PEPTIDES OBTAINED BY PEPTIC HYDROLYSIS OF PERFORMIC ACID-OXIDIZED RIBONUCLEASE

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In a continuation of the structural studies on ribonuclease reported in earlier papers (1-3), the products resulting from the action of pepsin on performic acid-oxidized ribonuclease have been examined by means of ion exchange chromatography. Eight peptides have been isolated in purified form and their amino acid compositions have been determined. In a following communication (4), a partial structural formula for oxidized ribonuclease is proposed which is derived by a joint consideration of the peptides obtained after hydrolysis by pepsin, trypsin (3), and chymotrypsin (4).

EXPERIMENTAL

Materials—Oxidized ribonuclease was prepared in 200 mg. lots from Armour crystalline ribonuclease (Lot 381059) by the method previously described (3). The pepsin used was a twice recrystallized sample obtained from the Worthington Biochemical Corporation, Freehold, New Jersey (Lot PM538).

Hydrolysis of Oxidized Ribonuclease by Pepsin—Rates of hydrolysis were determined at 25° in a 0.2 M sodium citrate buffer at pH 1.9 and at pH 2.2. The buffer at pH 2.2 was prepared from 105 gm. of citric acid, 42 gm. of sodium hydroxide, and 80 ml. of concentrated hydrochloric acid made up to 5 liters; concentrated hydrochloric acid was added to bring the buffer to pH 1.9. The course of hydrolysis at an enzyme concentration of 0.02 per cent and at substrate concentrations of 1 and 4 per cent was followed by the ninhydrin procedure (5). Samples to be chromatographed were brought to pH 7 with 2 N NaOH to terminate the enzymatic action. After an interval of about 30 minutes, the sample was brought to pH 2.2 before it was transferred to the column.

Chromatography of Peptides on Columns of Dowex 50-X2—The resin used in this work was Dowex 50-X2, Lot 3328-12 (200 to 400 mesh), purchased from The Dow Chemical Company, Midland, Michigan. The method of preparing and operating the Dowex 50-X2 columns has been described in detail in a previous communication (3). Peptic hydrolysates from 40 mg. of oxidized ribonuclease were analyzed on 150 X 0.9 cm. columns. Larger columns (150 X 1.8 cm.) were used for the fractionation of hydrolysates of 200 mg. of oxidized ribonuclease. The N and 2 N sodium acetate-cit-
rate buffers at pH 5.1 were added to the upper reservoir of the mixing device at effluent volumes of 1256 and 2750 ml., respectively (cf. Fig. 2). At the conclusion of the experiment, the resin was flushed from the inverted column with distilled water, transferred to a Büchner funnel, and washed with 0.2 M NaOH. The alkaline eluate (60 ml.), containing any ninhydrin-positive materials still held by the resin at pH 5, was promptly neutralized with 6 N HCl.

When columns 0.9 cm. in diameter were used, alternate effluent fractions were analyzed directly by the modified ninhydrin method (5), and the remaining fractions were analyzed after alkaline hydrolysis (3). With columns 1.8 cm. in diameter, aliquots were pipetted for analysis both with and without hydrolysis. Thus peptides giving a low color yield in the ninhydrin reaction were not overlooked. Quantitative amino acid analysis was carried out (3) on an aliquot of the pooled fractions containing each peptide. The aliquot was of a volume calculated to yield about 0.5 to 1 μmole of each constituent amino acid present as a single residue.

Peptides 9 and 10 were desalted before analysis by exchange of sodium for ammonium acetate on a column of ammonium Dowex 50-X2, after which the ammonium salt was removed by lyophilization (cf. (3)). Most of the sodium ion was removed from Peptides 5 and 7 prior to acid hydrolysis by concentrating the solution to dryness, extracting the residue with concentrated HCl, and removing the precipitated sodium chloride by centrifugation. Peptides 2, 3, 6, 9, 10, and 11 were hydrolyzed with 6 N HCl under reflux for 22 hours. Peptides 5 and 7 were hydrolyzed for 22 hours at 115° with 6 N HCl in evacuated sealed tubes.

Chromatography of Peptides on Columns of Amberlite IRC-50 (XE-64)—Since short times of peptic hydrolysis yielded appreciable percentages of peptides which could be eluted from Dowex 50 only by NaOH, a study was made of the possibility of fractionating these strongly bound segments on a carboxylic acid resin. Columns 30 × 0.9 cm. and 60 × 0.9 cm. were prepared with resin (XE-64, batch No. 2165, 200 to 400 mesh) that had been pretreated in the manner described for the chromatography of ribonuclease (6). To obtain the chromatogram shown in Fig. 3, a 4 hour peptic hydrolysate of oxidized ribonuclease (2 ml. volume) was adjusted to pH 5.85 and applied to a column 54 × 0.9 cm. equilibrated at pH 6.02 with 0.2 M phosphate buffer made up as described in the preceding studies (7). Effluent fractions of 1 ml. were collected from which 0.1 ml. aliquots were taken for analysis by the ninhydrin method.

**Results**

The curves shown in Fig. 1 give the rates of hydrolysis of oxidized ribonuclease by pepsin under two experimental conditions. Chromatographic
analysis was performed upon peptide mixtures obtained after 4 hours of hydrolysis at an enzyme-substrate ratio of 1:230 (similar to hydrolysate for Curve A, Fig. 1) and after 24 hours of hydrolysis at an enzyme-substrate ratio of 1:50 (similar to hydrolysate for Curve B, Fig. 1). From the increase in ninhydrin color, it was calculated that seven and eleven peptide bonds, respectively, could have been split quantitatively (twelve are hydrolyzed by trypsin (3)). In actuality, as will appear later, incomplete hydrolysis of a larger number of bonds undoubtedly takes place.

![Graph](http://www.jbc.org/)

**Fig. 1.** The rate of hydrolysis of oxidized ribonuclease by pepsin at 25°. Curve A, pepsin concentration, 0.02 per cent; oxidized ribonuclease concentration, 4 per cent; pH 2.2. Curve B, pepsin concentration, 0.02 per cent; oxidized ribonuclease concentration, 1 per cent; pH 1.9. The curves have been corrected for the small amount of autodigestion occurring in control solutions of the enzyme.

Chromatography of the peptides obtained from 187 mg. of oxidized ribonuclease after a restricted treatment with pepsin (Curve A) gave the effluent curve shown by the solid line in Fig. 2. As indicated by the dash line, the peaks numbered 5, 6, 7, and 10 were found to rise when the enzyme-substrate ratio was increased to 1:50 (the pepsin concentration remaining constant) and the hydrolysis time was lengthened to 24 hours. The yield of Peptide 2 decreased under these conditions. The results obtained after hydrolysis of aliquots of each of the effluent fractions with alkali have not been included on the curve, inasmuch as no ninhydrin-negative peptides were found. The peak given by ammonia (accumulated from the reagents) was identified from its disappearance during alkaline hydrolysis.

The molar ratios summarized in Table I, considered together with the
chromatographic results (Fig. 2), indicated that the peaks labeled 2, 3, 5, 6, 7, 9, and 10 probably had arisen from individual peptides. The amino acid composition of each of the principal peptides is shown in Fig. 2 above the appropriate peak. Amino acid analyses of the fractions derived from the peaks labeled 1, 4, and 8 indicated that single peptides were clearly not present in these instances and no attempt was made to resolve these mixtures further.

When the less completely hydrolyzed sample (Curve A) was chromatographed on Dowex 50-X2, a considerable quantity of the ninhydrin-positive material applied to the column was found to be so strongly adsorbed that elution with alkali was required. An attempt was therefore made to fractionate the hydrolysate on a column of IRC-50 (54 × 0.9 cm.) equilibrated at pH 6.02, with the results shown in Fig. 3. Amino acid analyses suggested that the peak labeled 11 had arisen from a large peptide of 42 or 43 amino acid residues not found in the eluate from the Dowex 50 column at pH 5. The last component eluted from the IRC-50 column (at 58 ml.
in Fig. 3) had the same amino acid composition as Peak 9 (Fig. 2) and the two have been assumed to be identical.

In accordance with the terminology adopted in the previous communication (3), the series of peptides obtained by peptic hydrolysis of oxidized ribonuclease is referred to in Table I by numbers with the prefix O-Pep. The composition of the individual peptides in terms of the integral number of residues of the constituent amino acids per molecule is given in bold-faced type. All values for amino acids present at as much as 0.01 of a residue are included. Many of the peptides contain significantly greater amounts of impurities than did the products obtained from the tryptic hydrolysates (3). The less specific character of the peptic hydrolysis apparently produces a larger number of peptides in low yield and the resulting mixture is fractionated less effectively by the column. Values for amide ammonia were not sufficiently accurate to warrant inclusion in Table I.

Tentative formulae have been assigned to O-Pep 9 and O-Pep 6 (cf. Fig. 2). The distinctive empirical formula of O-Pep 9, Glu, Thr, Ala, Phe, Lys, indicates that it arises from the amino-terminal end of the ribonuclease molecule (3) and corresponds to the previously isolated terminal heptapeptide, O-Trypt 10, plus a phenylalanine residue. This conclusion is given additional support by the isolation of the same octapeptide (O-Chy 25) from chymotryptic digests of oxidized ribonuclease (4). The structure Lys, Glu, Thr, Ala, Ala- assigned to O-Pep 6 is logical, assuming this peptide...
### Table I

**Amino Acid Composition of Peptide Fractions Obtained from Peptic Hydrolysis of Oxidized Ribonuclease**

The amino acid composition of each peptide fraction obtained from chromatograms of the type shown in Figs. 2 and 3 is expressed in terms of the molar ratios of the constituent amino acids. The values for the principal components are given in bold-faced type. Values for amino acids present to less than 0.01 of a single residue are omitted.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>O-Pep 1</th>
<th>O-Pep 2</th>
<th>O-Pep 3</th>
<th>O-Pep 4</th>
<th>O-Pep 5</th>
<th>O-Pep 6</th>
<th>O-Pep 7</th>
<th>O-Pep 8</th>
<th>O-Pep 9</th>
<th>O-Pep 10</th>
<th>O-Pep 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>1.04</td>
<td>0.10</td>
<td>1.05</td>
<td>0.15</td>
<td>0.13</td>
<td>0.05</td>
<td>5.76</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Glutamic acid</td>
<td>0.16</td>
<td>0.92</td>
<td>1.08</td>
<td>1.06</td>
<td>1.14</td>
<td>0.98</td>
<td>3.96</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.06</td>
<td>1.14</td>
<td>0.16</td>
<td>0.11</td>
<td>0.14</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.99</td>
<td>1.03</td>
<td>1.01</td>
<td>1.97</td>
<td>0.12</td>
<td>3.19</td>
<td>2.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Valine</td>
<td>0.97</td>
<td>1.03</td>
<td>1.77</td>
<td>0.93</td>
<td>0.07</td>
<td>0.94</td>
<td>2.09</td>
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<tr>
<td>Leucine</td>
<td>0.04</td>
<td>0.01</td>
<td>0.11</td>
<td>1.00</td>
<td>0.05</td>
<td>0.98</td>
<td>2.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Isoleucine</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>0.02</td>
<td>0.04</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>0.95</td>
<td>0.12</td>
<td>0.22</td>
<td>1.18</td>
<td>0.24</td>
<td>1.09</td>
<td>7.31</td>
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<tr>
<td>Threonine</td>
<td>0.06</td>
<td>0.23</td>
<td>1.13</td>
<td>0.08</td>
<td>0.96</td>
<td>3.12</td>
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<tr>
<td>Cysteic acid</td>
<td>1.20</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>2.21</td>
<td>2.62</td>
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<tr>
<td>Methionine sulfone</td>
<td>1.88</td>
<td>0.11</td>
<td>0.86</td>
<td>0.99</td>
<td>1.13</td>
<td></td>
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<tr>
<td>Proline</td>
<td>0.92</td>
<td>0.11</td>
<td>0.94</td>
<td>0.11</td>
<td>1.06</td>
<td>2.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.06</td>
<td>0.93</td>
<td>0.94</td>
<td>0.97</td>
<td>0.07</td>
<td>1.97</td>
<td>3.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tyrosine</td>
<td>0.09</td>
<td>0.17</td>
<td>0.07</td>
<td>1.06</td>
<td>1.97</td>
<td>2.89</td>
<td></td>
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</tr>
</tbody>
</table>

* An approximate correction of 10 per cent for decomposition of serine during hydrolysis makes the molar ratio of serine doubtful in this instance. A value of 8 rather than 7 residues has been included in Fig. 2 and appears more likely to be the correct value on the basis of the formula given in the accompanying paper (4).

† Low recoveries of tyrosine have been observed when small quantities of tyrosine-containing peptides are hydrolyzed in the presence of large amounts of acetate or citrate buffers, as noted in the accompanying paper on the chymotrypsin series of peptides (4). The above value for tyrosine is based upon the amount of the tyrosine in the hydrolysate (molar ratio of 0.57) plus the quantity of the tyrosine decomposition product emerging near the position of monochlorotyrosine in the chromatographic analysis.

To be derived from O-Pep 9 during the more extensive hydrolysis. This assumption is strengthened by the fact that O-Pep 9 could no longer be detected after the more drastic hydrolysis by pepsin, while the yield of O-Pep 6 rises concomitantly to 100 per cent.
O-Pep 2 (Asp, Ala, Ser, Val) has the same amino acid composition and chromatographic behavior as O-Chy 4 (cf. (4)), and hence the two may be assumed to be identical. The same peptide has been isolated from peptic hydrolysates of unoxidized ribonuclease by Anfinsen (8). In the accompanying paper (4) it is concluded that this valine-containing tetrapeptide occurs at the carboxyl terminal end of the chain and that O-Pep 5, containing 12 residues, is the segment immediately preceding O-Pep 2 in the carboxyl-terminal sequence.

**DISCUSSION**

When the peptides isolated from peptic hydrolysates are considered in the light of the partial formula for oxidized ribonuclease proposed in the accompanying communication (4), it would appear that pepsin has acted predominantly at the two ends of the peptide chain. O-Pep 2 and 5 are derived from the last 16 residues in the carboxyl-terminal sequence, while O-Pep 3, 6, 7, 9, 10, and 11 all are split from the first 55 residues starting at the amino-terminal end. The central section of the chain has not been accounted for. The evidence from the present study for the individuality of O-Pep 11 is not strong. The likelihood that it represents a single peptide rests upon the fact that its amino acid composition fits a sequence of 43 amino acid residues following O-Pep 9 in the first half of the formula of the protein (4).

From the work with synthetic substrates (cf. (9) for a summary), pepsin would be expected to cleave oxidized ribonuclease preferentially at peptide bonds involving the 9 aromatic amino acid residues. It is clear from the present results, however, that a number of peptide bonds, in addition to those involving the aromatic amino acids, have been hydrolyzed and that not all of the peptides expected by splitting at tyrosine and phenylalanine residues have been formed. Moreover, several of the peptide fragments that have been identified are obtained in relatively poor yield. These facts indicate that the action of pepsin is rather complex, a conclusion that also emerges from the work of Sanger, Thompson, and Tuppy (10) on insulin and of Bell (11) on corticotropin. Pepsin is a less specific hydrolytic agent than trypsin or chymotrypsin for use in studies on the structure of proteins. Nevertheless, the peptides that have been isolated from peptic hydrolysates of oxidized ribonuclease have provided limited but useful information concerning the arrangement of amino acid residues in certain sections of the ribonuclease molecule.

**SUMMARY**

The mixture of peptides resulting from the hydrolysis of oxidized ribonuclease by pepsin has been fractionated on columns of Dowex 50-X2.
Eight peptides have been separated in purified form in yields of 10 to 100 per cent. About half of the 124 amino acid residues in the protein have been accounted for in terms of two tetra-, one hexa-, and one octapeptide and two large fragments of 12 and 43 amino acid residues, respectively. The results, although providing a less complete accounting for the amino acid residues in the molecule than experiments with trypsin and chymotrypsin, give evidence concerning the arrangement in both the amino-terminal and carboxyl-terminal sections of the peptide chain.

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