
<td>36.5</td>
<td>36.2</td>
<td>20</td>
<td>15.5</td>
</tr>
<tr>
<td>Fibrin</td>
<td>18.2</td>
<td>23.4</td>
<td>21.7</td>
<td>10</td>
<td>6.3</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>15.2</td>
<td>13.3</td>
<td>11.1</td>
<td>11</td>
<td>12.5</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>1.8</td>
<td>0.02</td>
<td>8.6</td>
<td>0.03</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* Sarcoplasmic protein from sardine muscle prepared as described in MATERIALS AND METHODS.
Table II. Hydrolysis of Protein Substrates by Acid Proteinase I and II from Sardine

Each of the proteinase solutions (25 μg/ml) was incubated at 37°C for 10 min with 0.6% protein substrates, which were preincubated at 100°C for 5 min. Buffers were: 0.1 M sodium acetate-HCl buffer, pH 2 for acid proteinase II and porcine pepsin; 0.1 M acetate buffer, pH 4 for acid proteinase I. Each enzyme activity is expressed as the percentage of each activity in hemoglobin.

<table>
<thead>
<tr>
<th>Protein substrate</th>
<th>Proteolytic activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>100</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>106.5</td>
</tr>
<tr>
<td>Milk casein</td>
<td>16.2</td>
</tr>
<tr>
<td>Fish protein*</td>
<td>24</td>
</tr>
<tr>
<td>Fibrin</td>
<td>8.3</td>
</tr>
<tr>
<td>Bovine albumin</td>
<td>8.8</td>
</tr>
<tr>
<td>Collagen</td>
<td>13</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* Sarcoplasmic protein from sardine muscle prepared as described in Materials and Methods.

Fig. 1. Effect of NaCl on the Stability of Alkaline Proteinases I, II and III from Sardine.
The purified enzyme (250 μg) was preincubated in 2 ml of 0.1 M phosphate buffer, pH 7.0 at 30°C for 24 hr with 0 to 25% NaCl and assayed toward casein after removing the NaCl by dialysis.

Fig. 2. Effect of NaCl on the Stability of Acid Proteinases I and II from Sardine.
The purified enzyme (250 μg) was preincubated in 2 ml of 0.1 M phosphate buffer, pH 7.0 at 30°C for 24 hr with 0 to 25% NaCl and assayed toward hemoglobin after removing the NaCl by dialysis.
concentration of NaCl.

Effect of NaCl on proteolytic activity

Alkaline proteinase. The proteolytic activity of each of the alkaline proteinases I, II and III was inhibited strongly in proportion to the amount of NaCl added to the reaction medium (Fig. 3). However, the extent of inhibition of the proteinases varied depending on the type of protein substrate used. Thus, an NaCl concentration indicating 50% inhibition ($I_{50}$) for each proteinase was compared using five different substrates. $I_{50}$ was 6~7% toward milk casein, 9~12% toward hemoglobin and 15% toward the sarcoplasmic protein from sardine muscle. A salt-soluble myofibril protein and a stroma protein from sardine muscle were similar to the sarcoplasmic protein in the effect of NaCl in their proteolysis. This result probably indicates that fish muscle proteins are more resistant to their conformational change in a high concentration of NaCl than casein and hemoglobin. Only a 19% inhibition in alkaline proteinase III was observed in the presence of 20% NaCl when a small substrate, benzoylarginine ethyl ester was used, whereas a 60 to 90% inhibition was seen in the same condition when protein substrates were used. This result shows that a high concentration of NaCl scarcely caused a change in the structure of the small substrate. Hayashi et al.\textsuperscript{14) also reported that the extent of inhibition of an Aspergillus proteinase by a high concentration of NaCl depended on the types of protein and peptide substrates.

Acid proteinase. The proteolytic activity of each of the acid proteinases I and II was also inhibited in the presence 15 to 20% NaCl; however, the inhibition of proteolysis was much less in the sarcoplasmic proteins from sardine than in casein and hemoglobin (Fig. 4). As observed in the alkaline proteinases, myofibril protein and stroma protein from sardine were also similar to the sarcoplasmic protein in the effect of NaCl in their proteolysis.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Effect of NaCl Concentration on Proteolytic Activity of Alkaline Proteinases I, II and III from Sardine. 
\textemdash\textemdash, milk casein; \textbullet\textbullet\textbullet, hemoglobin; \textsquare\textsquare\textsquare, sarcoplasmic protein from sardine. The purified enzyme (30 \mu g) was incubated in a 0.1 M phosphate buffer, pH 7.0 for 30 min at 30°C with each substrate (0.6%) in the presence of 0 to 20% NaCl.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Effect of NaCl Concentration on the Activity of Sardine Acid Proteinases I and II.
\textemdash\textemdash, milk casein; \textbullet\textbullet, hemoglobin; \textsquare\textsquare, sarcoplasmic protein from sardine. The purified enzyme (30 \mu g) was incubated in a 0.1 M acetate buffer pH 5.5 for 30 min at 30°C with each substrate (0.6%) in the presence of 0 to 20% NaCl.}
\end{figure}
**Proteinases from Sardine**

We tried a preliminary study to produce a fish sauce from fresh sardine and to identify the proteinases in the fish sauce periodically during an incubation of pieces of sardine in the presence of 22% NaCl at 30°C. Alkaline proteinase III and acid proteinase II were stable for three months after the beginning of fish sauce production, although all other proteinases in sardine disappeared almost completely within a week. These results are consistent with the ones described in "Effect of NaCl on proteinase stability" in this text.

The pH of the fish sauce during its production was 5.3~5.8 and fairly distant from the optimal pH values of alkaline proteinase III and acid proteinase II. Thus, the activity of both proteinases appearing in the fish sauce was very much suppressed by the pH shift from their optimal value and by the presence of a high concentration of NaCl. The suppressed proteinase activity causes a long period for fish sauce production.

The proteinases in the fish sauce were identified by their isoelectric point and elution pattern from DEAE- or CM-cellulose column as described in our previous papers. Details on the proteinases or amino acids and peptides appearing in fish sauce production will be published elsewhere.

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**REFERENCES**