CRYSTALLINE PEPSIN

V. ISOLATION OF CRYSTALLINE PEPSIN FROM BOVINE GASTRIC JUICE

BY JOHN H. NORTHROP

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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A crystalline protein having powerful proteolytic activity has been described in previous papers of this series (1). This protein was isolated from commercial pepsin preparations which, in turn, had been prepared from the gastric mucosa of swine. The possibility exists that this protein was formed from some more complicated compound during the process of extraction and does not represent the enzyme as secreted normally in the gastric juice. In order to determine whether the activity of the gastric juice is due to the same protein, experiments were undertaken to isolate the enzyme from gastric juice.

Preliminary determinations of the activity of swine gastric juice showed that if the activity were due to the crystalline enzyme isolated from the gastric mucosa, the quantity of this protein in the gastric juice was extremely small and that a very large quantity of gastric juice would be necessary before any attempt at isolation could be made. It was found to be impossible to obtain sufficient quantities of swine gastric juice but bovine gastric juice may be collected in quantity as described by Williams (2).

The contents of the fourth pouch of cattle was removed as soon as possible after the cattle had been killed in the slaughter house. About ½ liter of juice was obtained from each animal and larger quantities were found in those animals which had been recently fed. The juice obtained in this way contained more or less undigested food and fil-

1 The writer is indebted to Dr. F. O. Taylor of Parke, Davis and Company for this information.

2 The writer's attention was directed to this valuable method by Dr. Robert Loeb of the Presbyterian Hospital.
tered with difficulty. After filtration it was found to contain about 1 mg. of nitrogen and about 0.002 proteolytic units per ml. as determined by the hemoglobin method (3). This corresponds to about 0.01 mg. of nitrogen in the form of the enzyme protein previously isolated, assuming that the activity of the enzyme in the gastric juice is the same as that of the purified enzyme (4). This quantity of protein is too small to be determined and in fact the gastric juice gives no precipitate with trichloracetic acid and would ordinarily be considered to be protein-free.

Pekelharing (5) has described a method of obtaining a highly active protein from gastric juice by dialysis against dilute hydrochloric acid and this method was tried. It was found, however, that a large loss in activity occurred during the dialysis so that the yield of active material was too small to be practical for purposes of isolation. It is also evident from the value for the solubility of crystalline pepsin already determined (6) that it cannot precipitate from gastric juice under the conditions described by Pekelharing since the solubility of the crystalline pepsin was found to be about 0.3 mg. of nitrogen per ml. at the minimum point (pH 2.8) while from the value for the activity the gastric juice contains only about 0.01 mg. pepsin nitrogen per ml. There is no doubt, however, that an active precipitate appears upon dialysis, as described by Pekelharing, but it is probable that this precipitate consists of a compound or an adsorption complex of the enzyme, possibly with the mucin or a mucoprotein present in the gastric juice. This conclusion is further indicated by the fact that the analysis of Pekelharing's preparation showed higher carbon and hydrogen and lower nitrogen than does crystalline pepsin itself.

**Method of Isolation**

Fractionation with various salt solutions was tried and it was found that the activity could be concentrated in the protein fraction. This

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This mucilaginous material is probably the mucoprotein recently described by Webster and Komarov (J. Biol. Chem., 1932, 96, 133) who have suggested the possibility of the existence of a complex between this mucoprotein and the pepsin. This compound does not exist in solution, however, since the diffusion coefficient of the enzyme in the gastric juice is the same as that of the purified enzyme. These experiments are described later in the paper.
### TABLE I

**Preparation of Crystalline Pepsin from Bovine Gastric Juice**

<table>
<thead>
<tr>
<th>No.</th>
<th>Vol. (ml)</th>
<th>n/ml</th>
<th>P.T. (mg)</th>
<th>Per ml</th>
<th>Total</th>
<th>Per mg N</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>5500</td>
<td>1.0</td>
<td>0.0025</td>
<td>14</td>
<td>0.0025</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>500</td>
<td>0.018</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>310</td>
<td>0.025</td>
<td>7.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>114</td>
<td>0.53</td>
<td>0.065</td>
<td>7.4</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>80</td>
<td>0.42</td>
<td>0.078</td>
<td>6.3</td>
<td>0.186</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>3 gm.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>5.6</td>
<td>1.0</td>
<td>10</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>2.8</td>
<td>0.5</td>
<td>5</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>0.77</td>
<td>0.146</td>
<td>2.8</td>
<td>0.19</td>
<td></td>
</tr>
</tbody>
</table>

Precipitate No. 9 + 10 ml. n/10 sodium acetate, titrated to pH 3.0 + n/2 sulfuric acid, slight precipitate in dark viscous liquid, could not be filtered nor centrifuged; 1 volume saturated magnesium sulfate added and suspension filtered with suction. Precipitate dissolved in 8 ml. n/10 sodium acetate, clear yellow solution.

Titrated to pH 3.0 + n/2 sulfuric acid, stood 18 hrs. 6°C., filtered with suction (slow). Precipitate dissolved at 45°C. with minimum quantity of water.

Cooled slowly while stirring. Crystals formed after about 1 hr. Kept at 20°C. for 24 hrs. and filtered. Precipitate normal pepsin crystals slightly yellowish—about 0.1 gm.

Crystals dissolved in n/10 sodium acetate...
fraction, however, contained a large amount of a mucilaginous substance which rendered the solutions extremely difficult to work with since they could be centrifuged or filtered only with the greatest difficulty. This difficulty has been encountered by other workers and it was found by Fenger and Andrew (7) that the active material could be freed from this mucilaginous impurity by precipitating in the cold with 75 per cent acetone. This process entails a loss of nearly half the total activity but no more satisfactory method could be found. A method of fractionation was eventually worked out, which consisted essentially in preliminary precipitation with saturated ammonium sulfate, solution in 60 per cent acetone, and precipitation with 75 per cent acetone. This process yielded a white amorphous precipitate free from most of the mucilaginous impurity and possessing about half of the total original activity. It was further purified by repeated precipitation with one-half saturated magnesium sulfate and finally crystallized from warm water. The details of the method are shown in Table I.

As the table shows, the activity per mg. of nitrogen, as determined by the hemoglobin method, increases from 0.0025, which is the value found in the original gastric juice, to about 0.19, which is the characteristic value already found for the crystalline protein isolated from swine (3). The yield of crystalline material is very poor and represents only a few per cent of the total original activity. Of this loss, about one-half occurs during the precipitation with acetone and the remainder during the repeated precipitation with magnesium sulfate. Actually about 100 mg. of crystalline material was obtained from 15 liters of gastric juice.

**Properties of the Crystalline Protein**

1. **Crystalline Form**

   The crystals are small, hexagonal, bipyramids and are indistinguishable by inspection from the crystals obtained previously from the gastric mucosa of swine.

2. **Specific Activity of the Crystals**

   The activity of the material was determined, as previously described (4), by a series of methods and compared with that of a purified prepa-
ration from swine mucosa. No significant difference in activity could be detected by any of the methods. The results of these experiments are shown in Table II.

It will be noted that the specific activity of both preparations differs in some cases from that already described for the swine pepsin but that the activity of the two preparations, as determined in this series of experiments, was not significantly different. This disparity between the activity found in these determinations and that found previously is due to the fact that the protein solutions were different from those in the previous experiments and, as usual, gave slightly different figures for the activity. The activity, as determined by the digestion of

### Table II

**Properties of Crystalline Pepsin from Bovine Gastric Juice and from Swine Stomach**

<table>
<thead>
<tr>
<th>Pepsin</th>
<th>Proteolytic activity per mg. nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gel V&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bovine gastric juice</td>
<td>10.6</td>
</tr>
<tr>
<td>Swine stomach standard pepsin solution</td>
<td>10.8</td>
</tr>
<tr>
<td>May 9, 1932</td>
<td>11.0</td>
</tr>
</tbody>
</table>

hemoglobin, however, agrees with that previously found since hemoglobin solutions are more reproducible than those of the other proteins.

Since only a small per cent of the original activity present in the gastric juice was actually isolated in the form of the crystalline protein it is possible that the original gastric juice contains other enzymes and that only a part of its activity is due to the fraction isolated. In order to check this point the activity of the gastric juice, as determined by various methods, was compared with the activity of crystalline swine pepsin. Since the gastric juice is too dilute for protein nitrogen determinations to be made, the specific activity per mg. of protein nitrogen cannot be calculated and it is necessary to express the results in terms of the activity per ml. solution. If the gastric juice contained other proteolytic enzymes it would be expected that the relative activity of the juice, compared to swine pepsin, would vary when
measured with different proteins. This, however, is not the case. If a solution of crystalline pepsin is made up so as to have the same activity per ml. as the gastric juice, as determined by the hemoglobin method, the two solutions are found to have the same activity when the activity is determined by any of the other methods. If the gastric juice, therefore, contains another enzyme beside that isolated, the relative activity of this hypothetical enzyme on the various proteins must be the same as that of the fraction isolated.

**Optical Activity**

The optical rotation of the material was determined in solution in N/10 sodium acetate, pH about 5.2 at 25°C. with the D line. The specific rotation was found to be about $-93^\circ$ while recrystallized swine pepsin gave a specific rotation of about $-70^\circ$. However, the optical activity of swine pepsin, which has only been crystallized once, varies from $-100^\circ$ to $-80^\circ$ so that the difference cannot be considered significant. Lack of material prevented further purification of the bovine pepsin. This variation in the optical activity is probably due to the presence of non-protein impurities.

**Diffusion Coefficient**

It was mentioned in discussing the method of isolation that a mucilaginous substance from which the active protein can be separated only with great difficulty is present in quite large amount in the original gastric juice. The possibility is suggested, therefore, that the enzyme is present in the gastric juice in some form of combination with this mucilaginous material. The methods used in the isolation would not be expected to split any chemical compound but might be considered to separate an adsorption complex. If such an adsorption complex existed in the gastric juice it would be expected that the size of the active particles (or molecules) in the juice would be considerably larger than that of the molecules of the purified enzyme. The diffusion coefficient of the active substance in the gastric juice should therefore be smaller than that of the purified enzyme. In order to determine the diffusion coefficient and, hence, the size of the active molecule in the unchanged gastric juice, the measurements were carried out as already described for the swine pepsin (8, 9) but owing
to the low concentration of enzyme present in the gastric juice they could not be made as accurately. The measurements gave a value for the diffusion coefficient in n/1 pH 4.5 acetate buffer at 6°C. of 0.049 ± 0.002 cm.²/day which agrees, within the experimental error, with that previously found (9) for the crystalline swine pepsin. These measurements are experimental evidence that the active molecule present in the gastric juice is the same or very nearly the same size as that in a solution of the purified crystalline protein and hence that the active molecule in the gastric juice does not exist in the form of a large complex.

**Solubility Experiments**

The experiments just described show no significant difference in properties between the bovine pepsin and the swine pepsin. A much more sensitive test for the identity of the two, however, consists in solubility measurements since the solubility of even closely related proteins is quite characteristic. This method, theoretically, is the same as the classical melting point method of organic chemistry and was first used as a method of establishing the difference between similar proteins by Landsteiner, and Heidelberger (10). The solubility of two different substances is, in general, independent of each other so that the solubility of a mixture of two different solids would be equal to the sum of the two solubilities separately. If the two solids, however, form a solid solution the solubility of a mixture of the two cannot be predicted with certainty but would be expected to lie between that of the two substances in pure solution (11). This result was obtained by Landsteiner and Heidelberger with hemoglobin from closely related animals and indicates that the hemoglobins are different but form solid solutions rather than mixtures. Owing to the small amount of material available it was not found possible to obtain accurate solubility figures for the bovine pepsin since it was necessary to use a precipitate obtained from the mother liquor of the crystallization. This mother liquor was precipitated with half-saturated magnesium sulfate, the precipitate redissolved in 0.02 molar pH 4.65 acetate buffer and precipitated by the addition of an equal volume of magnesium sulfate solution having a specific gravity of 1.294. This precipitate was stirred with about 10 ml. of a solution consisting of 1 volume of 0.02 molar pH 4.65 acetate and 1 volume saturated magnesium sulfate
specific gravity 1.294, the suspension centrifuged, and the supernatant solution analyzed for nitrogen. This process was repeated until a constant value was obtained on successive washings. Four or five washings are usually required before constant solubility is obtained. The precipitate was then dissolved by the addition of 5 ml. of 0.02 molar pH 4.65 acetate and precipitated by the addition of 5 ml.

TABLE III

| Solubility of Pepsin from Swine Mucosa and from Bovine Gastric Juice | 22 ° Specific gravity saturated magnesium sulfate = 1.294 |
|---|---|---|
| in [0.01 M pH 4.65 Acetate<br>0.50 Saturated Magnesium Sulfate] | at 26°C. |

<table>
<thead>
<tr>
<th>Swine pepsin</th>
<th>Bovine pepsin</th>
<th>Swine + bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>0.80</td>
<td>0.17</td>
<td>0.69</td>
</tr>
<tr>
<td>0.12</td>
<td>0.08</td>
<td>0.20</td>
</tr>
</tbody>
</table>

About 1 gm. amorphous pepsin stirred with 10 ml. solvent, centrifuge, and filter, repeat 4 times, filtrate. ........................................ 1
5 ml. 0.02 pH 4.65 acetate added to precipitate, clear solution. ........................................ 2
5 ml. sat. magnesium sulfate added (precipitate), centrifuge and filter supernatant. ........ 0.84
5 ml. supernatant No. 2, stir + precipitate No. 1 swine pepsin; filtrate. 0.85
5 ml. supernatant No. 2 from swine pepsin, added to precipitate No. 1 from bovine pepsin, stir, centrifuge and filter, supernatant. 1.60 0.32
Calculated if solubilities additive. .... 1.49 0.20

saturated magnesium sulfate. The solubility determined in this way agreed with that found by stirring the precipitate. This shows that the value is an equilibrium one since the same value is obtained from both sides. The experimental technique was the same as that previously described (6).

A solution of three times recrystallized swine pepsin was treated in the same way. The solubility of the bovine pepsin determined in this manner was found to be slightly less than that of the
swine pepsin but, owing to the small quantity available, this difference could not be considered significant. However, when the saturated solution of the swine pepsin was added to the solid bovine pepsin and the suspension stirred, the total protein and activity of the resulting solution was very nearly equal to the sum of these quantities as determined separately with the two preparations. The solubilities of the two preparations are, therefore, additive which shows that the proteins are chemically different and form mixtures in the solid phase. The results of these experiments are shown in Table III.

The solubility of the swine pepsin is slightly higher than that previously found (12) owing to slightly lower magnesium sulfate concentration and slightly higher temperature.

SUMMARY

1. A method has been described for isolating a crystalline protein with high proteolytic activity from bovine gastric juice by means of precipitation with magnesium sulfate and fractionation of the precipitate with acetone and magnesium sulfate.

2. The crystalline protein obtained in this way has the same crystalline form, optical activity, and specific activity, as determined by a number of methods, as does the crystalline protein previously isolated from swine gastric mucosa.

3. The solubility of the two preparations, however, is additive so that they are different although very closely related proteins.

REFERENCES