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The Heterogeneity of Bovine Pancreatic Ribonuclease S*

M. S. Doscher and C. H. W. Hirs

ABSTRACT: The initial proteolysis of native bovine pancreatic ribonuclease A by subtilisin BPN′ (Nagarse) occurs at either the bond between residues 21 and 22 or the previously established position between residues 20 and 21. As a result, the ribonuclease S produced contains a mixture of S-peptide molecules composed of residues 1–20 and 1–21 and of S-protein molecules composed of residues 21–124 and 22–124. Resolution of the S-peptide fraction was achieved by gradient elution from a column of Dowex 50-X2. Resolution of the intact S-protein fraction was not attempted. Instead, the fraction was fragmented by successive reaction with cyanogen bromide and performic acid and the products were fractionated by gel filtration through a column of Sephadex G-25. A zone containing a partially resolved mixture of peptides derived from residues 21–29 and 22–29 was obtained. Amino acid analyses of material along this zone provided an estimate of the yield of each peptide. Resolution of the various species of ribonuclease S has not yet been achieved. Examination of the S-peptide fraction of ribonuclease S prepared by the digestion of ribonuclease A with subtilopeptidase A revealed that the yield of the peptide composed of residues 1–20 is higher with this enzyme, although the peptide is still not the sole chromatographic component in the fraction. Additional components of unknown composition and comprising 16% of the fraction are also present.

The modification of native bovine pancreatic ribonuclease A by proteolytic enzymes from two strains of *Bacillus subtilis* has in both cases converted the protein in high yield to a product, designated ribonuclease S, which retains full enzymatic activity (Richards and Vithayathil, 1959; Gordillo *et al.*, 1962). Subtilisin (Güntelberg and Ottesen, 1954) was the enzyme first used to obtain this conversion (Richards and Vithayathil, 1959). The properties of ribonuclease S were consistent with the hypothesis that it was a homogeneous protein formed by the cleavage of the bond between residues 20 and 21 of ribonuclease A. Subsequently, the strain of *B. subtilis* from which subtilisin had been isolated was lost, and it became necessary to develop a procedure utilizing subtilisin BPN′ (Gordillo *et al.*, 1962), an enzyme isolated by Hagihara (1960) from a different strain of *B. subtilis*, and frequently referred to by its trade name (Nagarse). The course of the digestion and the properties of the ribonuclease S formed were very similar to those observed with sub-

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tilisin, with the exception that an unusually high serine content was reported for the S-peptide moiety (Gordillo *et al.*, 1962).[1]

A closer examination of the ribonuclease S obtained by digestion with subtilisin BPN′, reported on here, has revealed that it contains a significant amount of a peptide composed of residues 1–21 in addition to the peptide composed of residues 1–20 previously believed to be the sole component of the S-peptide fraction. Examination of the fragments derived by successive treatment of the S-protein fraction with cyanogen bromide (Gross and Witkop, 1962) and performic acid (Hirs, 1956) has revealed that polypeptides derived from both residues 21–124 and 22–124 are present in this fraction. This type of S-protein heterogeneity would arise if the initial proteolytic cleavage could occur at either the bond between residues 20 and 21 or the bond between residues 21 and 22. The amount of polypeptide 22–124 is higher than would have been predicted from the amount of peptide 1–21 in the S-peptide fraction, suggesting that some of peptide 1–20 is formed by further digestion of peptide 1–21. Recently, Gross and Witkop (1966) have stated that the S-peptide fraction isolated by them after digestion of ribonuclease A with subtilisin BPN′ is a mixture derived from residues 1–20 and 1–21.

A proteolytic enzyme designated subtilopeptidase A, which is "apparently identical to the original subtilisin of Güntelberg and Ottesen in every respect" (Johansen and Ottesen, 1964), is now commercially available. A ribonuclease S preparation was made with this enzyme and the S-peptide fraction examined. It was found that 84% of the fraction was the peptide derived from residues 1–20. This is a yield significantly higher than that obtained with subtilisin BPN′. The remainder of the fraction was composed of several components of as yet unknown composition.

Materials and Methods

Bovine pancreatic ribonuclease A, Lot RAF 6088, was a salt-free lyophilized preparation purchased from Worthington Biochemical Corp., Freehold, N. J. *Subtilisin BPN′* (Hagihara, 1960), sold as Nagarse, was Batch CC H2678 from the Biddle Sawyer Corp., New York, N. Y. *Subtilopeptidase A* (Johansen and Ottesen, 1964) was a gift from Novo Industri A/S, Copenhagen N, Denmark. The enzyme, which was received as a lyophilized powder, had been crystallized once and was reported to have a proteolytic activity of 20 units/g as measured by the Anson hemoglobin method. *Amberlite IRC-50* was purchased from Mallinckrodt and fractionated by the flotation method of Hamilton (1958). The resin particles had a diameter of 17–30 μ. *Dowex 50-X2* (200–400 mesh) was purchased from Bio-Rad Laboratories, Richmond, Calif., and prepared for chromatography by the method of Schroeder *et al.* (1962). *Sephadex G-25* (fine grade) and *Sephadex G-75* (coarse grade, bead form) were purchased from Pharmacia, Inc. The Sephadex G-75 was equilibrated overnight with 50% acetic acid prior to use. *Thiodiglycol* was a purified grade, no. 285000, available from the Pierce Chemical Co., Rockford, Ill. γ-Collidine (2,4,6-trimethylpyridine; Eastman no. 4815) was redistilled shortly before use and stored under nitrogen. *Pyridine*, Baker and Adamson, reagent grade, and *2-mercaptoethanol*, Eastman no. 4196, were used without further purification.

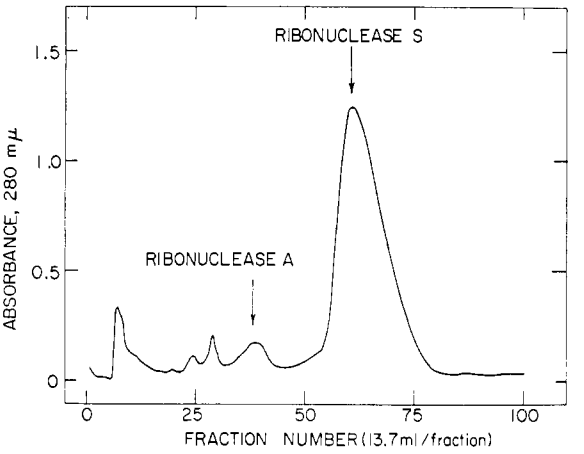

FIGURE 1: Chromatography of IRC-50 (particle size, 17–30 μ) of the mixture obtained by modification of ribonuclease A with subtilisin BPN′. Column, 3.7 × 45 cm; sample size, 400 mg; eluting buffer, 0.2 M sodium phosphate, pH 6.47; flow rate, 17 ml/hr; temperature, 5°.

Preparation of Ribonuclease S. A 2.0% solution of lyophilized, phosphate-free ribonuclease A (300–500 mg) in 0.1 M Tris–HCl, pH 8.0, was cooled to 5° and an appropriate volume (see Results and Discussion) of a freshly prepared 1.0% solution of subtilisin BPN′ or subtilopeptidase A in 0.1 M Tris–HCl, pH 8.0, was added with stirring. Stirring was discontinued when thorough mixing had occurred and the solution was held at 5° until, as determined by the tryptic digestion method of Gordillo *et al.* (1962), the maximum yield of ribonuclease S had been obtained. The cold solution was brought to pH 2.5 with 1 N HCl, stirred at room temperature for 1 hr, cooled to 5°, and brought to pH 6.0 by the dropwise addition of 5 N NH_4OH. Chromatographic fractionation of this solution on a column of IRC-50 was performed essentially as described by Richards and Vithayathil (1959). Details are given in the legend to Figure 1.

Effluent fractions which contained ribonuclease S were subjected to lyophilization and the resulting powder was desalted at 5° on a 3.7 × 50 cm column of Sephadex G-25. Elution was effected with 0.05 M pyridine–collidine acetate, pH 8.3, at a rate of 100 ml/hr. The buffer was prepared by diluting 50 ml of pyridine, 50 ml of γ-collidine, and 1.9 ml of glacial acetic acid to 10 l. with water. The protein was located

[1] We are grateful to Dr. A. L. Murdock for pointing out this fact to us.

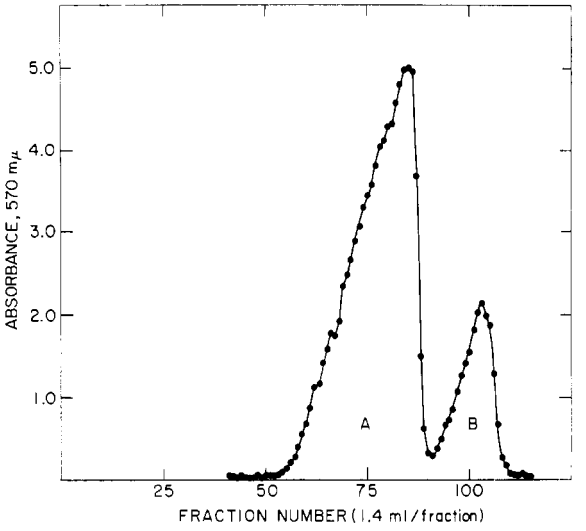

FIGURE 2: Separation of the S-protein and S-peptide fractions of ribonuclease S by filtration in 50% acetic acid through Sephadex G-75 (coarse grade, bead form). (A) S-protein fraction. (B) S-peptide fraction. Column, 1.8 × 70 cm; sample size, 179 mg; flow rate, 8 ml/hr; temperature, 23°. The absorbance at 570 mμ refers to the ninhydrin color value obtained after alkaline hydrolysis of 10-μl samples from the effluent fractions.

in the effluent fractions by the method of Lowry *et al.* (1951). Lyophilization of the protein-containing fractions provided the material from which the S-peptide and S-protein fractions were prepared.

Preparation of S-Peptide and S-Protein Fractions. Ribonuclease S (179–206 mg) was dissolved in 2.5 ml of water and 2.5 ml of glacial acetic acid was added with stirring. The solution was subjected to gel filtration at 25° on a 1.8 × 70 cm column of Sephadex G-75 which had been equilibrated with 50% acetic acid (Crestfield *et al.*, 1962). Details are given in the legend to Figure 2. Appropriate cuts were diluted tenfold with water, shell frozen, and subjected to lyophilization. The powders were stored at −20°.

Chromatography of the S-Peptide Fraction on Dowex 50-X2. The lyophilized S-peptide fraction (4–7 mg, 1.8–3.2 μmoles) was dissolved in 1.0 ml of 0.2 M acetic acid in a small test tube. The acetic acid solution had been thoroughly deaerated at 40° under vacuum from an aspirator. Mercaptoethanol (25 μl, 360 μmoles) was added, the tube was flushed out with nitrogen, and the solution was held at 40° for 72 hr. The solution was applied to a 0.9 × 150 cm column of Dowex 50-X2 (200–400 mesh), equilibrated with 0.50 M pyridine acetate, pH 4.10 (880 ml of glacial acetic acid and 322 ml of pyridine diluted to 8 l. with water). Elution was effected at a flow rate of 6 ml/hr with a linear gradient between 0.50 M pyridine acetate, pH 4.10, and 2.0 M pyridine acetate, pH 4.95 (1146 ml of glacial acetic acid and 1290 ml of pyridine diluted to 8 l. with water). The initial volume of each buffer in the reservoirs was 550 ml. Just prior to use the buffers were thoroughly deaerated at 40° under vacuum from an aspirator and thiodiglycol (5 ml/l.) was added. The effluent profile was developed by subjecting aliquot samples (250–400 μl) of the fractions to alkaline hydrolysis and treating with ninhydrin (Crestfield *et al.*, 1963). The samples were placed in polypropylene tubes and brought to dryness at 100° in an oven. Air was drawn through the oven *via* a tube fitted to an aspirator. After the addition of 0.18 ml (3 drops) of 5 N NaOH, hydrolysis was achieved in an autoclave under steam at 15 psi for 30 min. The cooled samples were neutralized by the addition of 0.25 ml of glacial acetic acid and subjected to reaction with ninhydrin.

Fragmentation of the S-Protein Fraction. The S-protein fraction (81 mg, 7.0 μmoles) from a ribonuclease S preparation obtained with subtilisin BPN′ was cleaved with cyanogen bromide according to the method of Gross and Witkop (1962) as modified by Steers *et al.* (1965). Performic acid oxidation of the cyanogen bromide cleavage product was performed according to the method of Hirs (1956).

Acid hydrolysates were prepared with twice-distilled, constant-boiling HCl in evacuated tubes at 110°. It was necessary to remove thiodiglycol from the peptides prior to hydrolysis to avoid loss of basic amino acids, presumably by reaction with products formed between thiodiglycol and HCl. This was achieved by gel filtration through a 0.9 × 300 cm column of Sephadex G-25 equilibrated with 0.2 N acetic acid.

TABLE I: Amino Acid Compositions[a] of the S-Peptide Fractions from Ribonuclease S Samples Prepared by Digestion with Subtilisin BPN′ and with Subtilopeptidase A.

Amino Acid	S-Peptide Fraction from		Residues 1–20 of Ribonuclease A[b]
	Subtilisin BPN′	Subtilopeptidase A	
Lysine	2.04	2.04	2
Histidine	1.03	1.00	1
Arginine	1.07	1.02	1
Aspartic acid	1.00	1.05	1
Threonine	1.91	1.95	2
Serine	3.07	2.85	3
Glutamic acid	2.98	2.97	3
Alanine	5.01	4.95	5
Methionine	0.96	0.99	1
Phenylalanine	1.01	1.03	1

[a] Values were determined after 22 hr of hydrolysis and are given as molar ratios of the constituent amino acids. No corrections for hydrolytic destruction have been applied. Values less than 0.03 residue are not included. [b] Smyth *et al.* (1963).

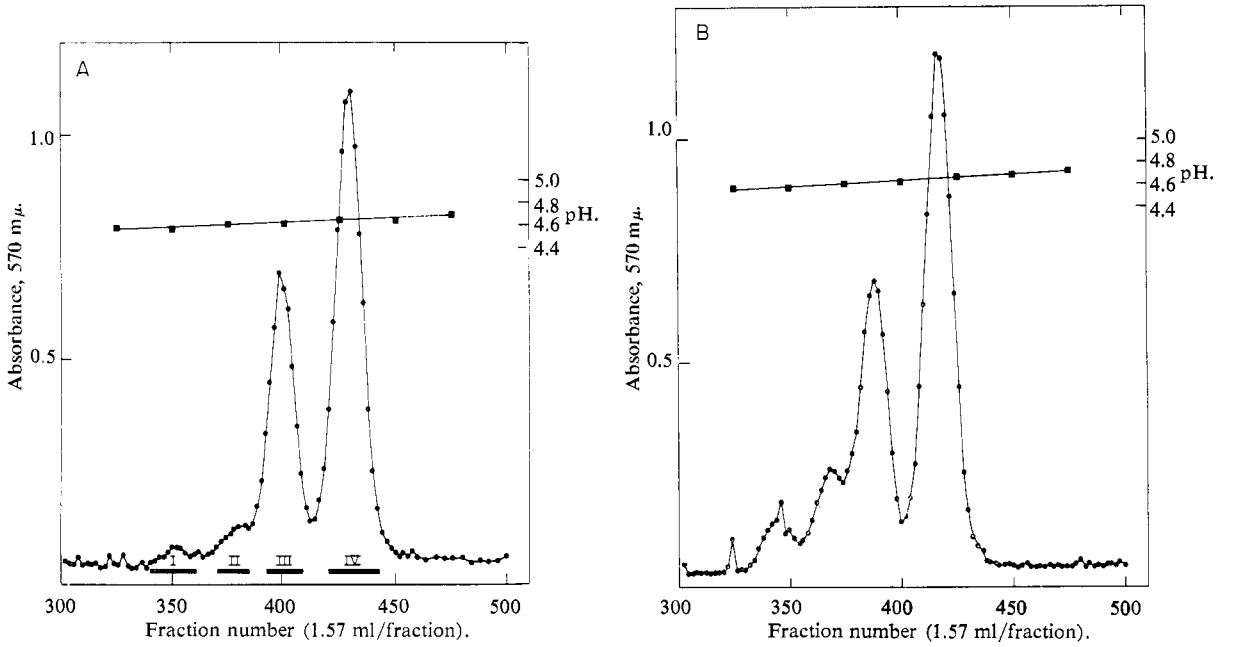

FIGURE 3: Chromatography on Dowex 50-X2 (200–400 mesh) of 4.2-mg samples of the S-peptide fraction from a ribonuclease S preparation obtained with subtilisin BPN′. (A) Column, 0.9 × 150 cm; flow rate, 6 ml/hr; temperature, 40°. Elution was effected with a linear gradient between 0.50 M pyridine acetate, pH 4.10, and 2.0 M pyridine acetate, pH 4.95. Both buffers contained thiodiglycol (5 ml/l.). A 0.4% solution of the sample in 0.2 M acetic acid was reduced with 25 μl of mercaptoethanol for 72 hr at 40° prior to chromatography. The absorbance at 570 mμ refers to the ninhydrin color value obtained after alkaline hydrolysis of 250-μl samples from the effluent fractions. The solid bands denote the cuts for which amino acid compositions are given in Table II. (B) The sample was not treated with mercaptoethanol prior to chromatography and no thiodiglycol was added to the buffers. The conditions were otherwise identical with those described for A.

Results and Discussion

In view of the nature of the present report a detailed description of the preparation of the ribonuclease S used in this work has been included. The procedure closely parallels the one described by Gordillo *et al.* (1962) and, indeed, is not known to depart from that procedure in any way which would alter the course of the proteolysis. The most obvious source of uncertainty is the use of *B. subtilis* protease preparations of unknown composition. A ribonuclease:subtilisin BPN′ weight ratio of 1000 was used in the present work. Under these conditions the maximum yield of ribonuclease S was obtained after 16–20 hr of digestion. In order to obtain a similar rate of digestion with the subtilopeptidase A preparation a weight ratio of 250 was required. Whether this difference in activity is a reflection of the purity of the protease preparations or of an intrinsic specificity difference is unknown at this time. The yield of ribonuclease S, as determined from the weight of the lyophilized, salt-free powders, was 65–75% with both preparations.

Gel filtration of salt-free ribonuclease S through a column of Sephadex G-75 equilibrated with 50% acetic acid caused the fractionation shown in Figure 2. The amino acid composition of the material in peak B is given in Table I for ribonuclease S samples prepared with subtilisin BPN′ and with subtilopeptidase A. For the ribonuclease S sample prepared with subtilopeptidase A the amino acid content of the material in peak B corresponds to the composition of S-peptide, *viz.*, residues 1–20 of ribonuclease A. The composition of the corresponding material from ribonuclease S prepared with subtilisin BPN′ also agrees with that of S peptide with the exception that the serine content is too high. A value of three residues is to be expected for a peptide composed of residues 1–20. Uncorrected serine values of between 3.1 and 3.2 residues were consistently obtained after 22 hr of hydrolysis. The amino acid content of the material in peak A corresponded, with both samples and within experimental error, to the composition of S-protein, *viz.*, residues of 21–124 of ribonuclease A.

The S-peptide fraction obtained from a subtilisin BPN′ digest was separated by chromatography on Dowex 50-X2 into the components shown in Figure 3A. The amino acid compositions and estimated percentages of the four components are given in Table II. The relative amounts of the components were estimated from the contribution of each to the total ninhydrin color obtained from the hydrolyzed aliquot samples taken for the development of the effluent profile. In view of the similarity in the compositions of the components these estimates are considered to be an accurate reflection of the amount of each one. The total

TABLE II: Amino Acid Compositions[a] of Chromatographic Components of the S-Peptide Fractions from Ribonuclease S Samples Prepared by Digestion with Subtilisin BPN′ and with Subtilopeptidase A.

	S-Peptide Fraction from						
	Subtilisin BPN′				Subtilopeptidase A Major	Residues 1–20 of Ribonuclease	Residues 1–21 of Ribonuclease
Amino Acid	I	II	III	IV	Component	A[b]	A[b]
Lysine	1.51	1.28	1.98	1.93	1.96	2	2
Histidine	0.87	0.86	0.99	0.98	1.03	1	1
Arginine	0.96	0.87	1.01	0.98	1.02	1	1
Aspartic acid	1.22	1.10	1.04	1.00	1.02	1	1
Threonine	2.00	1.92	1.95	1.94	1.94	2	2
Serine	3.60	3.07	3.63	2.78	2.82	3	4
Glutamic acid	3.00	2.91	2.99	2.96	2.99	3	3
Glycine	0.43	0.57	0.05	0.04	0.08	0	0
Alanine	4.84	4.97	4.99	5.07	5.01	5	5
Valine	0.14	—	—	—	—	0	0
Methionine	0.04[c]	—[c]	0.92	0.93	0.76[c]	1	1
Leucine	0.06	—	—	—	—	0	0
Phenylalanine	0.94	1.02	0.97	0.97	0.98	1	1
Methionine sulfoxides[c]	1.2	1.1	—	—	0.2	0	0
Percentage of total fraction[d]	2	6	32	60	84		

[a] Values were determined after 22 hr of hydrolysis and are given as molar ratios of the constituent amino acids. No corrections for hydrolytic destruction have been applied. Values less than 0.03 residue are not included. [b] Smyth *et al.* (1963). [c] For an unknown reason the methionine in these samples appeared partially or entirely in the form of the sulfoxide derivatives. This phenomenon cannot be viewed as a reflection of the sulfoxide content of the components because acid hydrolysis under vacuum normally converts methionine sulfoxides to methionine (Ray and Koshland, 1960). Insufficient amounts of the subtilisin BPN′ components were at hand to permit further investigation. A second hydrolysate of the subtilopeptidase A component gave values for methionine and the methionine sulfoxides indistinguishable from those obtained with the first hydrolysate. [d] The percentages were estimated from the contribution of each component to the total ninhydrin color obtained from the hydrolyzed aliquot samples taken for the development of the effluent profile.

recovery of the S-peptide fraction from the column was 60–65% as determined from amino acid analyses. No ninhydrin-positive material other than that shown in the figure was detected.

A study of the rate of destruction on hydrolysis of serine in the two major components revealed a linear decline at a rate equal to the one determined by Rees (1946) for free serine (Figure 4). The extrapolated value of 3.8 residues found for component III probably does not arise from contamination with neighboring components. The initial rate of serine destruction may be unusually high. It might be noted that this component contains a C-terminal serine, whereas the other does not. Contamination by the deamidated derivative of the shorter peptide, which might logically chromatograph at this position, would also lower the serine content. The amide content of the components, which in theory is one glutamine residue, was not measured.

Although the major component is the peptide composed of residues 1–20 of ribonuclease A, it is apparent that a substantial amount of the peptide composed of residues 1–21 is also present. The two minor components appear in positions occupied by what appear to be the methionine sulfoxide derivatives of the major components (see below). Before application, the sample had been exposed to conditions considered to be sufficient to reduce sulfoxides and was chromatographed under conditions chosen to minimize their re-formation. That the minor components are not composed entirely of the sulfoxide derivatives of the major components is also indicated by the low lysine content of both materials. The minor components may be composed in part of peptides lacking the N-terminal lysine. The appearance of such peptides is surprising in view of the homogeneity claimed for the starting material as well as the relative ease with which deslysylribonuclease A may be sepa

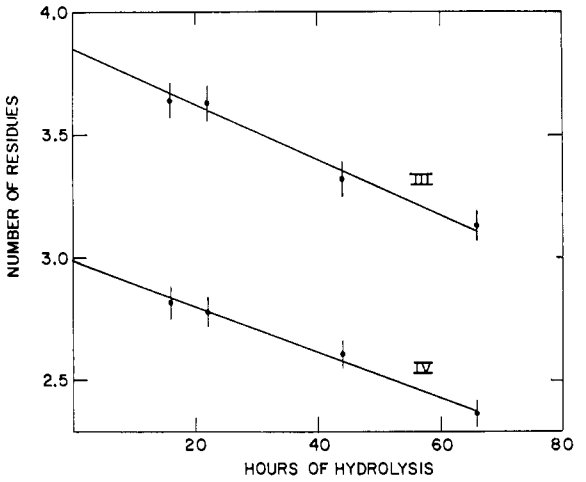

FIGURE 4: The rates of hydrolytic destruction of serine in components III and IV of Figure 3A. The hydrolyses were performed in evacuated tubes at 110° with twice-distilled, constant-boiling HCl. The vertical lines through the points span the region of 2% error.

rated from ribonuclease A (Gordillo *et al.*, 1962; Eaker *et al.*, 1965).

Although the S-peptide fraction obtained after digestion with subtilopeptidase A had an amino acid composition corresponding within experimental error to that of residues 1–20 of ribonuclease A, chromatography on Dowex 50-X2 separated the material into the components shown in Figure 5. The amino acid composition and estimated percentage of the major component are given in Table II. In this case, the estimate is based on the assumption of similarity in the compositions of the components. The materials running ahead of the major component were not isolated in amounts sufficient for amino acid analysis.

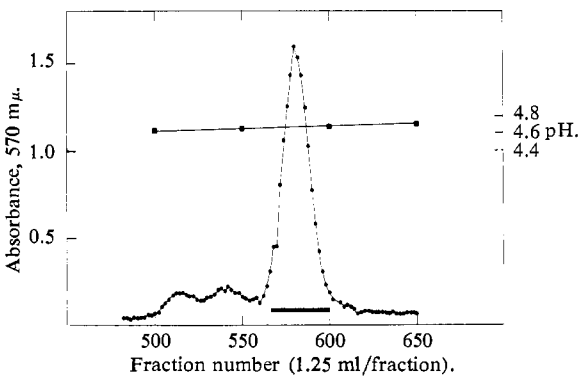

FIGURE 5: Chromatography on Dowex 50-X2 (200–400 mesh) of the S-peptide fraction from a ribonuclease S preparation obtained with subtilopeptidase A. The sample size was 4.1 mg and 400-μl samples were removed from the effluent fractions for the development of the profile. The conditions were otherwise identical with those described in the legend to Figure 3A.

The yield of the shorter S peptide is significantly higher when ribonuclease A is modified with subtilopeptidase A rather than with subtilisin BPN′. Whether the difference results from the activity of differing amounts of contaminants in the two protease preparations or from an intrinsic difference in specificity is not known. The products obtained from the partial digestion of ovalbumin by the two proteases are not identical (Ottesen and Østergaard, 1964; Haruna, 1960).

Chromatography of the S-peptide fraction in the absence of prior reduction with mercaptoethanol and with omission of thiodiglycol from the buffers afforded an effluent profile of the type shown in Figure 3B. The amino acid compositions of the minor components corresponded to those of residues 1–21 and 1–20 of ribonuclease A, with the exception that the lysine content was somewhat low. Inclusion of the reduction step and addition of thiodiglycol to the buffers produced material which gave an effluent profile of the type shown in Figure 3A. This behavior is consistent with the hypothesis that peptides containing the sulfoxide derivatives of the methionine at position 13 are present in the S-peptide fraction. The sample of the S-peptide fraction used for the chromatography shown in Figure 3A and

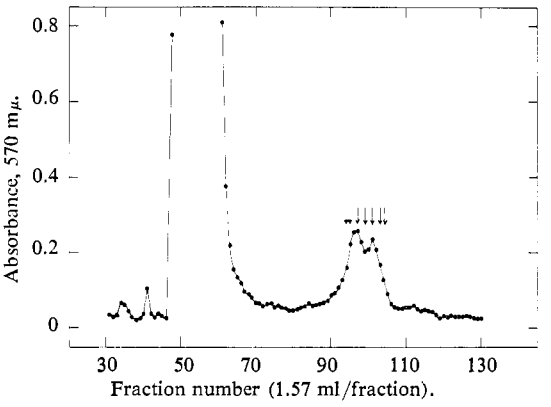

FIGURE 6: The fractionation by filtration through Sephadex G-25 (fine grade) of an S-protein fraction which had been fragmented by successive reaction with cyanogen bromide and performic acid. The fraction was from a ribonuclease S preparation obtained with subtilisin BPN′. Column, 0.9 × 300 cm; sample size, 82 mg (7.0 μmoles); solvent, 0.2 M acetic acid; flow rate, 8 ml/hr; temperature, 23°. The absorbance at 570 mμ refers to the ninhydrin color value obtained after alkaline hydrolysis of 25-μl samples from the effluent fractions. The arrows denote those fractions for which amino acid compositions are given in Table III.

TABLE III: Amino Acid Compositions[a] of the Material in Selected Tubes from the Gel Filtration Profile of the Fragmented S-Protein Fraction of a Ribonuclease S Sample Prepared by Digestion with Subtilisin BPN'.

	Tube Number								
	94	95	97	99	101	103	104	Residues 21–29 of Ribonuclease A[b]	Residues 22–29 of Ribonuclease A[b]
	Total μmoles in Tube								
Amino Acid	0.250	0.321	0.374	0.297	0.291	0.249	0.187		
Lysine	0.06	0.05	0.05	0.06	0.05	0.04	0.04	0	0
Cysteic acid	0.85	0.95	0.94	0.90	0.97	1.00	0.98	1	1
Aspartic acid	1.99	1.99	1.97	1.98	1.98	1.99	1.99	2	2
Threonine	0.05	0.03	0.05	0.04	—	—	—	0	0
Serine	2.50	2.50	2.43	2.19	1.96	1.89	1.89	3	2
Glutamic acid	1.01	1.01	1.03	1.02	1.02	1.01	1.01	1	1
Glycine	0.07	0.07	0.07	0.05	0.07	0.07	0.08	0	0
Alanine	0.11	0.05	0.05	0.03	0.03	—	—	0	0
Valine	0.15	0.06	0.06	0.09	0.15	0.07	0.04	0	0
Tyrosine[c]	0.07	0.06	0.08	0.08	0.07	0.06	0.07	1	1
Homoserine[d]	0.34	0.40	0.41	0.60[e]	1.40[e]	0.47	0.47		
Homoserine lactone[d]	0.58	0.55	0.62	0.79[e]	1.69[e]	0.60	0.51	1	1
Unknown[c]	1.03	0.91	0.98	0.96	0.91	0.86	0.99	—	—

[a] Values were determined after 22 hr of hydrolysis and are given as molar ratios of the constituent amino acids. No corrections for hydrolytic destruction have been applied. Values less than 0.03 residue are not included. [b] Smyth *et al.* (1963). [c] Bromide ions are a product of the reaction between cyanogen bromide and methionine (Gross and Witkop, 1962) and of the hydrolysis of cyanogen bromide. Oxidation of bromide counterions to bromine during the treatment of the modified S-protein with performic acid and resultant bromination of the tyrosine residues may have occurred. Ninhydrin-positive material, labeled unknown in the table, appeared where chlorotyrosine is found. The molar ratio of this material was calculated using the integration constant for tyrosine. [d] The molar ratios of homoserine and homoserine lactone were calculated using the integration constant for serine. [e] The reaction of cyanogen bromide with ribonuclease A produces free homoserine lactone owing to the presence of a methionylmethionyl sequence at positions 29 and 30 (Gross and Witkop, 1962). The high molar ratios of homoserine and homoserine lactone found in tubes 99 and 101 probably resulted from the appearance of this derivative in these tubes. Such an overlap with an octa- and a nonapeptide implies that strong adsorption of these peptides to the Sephadex occurred.

B had been freshly prepared and the apparent sulfoxide content was probably lower than that to be expected in samples stored for several months. The chromatographic behavior of such a stored sample indicated that 40% of the methionine had been converted to the sulfoxide forms.

Kenkare and Richards (1966) exposed a sample of the S-peptide fraction to conditions which might have caused oxidation to the sulfoxide forms. The apparent binding constants between this material and the S-protein fraction were measured both before and after treatment of the sample with mercaptoethanol. It was found that exposure to mercaptoethanol increased the apparent binding constant by a factor of nine.

Neumann *et al.* (1962) found no evidence that the methionine residues in native ribonuclease A are in the sulfoxide forms. One may infer that conversion to the ribonuclease S derivatives or, more likely, the separation of the S-peptide and S-protein moieties changes the environment of the methionine at position 13 sufficiently to permit oxidation.

The two major S-peptides obtained after subtilisin BPN' digestion of ribonuclease A could arise independently from cleavages between residues 20–21 and residues 21–22, or the shorter peptide could be produced by removal of the C-terminal serine of a portion of the longer peptide molecules. In the first case, a heterogeneous S-protein fraction would also be produced and the possibility of obtaining four species of ribonuclease S would arise. In the second case, a homogeneous S-protein fraction would be produced and only two species of ribonuclease S would be expected. Fractionation of the ribonuclease S mixture or of the S-protein fraction has not been achieved. A quantitative determination of the N-terminal residues in the S-protein fraction is not

informative owing to the presence of serine in both positions 21 and 22. However, fragmentation of the S-protein fraction by successive reaction with cyanogen bromide (Gross and Witkop, 1962) and performic acid (Hirs, 1956) provided an N-terminal fraction of manageable size.

Gel filtration of the fragmented S-protein fraction on a column of Sephadex G-25 caused the fractionation shown in Figure 6. The major unretarded fraction is presumably composed of a mixture of the two peptides derived from residues 31–79 and residues 80–124 as well as any unreacted material. The material in the minor fraction at about 150 effluent ml was analyzed at the several points indicated by the arrows in the figure. The amino acid compositions of the material in these tubes are given in Table III along with the compositions of the peptides derived from residues 21–29 and 22–29 of ribonuclease A. A progressive decline in serine content with increasing tube number is evident. Such a decline would be expected if the front portion of the fraction contained the peptide derived from residues 21–29 and the back portion contained the peptide derived from residues 22–29. The relative position of the peptides is consistent with their size. The amount of the two peptides in each of the tubes taken for analysis was calculated from: $3 \times \mu$moles of peptide 21–29 $+ 2 \times \mu$moles of peptide 22–29 = observed number of serine residues $\times$ total μmoles in tube. A standard correction for the hydrolytic destruction of serine was used (Rees, 1946). From the areas under the independent profiles so generated the fraction was estimated to be composed of $53 \pm 5\%$ peptide 21–29 and $47 \pm 5\%$ peptide 22–29. The combined yield of the two peptides was 59%.

The initial proteolytic cleavage evidently can occur at either the bond between residues 20 and 21 or the bond between residues 21 and 22. However, the relative amounts of peptides 21–29 and 22 and 29 do not correspond to the relative amounts of peptides 1–20 and 1–21 found in the S-peptide fraction (53:47 *vs.* 60:32) indicating that, in addition, a portion of peptide 1–20 is produced by the removal of the C-terminal serine from peptide 1–21.

An acidified sample of an 18-hr digestion mixture was subjected to chromatography on an amino acid analyzer in an attempt to detect and measure free serine. Although material appeared at the position occupied by free serine, there were approximately twenty additional ninhydrin-positive components, many present in quantities comparable to "serine." Richards (1955) found that approximately 30 of the peptide bonds of ribonuclease are ultimately cleaved by subtilisin. The rates of appearance of final digestion products, *i.e.*, free amino acids and small peptides, are apparently high enough to eliminate the possibility of detecting the serine from position 21 in this straightforward manner.

Klee has proposed a structure for residues 15–24 of ribonuclease A on the basis of the disparate susceptibility to proteolysis of the peptide bonds in this region (Klee, 1965). The observation that the bond between residues 21 and 22 serves as an initial cleavage point requires revision either of the structure or of the assumption that participation in such a structure renders the bond unavailable for proteolysis. In the present work there was no evidence of hydrolysis at bond 22–23 although this bond involves the same amino acid as bond 21–22, *viz.*, serine.

Whether all of the four major species of ribonuclease S which are potentially present in a subtilisin BPN′ digest are formed is not known at this time. A revision of the nomenclature for this system seems to be required in any case. It is suggested that a bracket, in which the composite segments are indicated, be appended to the term ribonuclease A, *e.g.*, ribonuclease A [1–20, 21–124], ribonuclease A [1–21, 22–124]. A simpler nomenclature in which the bracketed information indicates the bond cleaved would not accommodate the species formed by the alternate combinations of the segments, *viz.*, ribonuclease A [1–20, 22–124] and ribonuclease A [1–21, 21–124]. The suggested nomenclature would also accommodate the products of the digestion of ribonuclease A by other proteolytic enzymes (Li *et al.*, 1966; Klee, 1965; Scheraga and Rupley, 1962) as well as many of the species obtained by the synthetic approach (Hofmann *et al.*, 1966).

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