PREPARATION AND PROPERTIES OF CRystalline SALMON PEPSIN*

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The techniques of crystallization, developed in recent years, have permitted enzyme studies that are far more exact than were possible with the more crude preparations. Among the crystalline enzymes, Northrop's pepsin has been the object of widespread and intensive research (1) which has contributed to the knowledge of enzyme action and mode of operation. In order to further the understanding of pepsin, it seemed worth while to begin comparative studies on pepsins from sources other than beef and swine. In the work to be described the pepsin of the Pacific Coast king salmon (Oncorhynchus tschawytscha) was crystallized, and comparative studies have been begun.

Methods for crystallizing swine pepsin have been described by Northrop (2) and Philpot (3). Fortunately, the techniques for handling mammalian pepsin and pepsinogen (4) have been found applicable, with some modification, to fish pepsin.

Most of the fish used as sources of material were caught in traps at La Conner, Washington, and brought under ice to Seattle, where they were dressed, after having been about 18 hours out of water. The stomachs were removed, cleaned, and stored frozen; about 100 were required for each kilo of mucosa. The stomachs from actively feeding fish, caught at sea, were larger and contained more pepsin, but were difficult to ship successfully to Seattle.

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Preparation of Enzyme—The mucosae were stripped from the muscle, washed and brushed to remove all mucin, and frozen for storage. In a half thawed condition they were finely ground for the extraction. It was found advantageous to extract in alkaline solution, which yielded pepsinogen; acidification of this extract converted the zymogen to pepsin, and at the same time precipitated practically all of the contaminating proteins, which could then be removed by filtration. Denaturation of some of these foreign proteins probably occurred during the alkaline extraction and, to assure this action, more alkali was added than is used by Herriott in the extraction of swine pepsinogen (4). Mucin does not interfere with such a procedure in the extraction of salmon mucosa as it does in the case of beef or swine. The enzyme was salted-out of the clear filtrate by ammonium sulfate, and crystallized by dissolving this precipitate in 0.1 M acetate, pH 5.0, and allowing the solution to stand. The pepsin crystallized as double
refracting needles (Fig. 1). A practical outline of the preparation procedure with some improvements on our previously announced method (5) is given in Table I. The crystals may be stored under half saturated ammonium sulfate in the refrigerator. Solutions of the enzyme are most stable at pH 3.0.

**Evidences of Purity**—All crystalline preparations, even after three successive crystallizations, have shown activities, as measured by Anson and Mirsky's Hb method (6), of 0.23 to 0.26 unit per mg. of protein nitrogen.

That our preparation is homogeneous with respect to protein is indicated by the solubility curves. This criterion for purity has been described by Kunitz and Northrop (7). The amorphous form of salmon pepsin, precipitated from a fresh solution of fresh and washed crystals, gave the solubility curves of Fig. 2, which are consistent with the demands of the phase rule for one protein species within the limits of our experimental error.

**Composition of Enzyme**—Microchemical analyses of the crystal-

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1 These units are calculated to 35.5°, which is the temperature specified in Anson and Mirsky's definition of the Hb unit. Salmon pepsin is 1.18 times as active at 35.5° as at 25° under the conditions of the determination; this factor for swine pepsin is 1.82 (6). When the two enzymes are compared at 25°, the specific activity of crystalline salmon pepsin is about 1.8 times that of Northrop's pepsin.
line enzyme have shown an elementary composition typical of a protein. The percentage composition is as follows:

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>N_{\text{Dumas}}</th>
<th>N_{\text{Kjeldahl}}</th>
<th>S</th>
<th>P</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51.9</td>
<td>6.48</td>
<td>15.62</td>
<td>15.2</td>
<td>1.58</td>
<td>0.031</td>
<td>0.08</td>
</tr>
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</table>

The techniques used are described in Pregl (8), with the exception that sulfur was determined by sodium peroxide fusion, a micro modification of a method of the Association of Official Agricultural Chemists (9). Part of the filter cake of crystals used in these analyses was dissolved, and portions of the solution were tested with silver nitrate, barium chloride, and Nessler's reagent. Indications of chloride, sulfate, or ammonium ions were not discernible.

The percentages of tyrosine, tryptophane, and cystine have been determined by the methods of Folin and Marenzi (10). The association of peptic activity with the tyrosine residues is illustrated by the preparations of acetyl (11), nitroso (12), and iodopepsin (13). Our attention was drawn to cystine by the higher sulfur content of salmon pepsin compared to swine pepsin (1), which corresponds in a qualitative way with the higher specific activity of the former as measured by the Hb method. The amino acid percentages are as follows:

<table>
<thead>
<tr>
<th></th>
<th>Tyrosine</th>
<th>Tryptophane</th>
<th>Cystine</th>
</tr>
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<tbody>
<tr>
<td>Crystallized once</td>
<td>6.7</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>&quot; twice</td>
<td>6.8</td>
<td>1.1</td>
<td>2.1</td>
</tr>
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</table>

Salmon pepsin, compared to swine pepsin (14), contains about twice the percentage of cystine, half as much tryptophane, and about seven-tenths as much tyrosine.

The isoelectric point of the crystalline enzyme, prepared by the method described, was estimated by minimum solubility of the amorphous form in ammonium sulfate solution and in alcohol. The pH of least solubility was near 2.0. Crystals, suspended in citrate-phosphate buffer, changed their direction of migration in an electric field between pH 3.0 and 3.1.

**Kinetics**

The Hb method (6) was used for all routine assays; the calibration curve for crystalline salmon pepsin is expressed by the
empirical relation, pepsin units \(= 0.313 \ T - 0.0020 \ T^2 + 0.00068 \ T^3\), in which \(T\) represents the milliequivalents of tyrosine in 5 ml. of filtrate, after 10 minutes incubation at 25°. The equation is valid between the limits \(T = 0\) and \(T = 12\). The proteolytic unit of this equation is based on the liberation of 1 milliequivalent of tyrosine per minute in the standard digest at 25°. In order to obtain 35.5° units, the equation constants should be multiplied by 1.18.

*Influence of pH*—The effect of pH on the initial rate of digestion of 2 per cent Hb is shown in Figs. 3 and 4. In the former case, close correspondence with Northrop’s pepsin was secured. These data were obtained with a preparation of salmon pepsin which had been prepared by acid extraction of the mucosa and precipitation with acetone, according to the method of Fenger and Andrew (15). The first precipitate had been dried in vacuo over sulfuric acid and stored for 3 years over calcium chloride. At the beginning of this work, the dried preparation was dissolved and fractionated with cold acetone; the fraction soluble in 60 per cent but precipitated by 80 per cent of the organic solvent was retained. Owing to the small quantity of the material, only a few crystals were obtained; they appeared to have the same crystalline form as is illustrated in Fig. 1.

Crystalline salmon pepsin, prepared according to the outline of Table I, exhibited a pH versus activity relationship which is plotted in Fig. 4. We have consistently obtained this type of curve with preparations made by this method and, over a short period of time, we have been unable to prepare again by acid extraction a salmon pepsin with the type of function shown in Fig. 3.

The presence of salt markedly increased the rate of digestion. Denatured Hb was found only sparingly soluble in salt concentrations which approach the composition of sea water. However, the rate of digestion as a function of pH and in the presence of 0.1 N NaCl is shown in Fig. 4, Curve B. The same enzyme solution was used for both curves of Fig. 4.

The Hb solutions were prepared by titrating a solution of 5 per cent dialyzed Hb with dilute HCl to the desired pH, and diluting to 2 per cent Hb with distilled water. Sodium chloride was added as a 3.5 N solution. The final pH values were determined by means of a glass electrode, standardized against pH
3.97 phthalate buffer. In all other respects the determinations were performed according to the procedure of the Hb method.

**Fig. 3.** Dependence of initial rate of digestion of Hb on pH. Curve A, salmon pepsin which had been desiccated; Curve B, Northrop's pepsin, prepared in this laboratory.

**Fig. 4.** Dependence of initial rate of digestion of Hb on pH. Curve A, crystalline salmon pepsin in salt-free medium; Curve B, in presence of 0.1 N NaCl.

*Oxidation-Reduction*—In order to ascertain whether oxidation-reduction could account for the two types of activity *versus* pH
TABLE I

Preparation of Crystalline Salmon Pepsin

(1) 2500 gm. finely ground mucosa stirred 1 hr. in 7500 cc. water containing 3 moles sodium acetate and 3 moles sodium bicarbonate. Strained through cloth.
Volume 7500 cc.; Hb unit 0.023 per cc., 0.013 per mg. N, total 176;
% of enzyme 100

(2) To the opalescent pink extract were added slowly 240 cc. 2 N H₂SO₄, bringing the mixture to pH 2.0. To this solution 200 gm. Hyflo super-cel added and solution filtered on a large Buchner funnel whose paper was covered with a thin cake of super-cel. Filter cake washed with 200 cc. 0.001 N H₂SO₄.
Filtrate and washings,
Volume 9690 cc.; Hb unit 0.015 per cc., 0.13 per mg. N, total 158;
% of enzyme 90

(3) To the clear yellow filtrate from (2) were added 390 gm. ammonium sulfate per liter, bringing the solution to 0.6 saturation; solution stood overnight at 5°. 20 gm. of Hyflo added and solution filtered; filter cake washed with 120 cc. 0.6 saturated ammonium sulfate solution. Filtrate and washings discarded. Filter cake suspended in 300 cc. 0.1 M acetate buffer, pH 4.2, and filtered to remove Hyflo. Filter washed with additional 100 cc. buffer solution.
Filtrate and washings,
Volume 429 cc.; Hb unit 0.34 per cc., 0.14 per mg. N, total 146;
% of enzyme 83

(4) To the clear filtrate was added N H₂SO₄ to produce pH about 2.0; 390 gm. ammonium sulfate per liter added; allowed to stand overnight at 5°. Filtered with suction on hard paper without filter aid. Cracks of draining cake closed with spatula in order to remove as much mother liquor as possible. Filtrate discarded.

(5) Cake from (4) stirred to mush with a small amount of 0.1 M acetate buffer, pH 5.0; more buffer added with stirring until ppt. just dissolved; in all, 175 cc. buffer used. This solution filtered immediately with suction and transferred to a beaker in which it was stirred slowly for 2 or 3 days at room temperature. (It is best to seed with crystals from a former batch.) Crystals filtered off and washed with 50 cc. cold water which had been acidified to pH 4 or 5 with HCl.
Crystal cake,
Hb unit 0.24 per mg. N, total 27; % of enzyme 16

(6) Filtrate and washings from (5) treated by procedure (4).

(7) Recrystallization. Crystal cake from (5) dissolved in 8 cc. 0.1 N H₂SO₄. Solution centrifuged. Supernatant poured into 80 cc. 0.1 M acetate, pH 5.0. Crystalization rapid and complete in 1 hr.
Hb unit 0.23-0.26 per mg. N
curves obtained, the effects of iodoacetic acid and cysteine were investigated. The Hb method was adapted to the experiments. Since iodoacetic acid reacts very slowly with sulfhydryl groups at a pH less than 5.0 (16), the enzyme solution at pH 5.3 was diluted with an equal volume of 0.01 N iodoacetic acid of pH 5.3 and allowed to stand 20 minutes, after which it was mixed with the Hb substrate without further dilution. For comparison, a second aliquot of the same enzyme solution was diluted with an

| TABLE II |
| Effects of Iodoacetic Acid and Cysteine on Salmon Pepsin |

(1, a) 5 cc. enzyme solution, pH 5.3 (solution containing about 0.0008 Hb unit per cc.) added to 5 cc. 0.01 N iodoacetic acid, pH 5.3; allowed to stand 20 min. 1 cc. of this mixture + 5 cc. 2% Hb digested 10 min. at 25°. M.eq. tyrosine liberated per 6 cc. digest, at pH 1.2, 0.0045; at pH 2.2, 0.0034; at pH 3.4, 0.0038

(1, b) Same as (1, a) except water, pH 5.3, used instead of iodoacetic acid.
M.eq. tyrosine liberated per 6 cc. digest, at pH 1.2, 0.0044; at pH 2.2, 0.0034; at pH 3.4, 0.0034

(2, a) 5 cc. Hb + 0.5 cc. 0.01 M cysteine solution of same pH + 1 cc. enzyme (solution containing about 0.0003 Hb unit per cc.) digested 10 min. at 25°. M.eq. tyrosine liberated per 6 cc. digest,* at pH 1.2, 0.0036; at pH 2.2, 0.0028; at pH 3.4, 0.0029

(2, b) Same as (2, a) except that cysteine was added just after addition of trichloroacetic acid. M.eq. tyrosine liberated per 6 cc. digest,* at pH 1.2, 0.0036; at pH 2.2, 0.0026; at pH 3.4, 0.0027

* The color due to cysteine was inhibited by treating the 5 cc. aliquot of filtrate with 1 cc. of 40 per cent formaldehyde and 10 cc. of 0.5 N NaOH, and adding the phenol reagent 5 minutes later (17).

equal volume of water of pH 5.3. The effect of reduction was observed by adding cysteine directly to the Hb solution, as suggested by Anson (17). Results of these experiments comprise Table II. The only influence shown was a slight increase in activity with the addition of either of these reagents, which, in view of the effect of salt, may be attributed to increases in ion concentrations of the solutions.

Since the desiccated preparation of salmon pepsin gave a pH relationship very similar to that of crystalline swine pepsin, we
attempted to learn whether swine pepsin would show an analogous transition during purification. The dependence of activity on pH in the case of swine pepsin has been reported by Sørensen (18), Okuda (19), Ringer (20), Michaelis and Mendelssohn (21), Northrop (22), and Herriott and Northrop (23). These authors state, in most cases, that commercial or purified pepsin had been used, and in no case was the use of a crude mucosa extract reported.

A simple extract of swine pepsin was made as follows: about 2 sq. inches of mucosa, taken from the stomach of a pig just slaught-

![Figure 5. Dependence of initial rate of digestion of Hb on pH. Curve A, crude extract of fresh hog stomach mucosa; Curve B, in presence of 0.1 N NaCl; Curve C, extract of fresh stomach mucosa of beef.](http://www.jbc.org/)

tered, was pounded in a mortar with a little sand until thoroughly disintegrated, well mixed with 10 ml. of 0.07 N HCl, and centrifuged. The cloudy supernatant liquid was diluted to 7 volumes with water and, after standing half an hour, again centrifuged. This clear colorless pepsin solution gave the relationships plotted as Curves A and B in Fig. 5. Curve C of the same figure was obtained with beef pepsin prepared in a similar manner.

The tests with iodoacetic acid and cysteine and the solubility curves lead us to believe that the broad curves of Fig. 4 are not caused by a certain oxidation-reduction state nor by a mixture of enzymes. The curves of Fig. 5 show an analogous situation in
the case of swine pepsin. Whether the occurrence of two forms of dependence of activity on pH is related to the existence of a pepsin-inhibitor complex in the newly formed pepsin, as was demonstrated by Herriott (24), or whether other changes occur in the pepsin molecule, are questions yet to be answered.

Influence of Temperature—The ability of cold blooded animals to carry on digestive processes in winter months was pointed out by Hoppe-Seyler (25) and Rakoczy (26) who studied the pepsin of pike. Hykes, Mazanec, and Szecsenyi (27) showed that the perch can digest proteins at almost freezing temperatures. The environment of the salmon living in the North Pacific probably varies between the limits of 5-15°. In Fig. 6 is shown the progress of salmon peptic digestion at pH 2.0 and at various temperatures with 2 per cent Hb as substrate.

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E. R. Norris and D. W. Elam

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SUMMARY

The pepsin of the Pacific Coast king salmon has been crystallized; the method of preparation is described in detail.

An elementary analysis and determinations of tyrosine, tryptophane, and cystine contents have been made. The material is a typical protein, but the needle type crystalline form and the composition show that this enzyme is distinct from Northrop's pepsin.

The enzyme, when prepared by the method described, has a specific activity of about 0.24 Hb unit, and is active over a wide pH range. The presence of salt enhances its activity.

The activity of salmon pepsin is less responsive to changes in temperature than the activity of swine pepsin.

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454 Crystalline Salmon Pepsin

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