The Preparation of Subtilisin-modified Ribonuclease and the Separation of the Peptide and Protein Components

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The proteolysis of bovine pancreatic ribonuclease by subtilisin has been shown to involve the formation of an enzymically active intermediate which is further degraded to inactive products (1, 2). This paper describes the preparation of the modified active ribonuclease, RNase (S) and its fractionation into two components, a peptide (S-peptide) and a protein (S-protein). Neither S-peptide nor S-protein alone shows appreciable ribonuclease activity. When these components are mixed in equimolar proportions, almost the full enzymic activity is recovered. The only observed change in covalent structure during the conversion of RNase A to RNase S is the hydrolysis of the peptide bond between residues 20 and 21 as numbered from the NH₂-terminal end of the single chain of RNase A. A comparison of the enzymic properties of RNase A, RNase S and RNase S’ (the reconstituted enzyme) under a number of conditions is presented. A brief report of some of these observations has been published (3).

EXPERIMENTAL AND RESULTS

Materials—Bovine pancreatic ribonuclease, Lot No. 381-059, was obtained from Armour and Company. The subtilisin used in this work was kindly donated by Dr. Martin Ottesen. The preparation of this sample of the crystalline proteolytic enzyme from Bacillus subtilis has been described by Güntelberg and Ottesen (4). Crystalline trypsin, Lot No. 62330, was obtained from the Worthington Biochemical Corporation. The yeast nucleic acid, the barium uridine-2′, 3′-phosphate, and the barium cytidine-2′, 3′-phosphate were products of the Schwarz Laboratories.

Chromatography—Ribonuclease A was prepared from the commercial crystalline material by chromatography on the Amberlite resin IRC-50 (XE-64) as described by Hirs et al. (5). The thymol was omitted and the column was operated in a cold room at 2°C. A typical chromatogram is shown in Fig. 1, a. The ninhydrin color was determined on aliquots with the strong buffer reagent of Moore and Stein (6); for plotting, the data were normalized at the peak fraction of the elution curve obtained by means of absorbance measurements at 280 mµ. The fast running peak near the solvent front has no enzyme activity. The small peak emerging just ahead of the principal component is commonly designated RNase B and is enzymically active. The fractions containing RNase A were pooled as shown in the figure.

Desalting Procedure—The pooled peak fractions of RNase A (about 1.5 l.) were adjusted to pH 5 with acetic acid and then passed over a 2.4 x 20-cm. column of IRC-50 previously equilibrated with 0.2 M phosphate buffer pH 5.0. As much as 1 g of protein was quantitatively retained on this column. After washing with 50 ml. of .001 M acetic acid, the resin was eluted with a solution of 0.4 M ammonium hydroxide adjusted to pH 9 with CO₂. A forerun of 200 ml., containing no protein, was discarded. The protein was quantitatively eluted in the next 600 ml., the pH of the eluent varying from 6.5 to 9. Both the water and the volatile ammonium bicarbonate were removed by lyophilization. When necessary, the last traces of nonvolatile salt were removed by passage through columns of the Amberlite resins IR-120 (hydrogen cycle) and IRA-400 (hydroxide cycle), both resins 20 to 50 mesh. The over-all recoveries of both RNase A and RNase S were essentially quantitative in this desalting procedure with respect to both absorbance at 280 mµ and enzymic activity. Earlier attempts to desalt by dialysis frequently were attended by large losses through the membranes in spite of selection of the dialysis tubing (7).

Digestion with Subtilisin²—In a typical preparation 730 mg of RNase A were dissolved in 5 ml of 0.1 M KCl. This solution was placed in the cell of a pH-stat (8) employing a Radiometer TTT1a titrator. The temperature was lowered to 3° and the pH adjusted to 8.0 with 0.1 N NaOH. 1 mg of subtilisin (in 0.5 per cent aqueous solution) was added. The addition of 0.1 N NaOH was recorded as a function of time. After about 3 hours the rate of reaction was very slow, and the total alkali addition corresponded to 0.9 mole of OH⁻ ion per mole of RNase A. The pH values of the amino groups produced are not precisely known; however, this amount of alkali probably corresponds to

* Aided by grants from the United States Public Health Service and from the National Science Foundation.

² A number of digestions have been carried out with another sample of "subtilisin" (also kindly supplied by Dr. Martin Ottesen and referred to as Novo enzyme) which had been prepared from a different strain of B. subtilis. Work at the Carlsberg Laboratory had already shown this preparation to be different in certain respects from the original crystalline material. Although this enzyme also produces a modified RNase which resembles RNase S in its chromatographic behavior and in its sensitivity to trypsin, we have not yet succeeded in finding conditions in which the yield has been greater than 25 per cent. For this reason no further work with this sample of the bacterial proteinase has been attempted at this time.

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zymic activity with RNA as substrate and the use of 50-μl aliquots for assay. The solid line represents absorbance at 280 nm; +, ninhydrin phosphate, pH 6.35. The abscissa is the same for the two runs shown.

An average of 1 to 2 bonds cleaved per mole of protein (9). The total ninhydrin color value of the digest increased about 10 per cent during the same interval, again indicating roughly the same degree of proteolysis. There was no change in ribonuclease activity, as normally measured, during this digestion.

When examined by the Sanger I-fluoro-2,4-dinitrobenzene procedure (10), RNase A shows the presence of a single NH₂-terminal lysine residue (11). When this procedure is used to follow the appearance of NH₂-terminal residues in the subtilisin digest, at very early time intervals only serine is found in addition to lysine. As the digestion proceeds there appears aspartic or glutamic acid, or both, followed by alanine and threonine. The yields of dinitrophenyl amino acids were not determined quantitatively.

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The digestion was stopped by adjusting the solution with N hydrochloric acid to pH 3, where subtilisin is not only inactive but is rapidly destroyed. The digest was then put on the same IRC-50 column used for the preparation of RNase A. The resulting elution diagram is shown in Fig. 1, b. The major peak, designated RNase S, moves more slowly than RNase A. The fastest moving material has considerable ninhydrin color, no enzymic activity, and presumably represents further degradation products. The small peak appearing close to the position expected for RNase A has the same specific activity as the native enzyme, is active in 8 M urea, is unaffected by treatment with trypsin, and thus is probably undigested material, designated RNase A'. It will be noted from Fig. 1, b that digestion with trypsin completely abolishes the activity of RNase S and serves as a convenient means of detecting contamination with RNase A.

The fractions containing the modified enzyme (RNase S) were pooled as shown in Fig. 1, b, desalted by the technique described above, and lyophilized. The yields obtained in some typical preparations are given in Table I below. It has been possible to crystallize RNase S from ammonium sulfate solutions at pH 5.5. The crystals are grossly similar to those obtained with the native enzyme, but no detailed study of the morphology and optics has been made. A solution of the crystalline material showed full enzymic activity.

**Fractionation with Trichloroacetic Acid—**A 1 per cent solution of salt-free RNase S in water was cooled in 0° in an ices bath. One-fifth volume of freshly prepared 20 per cent trichloroacetic acid, precooled to 0°, was added and the mixture allowed to warm to room temperature (20-25°). Precipitation began at about 10 15°. After 1 hour at room temperature, the suspension was centrifuged, and the precipitate was redissolved in the original volume of water. (After first becoming a transparent gel, the precipitate slowly passed into solution without neutralization or the addition of any salt.) A second precipitation with trichloroacetic acid was then carried out exactly as before.

The combined supernatant fluids were continuously extracted with ether until no free acid could be detected in the ether extract. A small amount of 0.1 N hydrochloric acid was added to the aqueous solution, and the extraction was continued to remove the last traces of trichloroacetic acid. The aqueous solution was lyophilized and the resulting powder was designated S-peptide.

The precipitate was redissolved in water and the solution was passed over the IR-120 - IRA-400 deionizing column to remove the residual trichloroacetic acid. The desalted solution was lyophilized and the resulting powder was designated S-protein. The yields obtained are indicated in Table I.

### Table I

<table>
<thead>
<tr>
<th>Starting material</th>
<th>Recovered from column</th>
<th>Recovered from column after subtilisin digestion</th>
<th>Recovered from trichloroacetic acid fractionation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armour RNase</td>
<td>RNase A</td>
<td>RNase A'</td>
<td>RNase S</td>
</tr>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>200</td>
<td>130</td>
<td>115</td>
<td>500</td>
</tr>
<tr>
<td>1000</td>
<td>700</td>
<td>64</td>
<td>530</td>
</tr>
<tr>
<td>Average yields</td>
<td>70%</td>
<td>11% (of A)</td>
<td>72% (of A)</td>
</tr>
</tbody>
</table>

* The numbers in each case refer to the weights of salt-free dry powders obtained, except for the column headed RNase A' where the recovery was estimated from the absorbance at 280 nm measured on the fractions from the column chromatogram.
The amino acids for which no values are listed were present in amounts of less than .02 moles per mole of peptide. Half-cystine was assumed to contain 20 residues per mole. The validity of these assumptions is based on the following considerations. If a total of S-peptide. The agreement between the results for RNase A, RNase S, and (S-protein + S-peptide) is unequivocal for all residues with the possible exception of serine, which is the amino acid known to exhibit the highest losses upon acid hydrolysis of proteins (16).

Measurements of Ribonuclease Activity—With ribonucleic acid as the substrate, the production of acid-soluble nucleotides was measured as described previously (17). With uridine-2',3'-phosphate as the substrate, the assay was carried out by titration in the pH-stat at 26° (18). The solvent was 0.3 M NaCl and the pH, 7.0. These conditions represent the ionic strength (18) and pH for maximal ribonuclease activity toward this substrate. For each assay, 1.5 ml. of solution containing 5 mg. of barium uridine-2',3'-phosphate was used. The titration was carried out with .01 N NaOH when RNase was added in the range of 0 to 10 μg. The reciprocal of the time required to reach a given substrate conversion was taken as a measure of the activity of the enzyme.

The effect of substrate concentration was tested with samples of RNase A, RNase S and RNase S'. The results, presented as an Eadie plot (19) in Fig. 2, should be used only for comparing the three enzyme preparations. Because of uncertainty as to the purity of the substrate and the complication of product inhibition, the Michaelis constants and maximal velocities which can be estimated from the figure should only serve as rough indications of the true values. If one assumes a 5 to 10 per cent error in the estimations of the reaction rates, it would appear that RNase S behaves in a fashion similar to the native enzyme, whereas the reconstituted enzyme, RNase S', may have a significantly different Kₘ and Vₘₐₓ. Also included on the graph are single determinations with cytidine-2',3'-phosphate. The results indicate no marked change in the relative activity of the various preparations towards these two substrates. Measurements with the RNA substrate at different concentrations also showed RNase A and RNase S to have similar behavior, whereas the relative activity of RNase S' appeared to decrease as the substrate concentration increased.

![FIG. 2. Effect of substrate concentration on reaction velocity for various enzyme preparations. Solvent 0.3 M NaCl, pH 7.0, temperature 26°, enzyme concentration 2.2 μg. per ml. in each case. Velocities were estimated from the rate of alkali consumption at less than 4 per cent conversion of initial substrate. The pKₗ of the product in this solvent is 5.85. The circles O, RNase A; □, RNase S; △, RNase S'. The open symbols refer to uridine-2',3'-phosphate as substrate, the solid symbols to cytidine-2',3'-phosphate as substrate. The lines drawn correspond to Kₘ values of 0.20 M (lower line) and 0.26 M (upper line).](https://www.jbc.org/content/237/6/1461)
Let \((S\text{-Prot})_0\) = total concentration of \(S\text{-Prot}\) and \((S\text{-Pep})_0\) = total concentration of \(S\text{-Pep}\). Define: \(r = (S\text{-Pep})_0/(S\text{-Prot})_0\) = molar ratio peptide/protein, \(a = (S')/(S\text{-Prot})_0\) = fractional activity (activity is directly proportional to concentration of \(S\)'), and \(K' = K/(S\text{-Prot})_0\). Substitution of these definitions in Equation 1 leads to the following expression for the fractional activity:

\[
a = \frac{1}{2} \left[ r + 1 + K' - \sqrt{(r + 1 + K')^2 - 4r} \right]
\]

The curves drawn in Fig. 3 were calculated from Equation 2 for various values of \(K'\). The data indicate that \(K'\) is equal to or less than \(10^{-2}\). Since \((S\text{-Prot})_0\) in the assay mixture used is about \(5 \times 10^{-8} \text{ M}\), then \(K\), the dissociation constant, equals \(5 \times 10^{-9} \text{ M}\) or less.

Because of the very tight binding of the peptide and protein, the assays with both substrates are indistinguishable within the accuracy of the measurements, and the dissociation constant itself can be given only an upper limit. It has been observed that some activity is obtained when performic acid-oxidized ribonuclease (oxidized RNase A) is added to \(S\text{-Prot}\). Solutions were prepared containing \(S\text{-Prot}\) at the same concentration as those described above, and various amounts of oxidized RNase A (20) were added. The measured activities were expressed as fractions of that expected for the same amount of \(S\text{-Prot}\) with excess \(S\text{-Pep}\). The results are also shown in Fig. 3. In this case the “dissociation constant” appears to be much greater and there is a large difference in the relative activity with the low and high molecular weight substrates.

Stability of Subtilisin-modified Ribonuclease—it has already been indicated that \(S\text{-Prot}\) and \(S\text{-Pep}\) remain bound together as an active enzyme during chromatography, during the column desalting procedures, and during extensive dialysis against water. Additional evidence for the strong interaction is provided by studies of the precipitation of RNase S from a 1 per cent solution by ammonium sulfate and by ethanol at pH 5 to 6. The results are given in Table III. In each case the precipitate was redissolved and this solution assayed with RNA as the substrate at the same time as the supernatant fluid. From the assay measurements the weight of active enzyme in the supernatant fluid and the precipitate was calculated. The data are expressed as per cent of the weight of enzyme initially present. It will be noted that the recovery is 90 per cent or more in every instance. Additional evidence for the lack of any significant fractionation is provided by the assays in the presence of added \(S\text{-Prot}\) or \(S\text{-Pep}\). Thus the precipitate did not contain any free \(S\text{-Prot}\) which could be activated by \(S\text{-Pep}\), nor did the supernatant fluid contain any free \(S\text{-Pep}\) which could be activated by the added \(S\text{-Prot}\). With the conditions under which the precipitation of RNase S is essentially complete with either ammonium sulfate or ethanol, free \(S\text{-Pep}\) is completely soluble.

![Fig. 3. Enzymic activity as a function of peptide to protein ratio.](http://www.jbc.org/)

**TABLE III**

<table>
<thead>
<tr>
<th>Precipitation of subtilisin-modified ribonuclease at neutral pH*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Per cent of initial enzyme present estimated from activity measurements</strong></td>
</tr>
<tr>
<td><strong>Precipitate</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td><strong>Ammonium sulfate</strong></td>
</tr>
<tr>
<td>(% saturation)</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>66.7</td>
</tr>
<tr>
<td>67.7</td>
</tr>
<tr>
<td>71.4</td>
</tr>
<tr>
<td>77.4</td>
</tr>
<tr>
<td>84.0</td>
</tr>
</tbody>
</table>

* To a 1 per cent unbuffered solution of RNase S, pH 5 to 6, the precipitating agent was added to give the concentration indicated. The precipitate was removed by centrifugation and redissolved, and the solution was assayed along with the supernatant fluid.
The stability of the various preparations to heat was investigated. The test liquids were made up from the components of the universal buffer mixture of Britton and Robinson (see (21)). The starting solution was 0.5 M NaCl and 0.003 M in each of phosphate, citrate, Veronal, and borate. Portions of this solution were adjusted with NaOH or HCl to give the required pH between 1 and 11. Aliquots of the protein or peptide solutions in these various mixtures were heated for 20 minutes at 100°. The solutions were then cooled, adjusted to pH 5, and assayed with RNA. The RNase A and RNase S solutions were assayed directly, S-protein solutions were assayed in the presence of excess S-peptide, and the S-peptide solutions were assayed in the presence of excess S-protein. The results, expressed as per cent of the unheated controls, are shown in Fig. 4. The peptide is unaffected at any pH in this range; and RNase A, RNase S and S-protein give very similar, if not identical results. The characteristic heat stability of ribonuclease does not appear to be significantly affected either by the subtilisin-induced proteolysis or by the subsequent acid fractionation.

Because of the ability of native ribonuclease to catalyze the hydrolysis of RNA even in concentrated urea solutions (22), the behavior of the modified enzyme was investigated in this regard. For this purpose the Kunitz spectrophotometric assay was used (see (17)). Both the RNA substrate and the enzyme were dissolved in urea solutions of the desired concentration, and the decrease in absorbance at 300 mμ was followed as a function of time. Because of the change in the shape of the curves obtained in this assay at different urea concentrations, the activity of RNase S is expressed as per cent of that shown by RNase A in the same solvent. The activities at the different urea concentrations are thus not directly comparable with each other on an absolute basis. The results are given in Fig. 5. The solid curves shown merely indicate the sharpness of the decrease in activity that might be expected for a protein undergoing reversible denaturation. They have been calculated from Schellman's (23) equations for the stability of α-helices of 40 and 50 residues, with his values for ΔΗ° and ΔS° employed. It is not intended that one should infer the existence of such helices in the enzyme structure from the data in Fig. 5. It does appear reasonable, however, to conclude that the substrate cannot "reverse" the denaturing action of urea with RNase S, whereas it can with RNase A (23). The action of urea on RNase S is not irreversible, since mere dilution of a solution of RNase S in 8 M urea results in the recovery of full activity.

If a solution of RNase S in 8 M urea is dialyzed against 8 M urea, the presence of S-peptide in the liquid outside the bag can be demonstrated. It would appear that the strong urea solution results in the dissociation of S-peptide and S-protein. Whether this is due to a direct interference with the bonding of S-peptide to S-protein or whether it is due to effects of urea elsewhere in the S-protein structure cannot be decided at this time.

**DISCUSSION**

The available evidence indicates that subtilisin-modified ribonuclease, RNase S, contains all the amino acids originally present in the native enzyme. The only observed change in covalent structure is the hydrolysis of one peptide bond in the single chain of RNase A. The site of hydrolysis is the alanine-serine bond between residues 20 and 21 as numbered from the NH2-terminal end of the RNase A chain. This conclusion is based on the partial sequence of the RNase A chain reported by Hirs et al. (14).
The enzyme data indicate that the reconstituted enzyme, RNase S', is similar to but not identical with RNase S. Since the $K_m$ and $V_{max}$ values for these two preparations may be different, the exact amount of activity recovered on recombination will depend on the precise condition of assay. Some slight irreversible alteration in the protein part may be caused by the precipitation with trichloroacetic acid. The activity data in Fig. 3 for the recombination of S-peptide and S-protein show a sharp break where the molar ratio is exactly 1.0. If the lowered maximal activity (85 per cent of S) were caused by the denaturation of a portion of the S-protein, then it would be necessary to assume that the “denatured” portion bound S-peptide as firmly as did the “native” portion. If this were not the case then the break in the activity curve would not occur at a molar ratio of 1.0. This condition appears unlikely, and, thus it is assumed that all of the S-protein produces an active enzyme but that RNase S and RNase S’ are slightly different.

The question can be asked whether the binding of the substrate affects the binding of the peptide to the protein. This cannot be answered at present for the system S-protein - S-peptide. However, the data with oxidized RNase A do appear to bear on this point. Oxidized RNase A contains S-peptide as its N-terminal segment except that the single methionine residue in this region has been oxidized to the sulfone. When oxidized RNase A is mixed with S-protein, much more activity was regained with RNA as a substrate than with the cyclic phosphate. The simplest hypothesis to explain this result appears to be

$$\text{Protein} + \text{peptide} \rightleftharpoons \text{Active enzyme}$$

$$+ \quad \text{enzyme products}$$

$$\text{Substrate} \rightleftharpoons \text{substrate} \rightarrow + \quad \text{compound enzyme}$$

If two substrates are used at concentrations below saturation of the enzyme, the one having the higher affinity for the active enzyme will shift the first equilibrium farther to the right and thus produce relatively more enzyme than the substrate with lower affinity. A direct comparison of $K_m$ values for two substrates may not indicate, of course, the relative binding constants. However, Vandendriessche (25) has shown that polyanions such as heparin and polysaccaride are much more effective inhibitors of RNase activity toward the low molecular weight cyclic phosphates than toward RNA (26). This is presumptive evidence, on the assumption of a single catalytic site, that RNA is bound much more tightly than uridine-2',3'-phosphate and would thus fit in with the scheme proposed above.

It is hoped that further study of the interaction of S-peptide and S-protein may provide data bearing directly on the problem of the secondary structure of RNase and its relation to the activity of the enzyme. The effects of alteration in the functional groups of S-peptide and S-protein on the regeneration of enzymic activity will be the subject of future communications.

**SUMMARY**

Digestion of the principal component of bovine pancreatic ribonuclease with subtilisin gives in high yield a modified ribonuclease which can be separated from the native enzyme. The only change in covalent structure so far observed is the hydrolysis of peptide bond 20 of RNase A, a change having a negligible effect on the enzymic properties of the protein. Preliminary spectroscopic and immunochemical evidence would also tend to indicate an additional structural change.

The available data indicate that the reconstituted enzyme, RNase S', is similar to but not identical with RNase S. Since the $K_m$ and $V_{max}$ values for these two preparations may be different, the exact amount of activity recovered on recombination will depend on the precise condition of assay. Some slight irreversible alteration in the protein part may be caused by the precipitation with trichloroacetic acid. The activity data in Fig. 3 for the recombination of S-peptide and S-protein show a sharp break where the molar ratio is exactly 1.0. If the lowered maximal activity (85 per cent of S) were caused by the denaturation of a portion of the S-protein, then it would be necessary to assume that the “denatured” portion bound S-peptide as firmly as did the “native” portion. If this were not the case then the break in the activity curve would not occur at a molar ratio of 1.0. This condition appears unlikely, and, thus it is assumed that all of the S-protein produces an active enzyme but that RNase S and RNase S’ are slightly different.

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**Acknowledgments**—The authors wish to express their gratitude to Dr. K. Linderström-Lang at whose suggestion and under whose guidance this problem was started, to Dr. Martin Ottosen for much help during the early stages and for the gift of the subtilisin, to Dr. J. S. Fruton for his encouragement during the work at Yale and for his criticism of this manuscript, and to Miss M. Hubbard for some of the analyses reported.
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Frederic M. Richards and Paul J. Vithayathil