Amino Acid Composition of Stem Bromelain*

TAKASHI MURACHI

From the Department of Biochemistry, Nagoya City University School of Medicine, Nagoya, Japan

Received February 13, 1964

The amino acid composition of pineapple-stem bromelain has been determined for the purified enzyme prepared according to the method of T. Murachi, M. Yasui, and Y. Yasuda (1964), *Biochemistry 3*, 48. The data are expressed as number of amino acid residues per molecule of mw 33,000. From the nearest integral number of residues a molecular weight of 32,500 was calculated, including 285 residues of amino acids and four of hexosamine, with a nitrogen content of 15.9%. The number of basic amino acid residues exceeds that of acidic groups, which is in accord with the earlier data on titration and isoelectric point. The presence of four methionine residues per molecule is a distinctive feature of stem bromelain as compared to papain, which contains no methionine. The amino acid composition found also has been compared with the data of an independent analysis recently reported by S. Ota, S. Moore, and W. H. Stein (1964), *Biochemistry 3*, 180. Good agreement is noted in general between these two sets of analyses, with a few exceptions, i.e., discrepancies in the contents of amide ammonia, histidine, methionine, and half-cystine. These differences may depend upon variations in the sample of stem bromelain.

The recent development in this laboratory of methods for purifying stem bromelain from crude commercial product (Murachi *et al.*, 1964) has made available sufficient material for a correlative study of its physical, chemical, and enzymatic properties. The molecular weight of the enzyme protein has been calculated to be 33,000 from physical measurements (Murachi *et al.*, 1964), and the kinetics of bromelain-catalyzed hydrolysis of synthetic substrates have been studied (Inagami and Murachi, 1963). The present paper describes the data of amino acid analyses performed with the same preparation by means of the technique of ion-exchange chromatography (Spackman *et al.*, 1958).

Practically no information had been available as to the amino acid composition of bromelain before the present study was undertaken.[1] More recently Ota *et al.* (1964)[2] have reported amino acid composition of stem bromelain obtained by a method slightly different from the one developed in this laboratory. Their report also included data on fruit bromelain. It is interesting to note that the results of analyses on the stem enzyme, obtained concurrently but independently of each other in these two laboratories, agree well with only a few exceptions.

Experimental Procedure

Material.—Stem bromelain was isolated from commercial product, "Bromelain" (lots 181 and 182) from the Hawaiian Pineapple Co., Honolulu, Hawaii,[3] according to the method described in a previous communication (Murachi *et al.*, 1964). Fraction 6 was lyophilized and used for analysis.

Hydrolysis and Amino Acid Analysis.—Lyophilized samples were weighed (4–10 mg) into ignition tubes and the acid hydrolysis was carried out with three-times-glass-distilled 5.7 N HCl in evacuated sealed tubes for 20, 40, and 70 hours at 110° ± 0.5° (Crestfield *et al.*, 1963). The hydrolysates were rapidly evaporated to dryness under reduced pressure at 40–42°, and then dissolved in 10 ml of 0.2 N sodium citrate buffer, *p*H 2.2. Aliquots of this solution were analyzed in a Hitachi Model KLA-2 amino acid analyzer using a 150-cm column for acidic and neutral amino acids and a 15-cm column for basic amino acids. The procedure of analysis followed with this instrument is essentially the same as that of Spackman *et al.* (1958). Duplicate analyses were performed on each hydrolysate. Calibration runs with a Beckman-Spinco Type 1 standard amino acid mixture were made immediately before and after the analyses. In calculating the amino acid composition correction was made for moisture content of the lyophilized samples (Murachi *et al.*, 1964).

Determination of Cystine plus Cysteine as Cysteic Acid.—HBr was used to destroy excess performic acid prior to hydrolysis of the oxidized sample (Moore, 1963). The quantitation of cysteic acid in the analysis data was related to the recoveries of alanine and glycine.

Determination of Hexosamine.—Acid hydrolysis for the determination of hexosamine content was carried out in 5.7 N HCl at 110° ± 0.5° for 5, 12, and 22 hours. Aliquots of the hydrolysates were analyzed in an amino acid analyzer using a 15-cm column under conditions identical with those employed for the analysis of basic amino acids. A single, symmetrical peak for hexosamine emerged immediately before that for tryptophan. Duplicate runs for calibration were performed with a standard amino acid mixture to which a known amount of D-glucosamine hydrochloride had been added. A better resolution was obtained when tryptophan content was low, which in fact was the case with the acid hydrolysate of stem bromelain. Since the technique gives only incomplete resolution of glucosamine from galactosamine (*cf.* Liu and Gotschlich, 1963), it was uncertain whether the hexosamine found in the chromatogram was glucosamine, galactosamine, or a mixture of both. While no further identification experiment was carried out in this laboratory, Ota *et al.* (1964) have identified the material as glucosamine by using a 150-cm column for analysis.

Determination of Tryptophan.—The tryptophan content of the sample was determined by the method of Spies and Chambers (1948).

* 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] The results of a preliminary amino acid analysis on a partially purified stem bromelain were reported at the 34th annual meeting of the Japanese Biochemical Society, Osaka, November, 1961, and at the 14th Symposium on Enzyme Chemistry, Fukuoka, May, 1962.

[2] We are indebted to Dr. Stanford Moore for his generosity in permitting us to see his manuscript prior to publication.

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

TABLE I
AMINO ACID COMPOSITION OF STEM BROMELAIN

Amino Acid	Number Residues per Molecule[a] Found Hours of Hydrolysis 20	40	70	Average or Extrapolated Value[b]	Expressed as Nearest Integer	Ota *et al.* (1964), Nearest Integral Number Residues/ Molecule[c]
Lysine	19.6		19.8	19.7	20	23
Histidine	1.33		1.35	1.34	1	2
Ammonia	26.9		31.2	25.2	25[d]	42[d]
Arginine	10.0		9.75	9.89	10	12
Aspartic acid	26.5	26.2	25.8	26.7	27	29
Threonine	11.5	11.3	10.3	11.9	12	14
Serine	22.5	20.7	17.1	24.2	24	28
Glutamic acid	20.1	20.4	19.8	20.1	20	23
Proline	12.9	13.1	13.8	13.3	13	14
Glycine	28.8	29.0	28.2	28.7	29	35
Alanine	30.1	30.3	29.5	30.0	30	35
Half-cystine	10.5	10.2	(7.05)	10.9[e]	11	10
Valine	18.6	19.1	18.7	19.1[f]	19	22
Methionine	3.60	(0.92)	3.19	3.81	4	5
Isoleucine	18.5	19.6	19.3	19.6[f]	20	21
Leucine	8.87	9.19	8.92	8.99	9	10
Tyrosine	18.4	17.3	17.5	18.7	19	21
Phenylalanine	8.77	8.39	8.08	8.98	9	9
Tryptophan				8.01[g]	8	8
Total					285	321
Hexosamine[h]	(2.91)	3.27	3.04	3.55	4	6[i]

[a] Calculated number residues per molecule of mw 33,000 (Murachi *et al.*, 1964). [b] The values in parentheses are omitted from average or extrapolation. [c] Calculated number residues per molecule of mw 35,730. [d] Not included in the total. [e] A value of 10.8 was obtained in a separate experiment with a performic acid–oxidized sample. [f] Value of 40-hour hydrolysate (see text). [g] Determined by the method of Spies and Chambers (1948). [h] Hours of hydrolysis for the determination of hexosamine content were, from left to right, 5, 12, and 22 hours. [i] Glucosamine.

RESULTS

Amino Acid Composition.—The results of the amino acid analyses are given in Table I. The values are expressed as number of residues per molecule, assuming a molecular weight of 33,000 (Murachi *et al.*, 1964). The values for 20-, 40-, and 70-hour hydrolysis are averages of duplicate determinations. A separate determination of cystine plus cysteine as cysteic acid gave a value of 10.8 residues per molecule, which is in good agreement with the extrapolated value, 10.9, shown in Table I. The values for valine and isoleucine were found to increase with time during the initial 40 hours of hydrolysis. Such an increase has been also observed by Bargetzi *et al.* (1963) with carboxypeptidase, and in that case the data were corrected by making an extrapolation to a longer time of hydrolysis. With valine and isoleucine in the present study, however, the values of 40-hour hydrolysates were used as such for further calculation, since these seemed to best represent the highest yields, a slight decrease being encountered by prolonged hydrolysis. The values for ammonia increased with time and a linear extrapolation to zero time of hydrolysis was made for approximation (Smith *et al.*, 1954).

The hexosamine content showed a minimal value at 5-hour hydrolysis and this value was omitted from extrapolation because of a possibility of incomplete hydrolysis of the material. A linear extrapolation of the other two values yielded a value of 3.55 residues hexosamine at zero time of hydrolysis, which, in turn, means that a 14% destruction of hexosamine had occurred during 22 hours of hydrolysis. The latter figure is far smaller than a value of 66% destruction of glucosamine which was reported to occur after 20 hours of acid hydrolysis of ribonuclease B (Plummer and Hirs, 1963) and employed by Ota *et al.* (1964) for their data of stem bromelain. On the other hand, the reported time-course of the decomposition of glucosamine in pneumococcal-C-polysaccharide-containing peptides during hydrolysis at 110° in 6 N HCl (Liu and Gotschlich, 1963) corresponds to a 21% destruction of glucosamine after 22 hours of hydrolysis. If this latter factor be applicable to the present result, the calculated hexosamine content at time zero would be 3.85 residues per molecule, which again is close to 4 residues per molecule shown in Table I.

The nitrogen content of stem bromelain calculated on the basis of either found number residues (column 5, Table I) or integral number residues (column 6, Table I) is 15.83% or 15.88%, respectively, both assuming a molecular weight of 33,000.

Molecular Weight.—Table I shows that one molecule of stem bromelain contains 285 amino acid residues and four hexosamine residues. The corresponding molecular weight, including four *N*-acetylhexosamine residues[4] and 25 amide groups, would be 31,839 or close to 31,800. This value does not include the carbohydrate component other than hexosamine. Since stem bromelain appears to be a glycoprotein, containing 2.1% carbohydrate as determined by the orcinol-H_2SO_4 method (Murachi *et al.*, 1964), inclusion of the carbohydrate component increases the molecular weight by approximately 700. The resultant molecular weight amounts to 32,500, which is in good agreement with a value of 33,200 obtained by sedimentation and diffusion methods (Murachi *et al.*, 1964). A molecular weight of 32,100 was computed from sedimentation constant and intrinsic viscosity, and a value of 33,500 was obtained by the Archibald method of sedimentation

[4] It is assumed that all the hexosamine residues occurred as *N*-acetylated form in the original molecule, although no experimental proof has been made.

analysis (Murachi *et al.*, 1964). Ota *et al.* (1964) have used a value of mw 35,730 in calculating amino acid composition, because this figure best fitted their data, assuming two residues of histidine and five of methionine per molecule.

Discussion

Ota *et al.* (1964) have reported the amino acid composition of stem bromelain. Their data are listed in Table I in comparison with the results of the present study. In general, the values of number residues per molecule reported by Ota *et al.* (1964) are proportionately higher than those obtained in the present study. This is readily explicable in terms of difference in molecular weights assumed: 35,730 by Ota *et al.*, and 33,000 in the present study. It is apparent, therefore, that when mole ratios of amino acid residues in both data are compared a marked similarity can be found. Notable exceptions are the values for amide ammonia, histidine, and methionine, which are higher in the results of Ota *et al.* (1964), and the value for half-cystine, which is higher in the present result. These discrepancies may depend upon differences in the samples of purified bromelain used, since different lots of the crude enzyme were employed as starting materials and the procedures of purification were different. The hazard of autodigestion is always present, possibly even at the stage of manufacturing the crude enzyme.

In view of a relatively large deviation of the experimetal value for histidine from the integral number (Table I), further work would be required to determine whether stem bromelain contains one histidine residue or two per molecule. It is difficult to determine the histidine, since it is the amino acid present in the smallest amount in the enzyme. The experimental value of 1.34 histidine residues per molecule, taken at face value, may indicate some heterogeneity of the preparation. The enzyme material used for the amino acid analysis had been found to be essentially homogeneous by several physicochemical criteria (Murachi *et al.*, 1964). However, strict homogeneity of the preparation could hardly be claimed in the present work or in the work by Ota *et al.* (1964).

Although the exact number of amide groups should be determined in a separate experiment in the future, a tentative calculation of the number of carboxyl groups could be made, assuming a value of 25 ammonia residues per molecule (Table I) to represent the number of residues of asparagine plus glutamine. It is also assumed that stem bromelain contains a single carboxyl terminus, since a single principal amino terminal residue, valine, was found to exist (Ota *et al.*, 1964).[5] Such calculation gives a value of twenty-three carboxyl groups per molecule, which is in accord with the titration data of stem bromelain (Murachi *et al.*, 1964), i.e., approximately twenty-five acidic residues per mole were titrated between *p*H 1.6 and 6.0. This value, as compared to the number of basic groups (Table I), is also consistent with the fact that stem bromelain is a basic protein with an isoelectric point of *p*H 9.55 (Murachi *et al.*, 1964).

Stem bromelain contains one "free" sulfhydryl group per molecule as determined by the spectrophotometric titration with *p*-mercuribenzoate (Boyer, 1954).[6] Therefore, from the data shown in Table I, it can be supposed that stem bromelain has one cysteinyl residue and five disulfide linkages per molecule.

From a comparison of the amino acid composition of stem bromelain to that of papain (Smith and Kimmel, 1960), it is evident that the most distinctive difference between these two sulfhydryl proteinases is in the content of methionine. Thus stem bromelain contains four residues of methionine per molecule, whereas no methionine is present in papain. The abundance of basic amino acid residues is more apparent in stem bromelain than in papain. This is in accord with the finding that stem bromelain has an isoelectric point higher than that for papain, *p*H 8.75 (Smith and Kimmel, 1960).

[5] We have also observed that dinitrophenylvaline was recovered as the principal dinitrophenylamino acid after acid hydrolysis of the dinitrophenylated sample of partially purified stem bromelain (Murachi, T., and Yasui, M., 34th annual meeting, Japanese Biochemical Society, Osaka, November, 1961).

Acknowledgment

The author wishes to express his appreciation to Mrs. Keiko Ikeda, Department of Public Health, Faculty of Medicine, Kyoto University, for her operation of the amino acid analyzer.

References

Bargetzi, J.-P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Inagami, T., and Murachi, T. (1963), *Biochemistry 2*, 1439.

Liu, T.-Y., and Gotschlich, E. C. (1963), *J. Biol. Chem. 238*, 1928.

Moore, S. (1963), *J. Biol. Chem. 238*, 235.

Murachi, T., Yasui, M., and Yasuda, Y. (1964), *Biochemistry 3*, 48.

Ota, S., Moore, S., and Stein, W. H. (1964), *Biochemistry 3*, 180.

Plummer, T. H., Jr., and Hirs, C. H. W. (1963), *J. Biol. Chem. 238*, 1396.

Smith, E. L., and Kimmel, J. R. (1960), *Enzymes 4*, 133.

Smith, E. L., Stockell, A., and Kimmel, J. R. (1954), *J. Biol. Chem. 207*, 551.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spies, J. R., and Chambers, D. C. (1948), *Anal. Chem. 20*, 30.

[6] Murachi, T., unpublished observation.