Evidence for Glycoprotein Nature of Stem Bromelain. Isolation of a Glycopeptide*

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ABSTRACT: The purified preparation of stem bromelain was known to contain 2.1% carbohydrate in addition to four hexosamine residues per molecule of molecular weight 33,000. Attempts have been made to demonstrate that these carbohydrates are covalently linked to the enzyme protein, *i.e.*, stem bromelain is a true glycoprotein. Heat coagulation test and acid precipitation have provided no conclusive evidence, but gel filtration techniques which involved the use of dextran gels with various degrees of cross-linkage, filtration experiments with varying the pH of the medium, and comparison of filtration pattern before and after the proteolytic digestion of the material have proved useful in demonstrating the glycoprotein nature of stem bromelain. Further evidence has been obtained by isolation of a glycopeptide from the proteolytic digest. Approximately 30 mg of the glycopeptide was obtained from 1 g of stem bromelain.

The isolated product was of reasonably high purity and showed a weakly acidic character on paper electrophoresis. Acid hydrolysis of the glycopeptide gave 3 moles of aspartic acid, three serines, two glutamic acids, four glucosamines, three mannoses, one xylose, and one fucose per mole of peptide. From the yield and composition of this octaglycopeptide as compared to the carbohydrate content of the parent protein, it is concluded that stem bromelain contains one sugar moiety per molecule.

The glycoprotein nature of stem bromelain was first suggested by Murachi *et al.* (1964) who found that the purified enzyme preparation contained approximately 2.1% carbohydrate. This observation was confirmed by Ota *et al.* (1964); their purified preparation contained 1.5% carbohydrate in addition to six glucosamine residues per mole of protein. Murachi (1964) also reported that his preparation contained four hexosamines per molecule of molecular weight 33,000. Although the carbohydrates, inclusive of hexosamine, in these preparations appear to be firmly bound to the enzyme protein, no direct evidence was available to indicate that the sugar moiety is covalently linked to the polypeptide chain and hence that stem bromelain is a true glycoprotein. Usually, evidence for a true glycoprotein is provided by heat coagulation test if it demonstrates removal of the carbohydrate from the solution concomitant with precipitation of the heat-denatured protein (see, for example, Neuberger, 1938; Hanafusa *et al.*, 1955). With a proteolytic enzyme, however, heat coagulation test would give variable results because of the accelerated autodigestion that might be associated with heating of the sample. Consequently, additional experimentation is needed to establish with certainty that stem bromelain contains carbohydrates as part of its molecular structure.

The objective of the present study was first to develop a method for providing evidence for the glycoprotein nature of stem bromelain and secondly to apply the method to the isolation of a glycopeptide from the enzyme molecule. The composition of the isolated glycopeptide is also described.

Materials and Methods

Stem Bromelain. The enzyme preparation used was isolated from crude "Bromelain" (lots 181 and 182) from the Hawaiian Pineapple Co., Honolulu, Hawaii,[1] by the method of Murachi *et al.* (1964). Fraction 6 was employed for the present experiments. The preparation was found to be essentially homogeneous by several independent physical criteria as well as by rechromatography (Murachi *et al.*, 1964). The molecular weight of the enzyme protein was assumed to be 33,000 (Murachi *et al.*, 1964). The protein concentration was determined by measuring the absorbancy at 280 mμ, using a molar absorbancy of 6.68×10^4 (Murachi *et al.*, 1965). A Hitachi Perkin-Elmer Model UV-VIS-139 spectrophotometer was used.

Streptomyces griseus Proteinase. "Pronase P" was obtained from Kaken Chemical Co., Tokyo, Japan. The enzyme was partially purified according to the procedure described in the previous communication

* From the Department of Biochemistry, Nagoya City University School of Medicine, Nagoya, Japan. *Received August 11, 1967.* A part of this work was presented before the 36th Annual Meeting of the Japanese Biochemical Society, Tokyo, Oct 29–31, 1963, and before the 16th Symposium on Protein Structure, Fukuoka, Japan, Oct 15–16, 1965 (Takahashi *et al.*, 1965). This work was supported in part by a grant from the Ministry of Education, Japan, and by a U. S. Public Health Service research grant (GM 08714) from the National Institute of General Medical Sciences.

[1] We are indebted to Dr. Ralph M. Heinicke for generous supply of this material.

TABLE I: Heat and Acid Denaturation of Stem Bromelain, Ovalbumin, and Taka-amylase A.

Protein	Carbohydrate Content (g/g of protein)	Method of Denaturation	Concn in Supernatant Fluid after Denaturation (%)[a]	
			Protein	Carbohydrate
Stem bromelain	0.021	Heat	33	54
		Acid	13	18
Ovalbumin	0.018	Heat	12	12
		Acid	2.4	2.0
Taka-amylase A	0.027	Heat	5.9	6.6
		Acid	3.7	3.8

[a] Protein and carbohydrate concentrations of the solution before denaturation were taken as 100%. Protein concentration was determined from absorbancy at 280 mμ. Carbohydrate was determined by the orcinol–H_2SO_4 method (Winzler, 1955).

(Murachi *et al.*, 1965). The partially purified preparation contained no carbohydrate.

Bacillus subtilis Proteinase. The crystalline enzyme was obtained from Nagase Co., Osaka, Japan, and used without further purification. The enzyme preparation contained no carbohydrate.

Determination of Carbohydrates. The total carbohydrate content of various samples was determined by orcinol–H_2SO_4 method of Winzler (1955). The samples were heated at 80° for 15 min and their absorbancy was determined at 540 mμ. Reagent grade D-mannose was used as standard. The present experiments revealed that the glycopeptide isolated from an enzymatic digest of stem bromelain contained four residues of glucosamine, three of mannose, one xylose, and one fucose (see below). Since glucosamine does not give an orcinol reaction, while fucose gives some 80% of the hexose color, the result of the present assay obtained by using mannose as the standard may represent fairly exactly the sum of three kinds of neutral sugars present. To be exact, a mixture of 3 moles of mannose, 1 mole of xylose, and 1 mole of fucose should give a color value equivalent to that of 4.8 moles of mannose.

Determination of Individual Neutral Sugars. Mannose, xylose, and fucose were separated from each other by paper chromatography and determined by specific color reactions. Carbazole–H_2SO_4 or cysteine–H_2SO_4 reaction (for mannose) was used for mannose, orcinol–HCl reaction for xylose, and cysteine–H_2SO_4 reaction (for methylpentose) for fucose (Ashwell, 1957).

Amino Acid Composition. The amino acid composition of the acid hydrolysate was determined by the method of Hamilton (1963), using a Technicon Auto-Analyzer. With the buffer system used glucosamine appeared as a well-separated single peak between alanine and valine, making a simultaneous determination possible.

Gel Filtration. Sephadex G-200 (140–400 mesh, lot To 39), G-100 (140–400 mesh, lot To 1291), G-75 (medium), and G-25 (fine) were products obtained from Pharmacia, Uppsala, Sweden. Gel filtration was carried out at cold room temperature (approximately 4°).

Others. Ovalbumin was crystallized by the method of Kekwick and Cannan (1936). Crystalline Taka-amylase A was prepared by Dr. T. Takagi[2] according to Akabori *et al.* (1954). Ninhydrin color value was estimated according to the method of Yemm and Cocking (1955). Toyo no. 51 filter paper was used for paper chromatography and electrophoresis. Buffers used for electrophoresis were pyridine–acetic acid–water (5:0.2:95) at pH 6.5, pyridine–acetic acid–water (1:10:89) at pH 3.5, and formic acid–pyridine–water (50:3:950) at pH 2.2.

Results

Coagulation by Heat. Stem bromelain was dissolved in 0.1 M KCl to make a 5.6% solution. The solution (2 ml) was heated in a boiling water bath for 5 min, after which the solution was rapidly cooled and then allowed to stand at 0° for 1 hr. The centrifuged supernatant fluid was analyzed for absorbancy at 280 mμ and for carbohydrate content. The heat coagulation test was carried out also with 4% Taka-amylase A and 4% ovalbumin. The results obtained are expressed in terms of per cent found in the supernatant as compared to the original protein solution. It is obvious from Table I that the test is applicable to demonstrating the glycoprotein nature of ovalbumin and Taka-amylase A but not of stem bromelain, since both values for stem bromelain are too high and do not agree with each other.

Acid Denaturation. To 1.0 ml of a 1% protein solution was added 1.0 ml of 20% trichloroacetic acid. The mixture was allowed to stand at 0° for 1 hr. After centrifugation, the supernatant was analyzed in the same way as described for the heat coagulation test.

[2] We are indebted to Dr. Toshio Takagi, Institute for Protein Research, Osaka University, Osaka, for supplying this preparation as a gift to us.

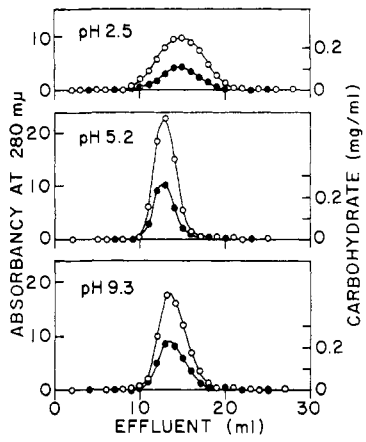

FIGURE 1: Gel filtration of stem bromelain with Sephadex G-100 at various pH values. (O) Absorbancy at 280 mμ. (●) Carbohydrate content. One milliliter of 3% stem bromelain was filtered through a 1.12 × 20 cm column with 0.05 M glycine–HCl buffer at pH 2.5, 0.05 M sodium acetate buffer at pH 5.2, or 0.05 M glycine–NaOH buffer at pH 9.3. Fractions of 1 ml were collected.

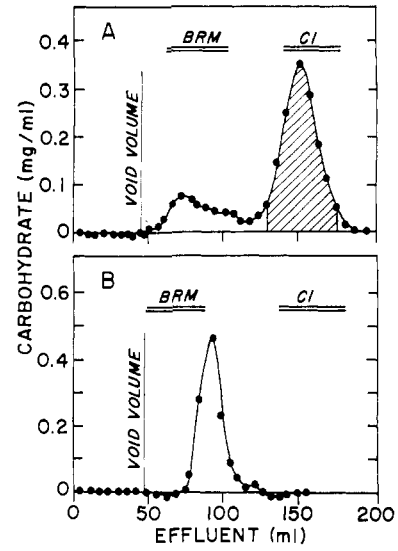

FIGURE 2: Gel filtration of the proteolytic digest of stem bromelain with Sephadex G-100 (A) and G-25 (B). A 2.5 × 33 cm column was used with 0.05 M sodium acetate buffer at pH 5.2. Fractions of 3 ml were collected. (A) Filtration of the digest obtained from 580 mg of stem bromelain. (B) The carbohydrate-containing fractions (hatched area) were combined, concentrated, and used for refiltration. Intact stem bromelain (BRM) and chloride ion (Cl) should appear through an identical Sephadex column at respective effluent volumes indicated in the figures.

The results obtained are also shown in Table I. The treatment with acid precipitated more than 96% of ovalbumin and Taka-amylase A, whereas in the case of stem bromelain a considerable portion of the carbohydrate remained soluble, again showing the failure of this method to demonstrate the glycoprotein nature of stem bromelain.

Gel Filtration. A 3% solution of stem bromelain in 0.05 M sodium acetate buffer at pH 5.2 (1 ml) was applied to a 1.12 × 20 cm (20 ml) column with Sephadex G-25 which had been washed with the same buffer. The column was washed with the same buffer and 1-ml fractions were collected. The absorbancy at 280 mμ of each fraction was read and the carbohydrate content was determined. Similar types of experiments were carried out also with Sephadex G-75, G-100, and G-200, respectively. No separation of carbohydrate from the protein occurred with any of the four kinds of dextran gels used, suggesting three possibilities: (1) the enzyme was a true glycoprotein, having covalently linked oligosaccharide moiety in the molecule; (2) the binding of oligosaccharide to the enzyme protein was not of covalent nature but it happened to be fairly strong at pH 5.2; and (3) there was a polysaccharide contamination, the molecular size of which was almost identical with the size of stem bromelain.

The second of the three possibilities mentioned above was then ruled out by carrying out gel filtration experiments at such extreme pH values as 2.5 and 9.3. At pH 2.5 was used 0.05 M glycine–HCl buffer, and for pH 9.3, 0.05 M glycine–NaOH buffer. The procedures of gel filtration and analysis were the same as those described above. The results obtained are shown in Figure 1.

Digestion by Proteinases. In order to determine which one of the remaining two possibilities is more likely, the enzyme protein was subjected to proteolytic digestion followed by gel filtration analysis. The filtration pattern of the carbohydrate would be expected to vary before and after the digestion, depending upon whether the carbohydrate found in the original enzyme preparation had been a contaminant polysaccharide or was covalently bound. Thus, 580 mg of stem bromelain in 25 ml of 0.01 M calcium acetate was heated in a boiling water bath for 4 min. To the resultant suspension of the coagulated protein were added 50 mg of the partially purified preparation of *S. griseus* proteinase and 15 mg of *B. subtilis* proteinase. The mixture was adjusted to pH 8.3, and incubated at 37° for 4 days with a small piece of thymol crystal added as disinfectant. The coagulated protein was digested to yield a clear solution within a few hours of incubation. Finally, the digest was applied to a 2.5 × 33 cm (160 ml) column with Sephadex G-100 gel in 0.05 M sodium acetate buffer at pH 5.2. The column was washed with the same buffer at room temperature. Carbohydrate content of each 3-ml fraction was determined. The filtration pattern obtained is depicted in Figure 2A. In the figures references are made for those effluent volumes at which intact stem bromelain and chloride ion, respectively, should appear through an identical Sephadex column. It is apparent from Figure

2A that after the proteolytic digestion most of the carbohydrate appears in the low molecular weight fractions. The finding is evidently inconsistent with possibility 3, *i.e.*, the original enzyme preparation contained a polysaccharide of a molecular size similar to the protein. The low molecular weight fractions with positive sugar test were combined (51 ml) and concentrated, and the concentrate (5 ml) was further applied to a 2.5 × 33 cm (160 ml) column with Sephadex G-25 gel in 0.05 M sodium acetate buffer at pH 5.2. The fractionation and the analysis were carried out in the same way as above. The results obtained are shown in Figure 2B. From the position of the carbohydrate-containing fractions in the filtration pattern shown in Figure 2B, the occurrence of a glycopeptide or glycopeptides of a molecular size of 2000–3000 may be suggested. Essentially the same result was obtained when the ratio of *S. griseus* proteinase to stem bromelain was reduced to 1:100 (w/w) and the use of *B. subtilis* proteinase was omitted. These observations strongly support possibility 1, *i.e.*, stem bromelain contains carbohydrate as a covalently linked part of its molecule.

Isolation of Glycopeptide. On the basis of the findings described above, a routine procedure for the isolation of glycopeptide from stem bromelain was developed. Thus, the 48-hr digest of 1 g of stem bromelain with 10 mg of Pronase P was passed through a 20-ml column with Amberlite IR-120 (H^+ form) resin. The column was washed with 100 ml of water. The combined effluent and the washing were concentrated *in vacuo* to 10 ml and the concentrate was applied to a 500-ml column with Sephadex G-25 which had been washed with 0.05 M sodium acetate buffer at pH 5.2. The column was washed with the same buffer and the effluent was collected in 10-ml fractions. The carbohydrate-containing fractions, usually with tube numbers from 20 to 25, were pooled. The recovery of carbohydrate as determined by the orcinol–H_2SO_4 method (Winzler, 1955) was 91.5% of the original protein. The combined effluent was passed through a 20-ml column with Amberlite IR-120 (H^+ form) resin. The effluent that contained the carbohydrate was concentrated *in vacuo* to dryness and the residue was dissolved in a minimum amount of water. The solution was applied in parts to six sheets of 60 × 60 cm filter paper, and the paper electrophoresis was carried out at pH 6.5, at 3000 v, for 1 hr. Figure 3A,B shows the tracings of guide strips as stained with ninhydrin and alkaline silver nitrate reagents, respectively. As shown in the figure, the sample is devoid of contaminating free amino acids and monosaccharides, and contains a material that migrates slowly toward the anode and shows positive ninhydrin test with no reducing power. The material was located on the unstained paper sheets by the aid of guide strips, and eluted from the paper with water. The eluate was concentrated *in vacuo* to dryness. A preliminary analysis showed that this material is a glycopeptide which upon acid hydrolysis gives three kinds of amino acids, three kinds of neutral sugars, and glucosamine. The yield of the material as calculated in terms of the recovery of carbohydrate was 49.8% from the original protein.

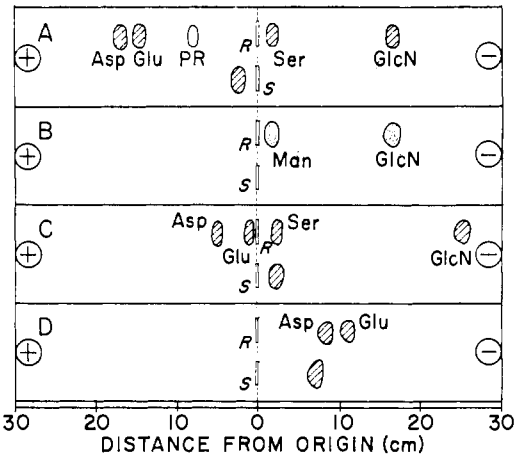

FIGURE 3: Tracings of electrophoretograms of the glycopeptide. (A and B) At pH 6.5, at 3000 v, for 1 hr. (C) At pH 3.5, at 2000 v, for 1 hr. (D) At pH 2.2, at 2000 v, for 1 hr. Authentic reference compounds and the sample were applied at R and S, respectively, on the line of the origin. For staining, ninhydrin was used in A, C, and D (hatched areas), and alkaline silver nitrate reagent in B (dotted areas). PR stands for phenol red used as a marker. The positions of the glycopeptide after electrophoresis runs are indicated by hatched areas in each lower half of tracings A, C, and D. The position of the glycopeptide in B should be identical with that in A, but the material gives no positive staining with silver nitrate.

This corresponds to a value of approximately 30 mg of the glycopeptide from 1 g of stem bromelain, provided that the composition of sugars and amino acids (see below) is in fact correct.

Homogeneity of Glycopeptide. The isolated glycopeptide was examined for homogeneity on paper electrophoresis at pH 3.5 and 2.2. Figure 3C,D represents tracings of electrophoretograms as stained with ninhydrin reagent. The homogeneity was further confirmed by identifying the amino-terminal residue with aspartic acid by the dinitrophenylation method (Sanger and Thompson, 1953). No other DNP-amino acid than DNP-aspartic acid was found to be present in the hydrolysate of the dinitrophenyl (DNP) peptide. The details of the end-group analyses will be described elsewhere.

Composition of Glycopeptide. The glycopeptide isolated as described above was analyzed for its component sugars and amino acids. Since the material was not obtained in salt-free, crystalline state, reference was always made to the content of neutral sugars in the sample as determined by the orcinol–H_2SO_4 method (Winzler, 1955). Thus, a sample which gave a color value equivalent to that of 4.8 μmoles of mannose was taken as containing 1 μmole of glycopeptide. The justification for the use of the value 4.8 was given in Materials and Methods.

For identification of component sugars, approximately 0.2 μmole of the glycopeptide was hydrolyzed with 1 ml of 1 N HCl at 100° for 3 hr and the concentrated hydrolysate was chromatographed on filter paper with a solvent system of ethyl acetate–acetic acid–water (3:1:3). Authentic specimens of frequently occurring monosaccharides were taken as references. Staining of the guide strips either with alkaline silver nitrate or with aniline–hydrogen phthalate reagent revealed the occurrence of three kinds of sugar which were identified with mannose, xylose, and fucose. Galactose was absent, while only a trace amount of glucose was detected. No positive staining was found in the chromatogram that corresponded to glucuronic acid. Neutral sugars on the unstained paper were located by the aid of guide strips and eluted from the paper with water. Further identification of each sugar was made by carrying out the specific color reaction for each sugar and comparing the absorption spectrum of the product with that for the authentic specimen (Ashwell, 1957). The color reaction also provided quantitative information on the content of each sugar. For the loss of neutral sugars throughout these procedures correction was made by the use of galactose added to the sample as an internal standard. The conditions for acid hydrolysis described above were found to give highest values for neutral sugar contents. The results obtained are shown in Table II with those for other constituents.

In a separate experiment, each 0.1 μmole of the glycopeptide and stem bromelain was reacted with 2 ml of Bial's reagent (Svennerholm, 1957a,b). Both samples gave a greenish color for pentoses but failed to give a reddish-purple color for sialic acid. The glycopeptide was also found to give no positive Ehrlich test for sialic acid (Kabat and Mayer, 1961). These facts indicate the absence of sialic acid either in the isolated glycopeptide or in the original enzyme protein.

For amino acids and glucosamine, approximately 0.2 μmole of the glycopeptide was hydrolyzed with 0.2 ml of redistilled 5.7 N HCl at 100° for 20 hr. The hydrolysate was evaporated *in vacuo* to dryness and was analyzed on an Auto-Analyzer. The data for glucosamine were corrected for the destruction during hydrolysis at 100° for 20 hr. Since the degree of destruction was found to be dependent on the amount of amino acids simultaneously present in the sample, different correction factors should be used for samples with different amino acid contents. Figure 4 illustrates a working curve from which such correction factors can be obtained. No reasonable explanation is possible for the observed phenomenon shown in the figure. Although better correction factors could be obtained, for example, by studying the time course of the destruction under various conditions, the use of the factors obtained from Figure 4 has given practically satisfactory results which agree well with the earlier report on the glucosamine content of stem bromelain (Murachi, 1964).

From the data summarized in Table II, one may conclude that the glycopeptide is composed of three aspartic acids, two glutamic acids, three serines, four glucosamines, three mannoses, one fucose, and one xylose.

TABLE II: Composition of the Glycopeptide from Stem Bromelain.

Constituent[a]	No. of Residues/Mole of Peptide[b] Found[c]	Expressed as Nearest Integer
Aspartic acid	2.97 ± 0.023	3
Serine	3.10 ± 0.046	3
Glutamic acid	2.02 ± 0.069	2
Glucosamine	4.16 ± 0.116	4
Mannose	2.74 ± 0.110	3
Xylose	1.06 ± 0.050	1
Fucose	1.01 ± 0.015	1

[a]Conditions of acid hydrolysis and methods of analysis are described in text. Ammonia from amide groups and acetic acid from *N*-acetyl groups were not determined. [b]Calculation was made by assuming that 1 mole of the glycopeptide has an orcinol–H_2SO_4 color value equivalent to that of 4.8 moles of mannose. [c]Mean value of 11 separate determinations with standard error of the mean.

Discussion

Evidence has been presented for the glycoprotein nature of stem bromelain. This is the first example of a plant proteinase being a glycoprotein (see, for other examples, Gottschalk, 1966). Although the isolation of a glycopeptide from the protein could be the final proof, as it was in the present experiment, gel filtration techniques have been shown to be most useful in demonstrating the glycoprotein nature of the protein. The techniques involved (1) use of dextran gels with various degrees of cross-linkage, (2) filtration experiments at varying pH of the medium (Figure 1), and (3) comparison of filtration patterns before and after the proteolytic digestion of the protein to be studied (Figure 2). The techniques are simple and have the advantage that one can readily interpret the results quantitatively. The isolation experiment, on the other hand, usually involves a number of steps of procedures and considerable losses of the material at these steps are unavoidable; consequently one could interpret the result only in a less quantitative way.

The results of the present experiments on the isolation and analysis of glycopeptide from stem bromelain indicate the fact that the original protein contains carbohydrates as one unit. Major factors of the evidence are (1) only one kind of octaglycopeptide was obtained with apparent homogeneity and reasonably high yield; (2) the isolated glycopeptide contains four glucosamine

residues per mole of peptide while the original protein has four glucosamine residues per mole of protein; (3) the neutral sugars of the isolated glycopeptide are mannose, xylose, and fucose, and these are all of the kinds of sugars found in the original protein;[3] and (4) the composition of neutral sugars found for the glycopeptide corresponds to 854 g/mole of peptide which is in fairly good agreement with an estimated value of 2.1% carbohydrate for the original protein of molecular weight 33,000 (Murachi, 1964; Murachi *et al.*, 1964). It follows that stem bromelain as a glycoprotein resembles ovalbumin (*e.g.*, Cunningham *et al.*, 1957; Johansen *et al.*, 1961; Yamashina and Makino, 1962) and Taka-amylase A (Anai *et al.*, 1966), and differs from plasma α_1-acid glycoprotein and submaxillary gland glycoprotein (Gottschalk and Graham, 1966). The glycoproteins of the former group contain the carbohydrate moiety as one unit chain per mole, whereas those of the latter group carry a number of heterooligosaccharide chains attached to one protein molecule.

Although in the present investigation no determination was made on the content of amide groups of the glycopeptide, it was later found from a study of the amino acid sequence that three β- and one γ-carboxyl groups are in amide linkages, while only one glutamic acid residue is present with a free γ-carboxyl group (Takahashi *et al.*, 1967). A separate experiment has also demonstrated that all the amino groups of glucosamine residues are acetylated (Takahashi *et al.*, 1967). These facts are consistent with the slightly acidic nature of the octaglycopeptide as was revealed by paper electrophoresis at different pH values (Figure 3). The observed mobilities are also compatible with the finding that the glycopeptide contains no such acidic sugars as sialic acid and glucuronic acid.

It is interesting to note that the use of a larger amount of *S. griseus* proteinase together with *B. subtilis* proteinase resulted in the same sort of glycopeptide as was obtained by the routine procedure employing the *S. griseus* proteinase alone. This may reflect a strong resistance against proteolytic enzymes of the octapeptide around the carbohydrate moiety. Attempts to digest further the isolated glycopeptide by various proteinases have proved unsuccessful (Takahashi *et al.*, 1965). In addition to this glycopeptide, small amounts of more acidic glycopeptides also appeared in some earlier experiments in which more drastic treatment of the material was employed such as heating of the stem bromelain solution at pH 2.0 at 100° for 1 hr before proteolytic digestion. Two of these minor products were separated by paper electrophoresis at pH 3.5 and each was found to have the same amino acid and carbohydrate composition, except for partial loss of fucose, as had the major product described above (Takahashi *et al.*, 1965). These findings indicate that the occurrence of the minor products does not imply polymorphism of the original protein, but it may represent a single origin of these glycopeptides which, in the course of isolation, become different in content of the amide groups, in *N*-acetyl groups, or in both.

[3] It was reported by Murachi *et al.* (1964) that the partially purified preparation of stem bromelain, *i.e.*, fraction 4 in the method employed, contained mannose, arabinose, and xylose, while further purification to the stage of fraction 6 eliminated arabinose-containing polysaccharides. The presence of fucose in the preparation of fraction 6 was later demonstrated (Takahashi *et al.*, 1965).

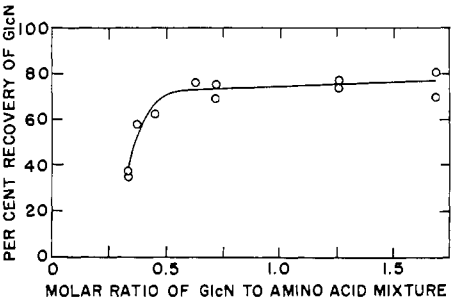

FIGURE 4: The recovery of *N*-acetylglucosamine by acid hydrolysis in the presence of amino acids and neutral sugars. *N*-Acetylglucosamine (0.5 μmole) was hydrolyzed in 100 μl of 5.7 N HCl at 100° for 20 hr with varying amount of amino acids in the presence of 0.625 μmole of neutral sugars. Neutral sugars were composed of mannose, xylose, and fucose, with a molar ratio of 3:1:1. The amino acids were used as a mixture of aspartic acid, glutamic acid, and serine with a molar ratio of 3:2:3. The hydrolysate was evaporated *in vacuo* to dryness and analyzed for glucosamine content on a Technicon Auto-Analyzer.

Acknowledgment

We wish to thank Miss Mihoko Nawa for her technical assistance.

References

Akabori, S., Ikenaka, T., and Hagihara, B. (1954), *J. Biochem. (Tokyo) 41*, 577.

Anai, M., Ikenaka, T., and Matsushima, Y. (1966), *J. Biochem. (Tokyo) 59*, 57.

Ashwell, G. (1957), *Methods Enzymol. 3*, 73.

Cunningham, L. W., Nvenke, B. J., and Nvenke, R. H. (1957), *Biochim. Biophys. Acta 26*, 660.

Gottschalk, A. (1966), *in* Glycoproteins, Gottschalk, A., Ed., Amsterdam, Elsevier, p 1.

Gottschalk, A., and Graham, E. R. B. (1966), *Proteins 4*, 95.

Hamilton, P. B. (1963), *Anal. Chem. 35*, 2055.

Hanafusa, H., Ikenaka, T., and Akabori, S. (1955), *J. Biochem. (Tokyo) 42*, 55.

Johansen, P. G., Marshall, R. D., and Neuberger, A. (1961), *Biochem. J. 78*, 518.

Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunology, 2nd ed, C. C Thomas, Springfield, Ill., p 511.

Kekwick, R. A., and Cannan, R. K. (1936), *Biochem. J. 30*, 227.
Murachi, T. (1964), *Biochemistry 3*, 932.
Murachi, T., Inagami, T., and Yasui, M. (1965), *Biochemistry 4*, 2815.
Murachi, T., Yasui, M., and Yasuda, Y. (1964), *Biochemistry 3*, 48.
Neuberger, A. (1938), *Biochem. J. 32*, 1435.
Ota, S., Moore, S., and Stein, W. H. (1964), *Biochemistry 3*, 180.
Sanger, F., and Thompson, E. O. P. (1953), *Biochem. J. 53*, 353.
Svennerholm, L. (1957a), *Arkiv Kemi 10*, 577.
Svennerholm, L. (1957b), *Chem. Abstr. 51*, 9400*h*.
Takahashi, N., Sato, M., and Murachi, T. (1965), Abstracts, 16th Symposium on Protein Structure, Fukuoka, Japan, p 22.
Takahashi, N., Yasuda, Y., Kuzuya, M., Suzuki, A., and Murachi, T. (1967), Abstracts, 7th International Congress of Biochemistry, Tokyo, Japan, p 706.
Winzler, R. L. (1955), *Methods Biochem. Anal. 2*, 279.
Yamashina, I., and Makino, M. (1962), *J. Biochem. (Tokyo) 51*, 359.
Yemm, E. W., and Cocking, E. C. (1955), *Analyst 80*, 209.

Intermediate Stages in the Thermally Induced Transconformation Reactions of Bovine Pancreatic Ribonuclease A*

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ABSTRACT: The nature of the thermally induced unfolding reactions of bovine pancreatic ribonuclease has been studied using proteolytic enzymes as conformational probes. The initial rates of proteolysis, as catalyzed by aminopeptidase, trypsin, chymotrypsin, and carboxypeptidase, were studied both with ribonuclease and performic acid oxidized ribonuclease as substrates. The latter protein served as a control substrate so as to take account of the effect of temperature on the proteases.

The temperature range studied was between 30 and 60° at pH 8 in 0.1 M NaCl. The results of these studies indicate that the conformation of ribonuclease changes in a gradual manner with increasing temperature and that some central regions become disordered at lower temperatures than does either end of the molecule. Concomitant optical rotatory dispersion studies also indicate that the thermally induced conformation changes of ribonuclease take place in a gradual rather than a two-step process.

The reversible thermal unfolding of bovine pancreatic ribonuclease (RNase) has been the object of much study since its discovery by Harrington and Schellman (1956). This process has often been treated as the reflection of a simple equilibrium between a native and a denatured state, with no thermodynamically stable intermediates (Harrington and Schellman, 1956; Foss and Schellman, 1959; Kalnitsky and Resnik, 1959; Hermans and Scheraga, 1961; Brandts, 1965; Lumry *et al.*, 1966). However, it has been observed by some that much of the data is not consistent with this simple scheme and that intermediate stages of unfolding probably exist (Foss, 1961; Holcomb and Van Holde, 1962; Scott and Scheraga, 1963; Poland and Scheraga, 1965; Beck *et al.*, 1965; Ginsburg and Carroll, 1965). It has been the object of this investigation to demonstrate in a more clear-cut manner than heretofore that the thermal unfolding of RNase is a gradual or multistate rather than an abrupt or two-state process and to learn something of the pathway which the molecule follows as its conformation is changed from the native or low-temperature form, to that which exists at high temperatures. The approach used has been to study the kinetics of the proteolysis of ribonuclease, with aminopeptidase, chymotrypsin, trypsin, and carboxypeptidase, as a function of temperature throughout the transition region. The specificity of these enzymes provides the means of following changes in RNase conformation at the amino terminus, in a central region or regions and at the carboxyl terminus, respectively. To take account of changes in the properties of these proteases with temperature, all experiments were performed both with RNase and with performic acid oxidized RNase (Ribox)[1] as substrates. The latter

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[1] Abbreviation used: Ribox, performic acid oxidized RNase.