An Improved Method for Purification of Ovomucoid from Japanese Quail Egg White.

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Ovomucoid was purified from Japanese quail egg white by a method including alcohol fractionation and column chromatographies on Sephacryl S-200 and SP-Sephadex C-25. The ovomucoid preparation obtained by the improved purification method was shown to be homogeneous by electrophoresis. Purified ovomucoid had a molecular weight of 26,000. The amino acid composition was characterized by a high content of aspartic acid and low contents of methionine, isoleucine and histidine. This ovomucoid inhibited trypsin but not α-chymotrypsin. These properties were in good agreement with those deduced from the amino acid sequence of Japanese quail ovomucoid, indicating that the method used here is very effective for the rapid purification of ovomucoid.

Abbreviation
SDS, sodium dodecyl sulfate

Introduction

Ovomucoid is a major component of avian egg whites and is responsible for most of the inhibitory activity against serine proteinases in the egg white. Concerning the purification of ovomucoid, the Lineweaver and Murray trichloroacetic acid-aceton method is well known. Davis et al. have also purified ovomucoid from chicken egg white by ammonium sulfate fractionation followed by successive batch treatment with anion and cation exchangers. However, preparations of ovomucoid obtained by these methods often contain other egg white proteins as impurities. We, therefore, tried to improve a method for purification of ovomucoid.

In this paper, we describe the improved purification method and properties of ovomucoid obtained by this method from Japanese quail egg white.

Materials and Methods

Materials.

Commercial eggs of Japanese quail were obtained from a local store, and fresh egg white was separated from the eggs. Enzymes and substrates were purchased from the following companies: bovine trypsin (Type III) and bovine α-chymotrypsin (Type II) from Sigma Chemical Co. (St. Louis, U.S.A.) and α-N-benzoyl-DL-arginine-p-nitroanilide HC1 (Bz-DL-Arg-pNA) and α-N-benzoyl-L-tyrosine-p-nitroanilide (Bz-Tyr-pNA) from the Peptide Institute, Inc. (Osaka). Sephacryl S-200 and SP-Sephadex C-25 were products of Pharmacia Fine Chemicals. (Uppsala, Sweden).
Measurement of inhibitor activity.

Trypsin inhibitory activity\(^9\) was estimated from the residual trypsin activity in the presence of the inhibitor and measured as follows: the reaction mixture consisted of 2.2ml of inhibitor solution in 0.1M Tris-HCl buffer (pH8.0) containing 10mM CaCl\(_2\), 0.1ml of bovine trypsin solution and 0.1ml of Bz-DL-Arg-pNA solution (10mg/ml in dimethylsulfoxide). After incubation for 10 min at 37°C, the reaction was stopped by the same procedure as described above.

\(\alpha\)-Chymotrypsin inhibitory activity was measured by the following method: the reaction mixture consisted of 1.7ml of inhibitor solution in 0.1M Tris-HCl buffer (pH 8.0) containing 10mM CaCl\(_2\), 0.1ml of chymotrypsin solution and 0.2ml of Bz-L-Tyr-pNA solution (1.22mg/ml dimethylformamide). After incubation for 10 min at 37°C, the reaction was stopped by adding 1.0ml of 10% acetic acid and the absorbance of the mixture was measured at 410nm.

Electrophoretic analysis and estimation of molecular weight.

SDS-PAGE was done by the method of Laemmli using 15% acrylamide gel at pH8.8. The molecular weight of ovomucoid was estimated by SDS-PAGE. Marker proteins used were: phosphorylase b (MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), carbonic anhydrase (MW 30,000), soybean trypsin inhibitor (MW 20,100), \(\alpha\)-lactalbumin (MW 14,400).

Amino acid analysis.

The protein was hydrolyzed with 6N HCl in sealed and evacuated tubes at 110°C for 24hr. Amino acid composition was analyzed on a Hitachi 835 amino acid analyzer by the procedure of Spackman et al.\(^9\)

Results

Purification

The egg white (100ml) of Japanese quail eggs was diluted 5-fold with distilled water. Cold 99.5% ethyl alcohol was added to the diluted egg white to a final alcohol concentration of 68% at 4°C with stirring.

The suspension was centrifuged at 25,500 xg for 60 min.

To the supernatant obtained, ethyl alcohol was added to give a final alcohol concentration of 75% as mentioned above.

After centrifugation of the suspension at 25,500 xg for 60 min, the precipitate obtained was dissolved in distilled water. After the insoluble materials had been removed by centrifugation at 25,500 xg for 60 min, the supernatant was then lyophilized to obtain the crude ovomucoid. The procedure of purification of the crude ovomucoid is shown in Fig. 1.

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Fig. 1. Preparation Procedure of Crude Ovomucoid.

Quail Egg White

- 5 fold-dilution with water
- 55% Et-OH precipitation
- centrifugation

\((16,000 \text{ xg}, 60\text{min}, 4^\circC)\)

Ppt. Sup.

- 68% Et-OH precipitation
- centrifugation

\((25,500 \text{ xg}, 60\text{min}, 4^\circC)\)

Ppt. Sup.

- 75% Et-OH precipitation
- centrifugation

\((25,500 \text{ xg}, 60\text{min}, 4^\circC)\)

Ppt. Sup.

- dissolution in water
- centrifugation

\((25,500 \text{ xg}, 60\text{min}, 4^\circC)\)

Ppt. Sup.

- lyophilization

\[\text{crude ovomucoid}\]
An Improved Method for Purification Ovomucoid

This crude preparation was dissolved in 10 ml of 50 mM NH₄HCO₃, put on a Sephacryl S-200 column (2.5 x 90 cm) equilibrated with the same solution, and chromatographed (Fig. 2). The protein peak with trypsin inhibitory activity was collected and lyophilized. The lyophilized preparation was dissolved in 3 ml of 50 mM Acetate-HCl buffer (pH3.5), put on a column (2.5 x 45 cm) of SP-Sephadex C-25 equilibrated with the same buffer, and eluted first with this buffer and then with a linear gradient to 1M NaCl in the same buffer (Fig. 3). The inhibitor fraction eluted with the NaCl gradient was collected, desalted, and lyophilized (375 mg).

Fig. 2. Gel-chromatography of the Crude Preparation on a Sephacryl S-200 column (2.5 x 90 cm).
- • absorbance at 280nm;
- ○ trypsin inhibitory activity.

Fig. 3. Ion-exchange chromatography of the Inhibitor (ovomucoid) Fraction on a SP-Sephadex C-25 column (2.5 x 45 cm).
- • absorbance at 280nm;
- ○ trypsin inhibitory activity.
- ----- NaCl concentration (M)
Fig. 4. SDS-PAGE of Purified Japanese Quail Ovomucoid.

Table 1. Amino Acid Composition of Ovomucoid from Japanese Quail Egg White.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>mole % ovomucoid</th>
<th>mole % reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>15.41</td>
<td>15.05</td>
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<tr>
<td>Threonine</td>
<td>7.99</td>
<td>8.60</td>
</tr>
<tr>
<td>Serine</td>
<td>5.15</td>
<td>5.91</td>
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<tr>
<td>Glutamic acid</td>
<td>9.85</td>
<td>9.68</td>
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<tr>
<td>Proline</td>
<td>5.55</td>
<td>4.30</td>
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<tr>
<td>Glycine</td>
<td>8.35</td>
<td>8.06</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.88</td>
<td>2.69</td>
</tr>
<tr>
<td>Half-Cystine</td>
<td>9.27</td>
<td>9.68</td>
</tr>
<tr>
<td>Valine</td>
<td>8.38</td>
<td>9.14</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.93</td>
<td>1.61</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.68</td>
<td>1.61</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.38</td>
<td>4.30</td>
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<tr>
<td>Tyrosine</td>
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<td>4.30</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.13</td>
<td>3.23</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.09</td>
<td>6.99</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.31</td>
<td>2.15</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.64</td>
<td>2.69</td>
</tr>
<tr>
<td>a Tryptothan</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.00</strong></td>
<td><strong>100.00</strong></td>
</tr>
</tbody>
</table>

- not determined

The final preparation was shown to be a homogeneous protein by electrophoretic analysis as presented in Fig.4.

**Molecular weight**

The molecular weight of ovomucoid from Japanese quail was estimated to be 26,000 by SDS-PAGE as shown in Fig.4.

**Amino acid composition**

Table 1 shows the amino acid composition of Japanese quail ovomucoid purified in this study together with the composition calculated from its amino acid sequence. Ovomucoid from Japanese quail is rich in aspartic acid, and poor in methionine, isoleucine, and histidine. The amino acid composition of ovomucoid purified in this study was very similar to that of name with each other.

**Inhibitory activity.**

The inhibitory effects of ovomucoid on proteases were examined at enzyme concentrations of the order of 10^{-7}M. Ovomucoid inhibited only trypsin and showed no inhibitory activity against α-chymotrypsin (pH 8.0). Fig. 5 shows the titration pattern of bovine trypsin with ovomucoid.

The inhibition was shown to be linear up to 80% inhibition.

From the point of 100% inhibition obtained by linear extrapolation it was seen that 1 μg of the enzyme was inactivated by 0.61 μg of the inhibitor.
Fig. 5. Titration Curves of Bovine Trypsin with Ovomucoid from Japanese Quail Egg White.

A constant amount of trypsin was mixed with various amounts of Ovomucoid and the amount of free enzyme in each reaction mixture was measured.

Discussion

This paper describes the purification of ovomucoid from Japanese quail egg white. The purification method used here consists of alcohol fractionation, gel filtration on Sephacryl S-200, and ion-exchange chromatography on SP-Sephadex C-25.

On the other hand, the method by which Lineweaver and Murry\(^1\) isolated ovomucoid from chicken egg white contains trichloroacetic acid-aceton treatment.

Furthermore, the method of Davis et al.\(^2\) is ammonium sulfate fractionation followed by successive batch treatment with anion and cation exchangers. Preparations of chicken ovomucoid obtained by the latter two methods often contain other egg white proteins. However, the ovomucoid preparation obtained by the present method was shown homogeneous by electrophoresis.

References