SEQUENCE OF AMINO ACIDS IN THE VICINITY OF THE REACTIVE THIOL GROUP OF STEM BROMELAIN [1]

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Received March 6, 1967

Plant proteases such as papain, ficin, and bromelain have been generally regarded as SH-enzymes in which a cysteine residue plays a key role in catalysis (Smith and Kimmel, 1960; Lowe and Williams, 1965). The amino acid sequence in the vicinity of the active SH group of papain and ficin has been found to be remarkably the same (Light et al., 1964; Wong and Liener, 1964). We now wish to report that this similarity in the structure of the active site of plant proteases may now be extended to include stem bromelain [2].

Experimental and Results

Stem bromelain was purified from commercial bromelain (courtesy of Dr. Ralph Heinicke, Dole Pineapple Co., Honolulu, Hawaii) by the procedure of Murachi et al. (1964) and its homogeneity verified by disc gel electrophoresis (Reisfeld et al., 1962). Maximum activity on casein was obtained in the presence of dithiothreitol (1 mg per mg enzyme) dissolved in 2.5×10^{-3} M EDTA, pH 5.5. The dithiothreitol was removed by dialysis against 1.0×10^{-3} M EDTA which had been deoxygenated by boiling and flushed with nitrogen. The SH content of the enzyme was determined directly with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman,

1 This work was supported by Grant No. GM-4614 from the National Institutes of Health and Grant No. GB-4130 from the National Science Foundation.

2 For the difference in properties between fruit and stem bromelain, consult Ota et al., (1964).

1959) or as CM[1] -cysteine (Brigham et al., 1960) or (1,2-dicarboxyethyl-L-cysteine (Smyth et al., 1964) after treatment with an excess of iodoacetic acid and N-ethyl-maleimide respectively. All of these methods gave a value of 0.8 - 1.0 mole SH per mole of enzyme, assuming bromelain to have a molecular weight of 33,000 (Murachi et al., 1964).

To 45 ml of a solution containing 1.5% bromelain, activated as described above, was added an equal volume of a solution containing 0.3 M KCN and 2.5 x 10^{-3} M EDTA, pH 7.5. Iodoacetic-1-C^{14} acid (0.1 mmole; specific activity 1.2 mC per mmole) was added and the pH maintained at 7.9 for 2 hr at room temperature. The protein was precipitated with 80% ice-cold acetone and washed with cold acetone-1 N HCl (39:1) and finally with ether. Disulfide bonds were reduced with mercaptoethanol in the presence of 6 M guanidine HCl and subsequently aminoethylated with ethylenimine (Raftery and Cole, 1966). Excess reagents were removed by dialysis, and the protein was lyophilized. The AE-cysteine content of this preparation was 6 residues per mole. Since bromelain has been reported to contain 5 disulfide bonds (Murachi et al., 1964), complete reduction and/or aminoethylation had evidently not been achieved under our experimental conditions.

AE-C^{14} M-bromelain was dissolved in 0.1 M Tris buffer containing 2 M urea and 0.1% thiodiglycol, pH 8, and digested with 5% of its weight of 2X crystallized trypsin (Worthington) which had been treated with TPCK in order to eliminate its chymotryptic activity (Kostka and Carpenter, 1964). After 24 hr at room temperature, any insoluble material was removed by centrifugation, and the supernatant, containing 68% of the original radioactivity, was examined by high voltage paper electrophoresis (pH 3.7, 2000 volts, 1-1/2 hr). All of the radioactivity remained at the origin.

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[1] Abbreviations: CM-, S-carboxymethyl-; AE-, S-β-aminoethyl-; TPCK, L-(1-tosylamido-2-phenyl)-ethyl-chloromethyl ketone.

The digestion with TPCK-trypsin was repeated, but this failed to produce any chang in the electrophoretic pattern. A third digestion was carried out with 2X crystallize trypsin which had not been treated with TPCK and which had an activity against N-acetyl-L-tyrosine ethyl ester equivalent to 0.4% chymotrypsin. In this instance the enzyme to substrate ratio was 1:10. The distribution of radioactivity after passage of this digest through Sephadex G-25 is shown in Fig. 1. Paper electrophoresis at pH 6.4 revealed fractions T_1 and T_2 to consist of several radioactive components

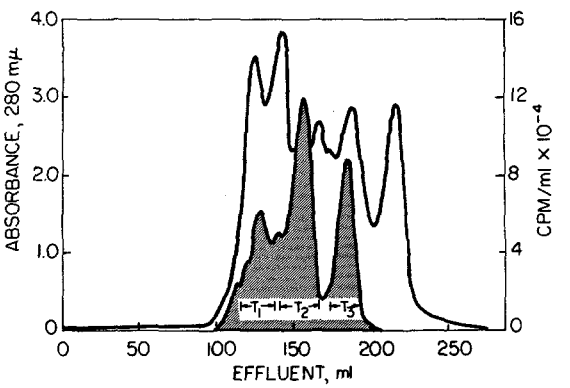

Figure 1 Chromatography of tryptic digest of AE-C^{14} M-bromelain on Sephadex G-25. Column (1.6 x 140 cm) was eluted with 2.5×10^{-3} M $(NH_4)_2CO_3$ containing 0.2% thiodiglycol. 3 ml fractions were collected at a flow rate of 9 ml per hr. Tubes displaying radioactivity denoted by cross-hatching. Solid line denotes absorbance at 280 mμ.

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which were not clearly resolved from each other. Essentially all of the radioactivit of fraction T_3, however, was associated with a single ninhydrin positive zone whic also gave a purple color with Ehrlich's reagent (Easley, 1965), indicative of tryptophan (Fig. 2A). When this zone was eluted with 0.3% mercaptoethanol and run at pH 3.7, it proved to be a pure radioactive peptide (peptide T_3-R, Fig. 2B). This peptide accounted for about 6% of the total radioactivity originally present in C^{14} M-bromelain.

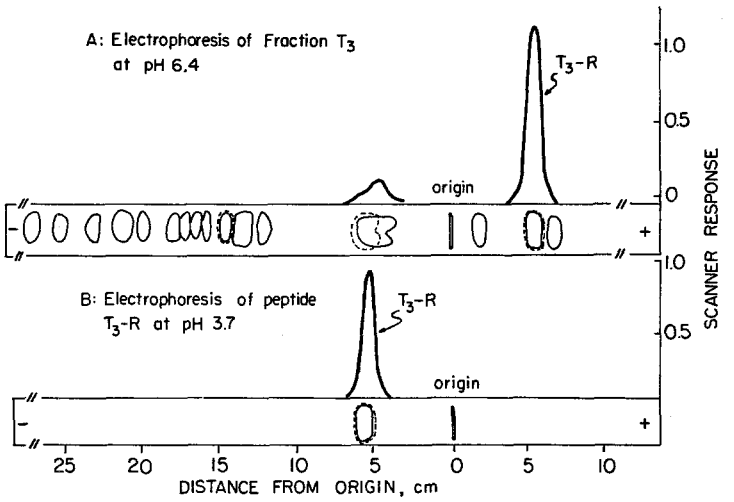

Figure 2. High voltage paper electrophoresis of fraction T_3 at pH 6.4 (A) and peptide T_3-R at pH 3.7 (B). Ninhydrin-positive zones denoted by solid lines. Dotted lines denote zones giving purple color with Ehrlich's reagent. Curves produced on chart of strip-scanner are shown above each strip of paper.

Table I.

Amino Acid composition of peptide T_3-R before and after Edman degradation. Column values which are underlined indicate a significant loss of that particular amino acid.

Amino Acid	Stage			
	0	1	2	3
Glycine	0.88	<u>0.54</u>	0.42	0.36
Alanine	1.03	0.98	<u>0.62</u>	0.52
CM-cysteine*	1.00	1.00	0.90	<u>0.76</u>

Amino acid analysis (Spackman et al., 1958) of an acid hydrolysate (6 N HCl, 22 hr, 110°) of peptide T_3-R, together with tryptophan analysis by the method of Spies and Chambers (1949), gave a molar ratio of CM-cysteine:glycine:alanine: tryptophan of 1.00:0.88:1.03:0.80. Three successive Edman degradations

(Konigsberg and Hill, 1962) yielded the data shown in Table I which establish the sequence as Gly-Ala-CM-Cys. The presence of tryptophan at the C-terminal end of the peptide was confirmed by the fact that only tryptophan (0.35 mole per mole of peptide) was released after 2 hr digestion with carboxypeptidase A (enzyme: substrate, 1:33, pH 8.5, 37°).

Discussion

While this work was in progress, Miake et al. (1966) reported the partial sequence of a peptide containing 21 amino acids isolated from a peptic digest of stem bromelain in which the SH group had been labeled with N-(4-dimethylamino-3,5-dinitrophenyl)-maleimide. The only sequence found to contain the active cysteine residue was Gly-Ala-Cys, which is in agreement with our work. From the partial sequence which they give for the remainder of their peptide, it can be deduced that the only way in which peptide T_3-R could have been produced in our experiment was by the tryptic cleavage of a peptide bond between AE-cysteine and glycine.

The occurrence of tryptophan at the carboxyl end of peptide T_3-R is most likely the result of the residual chymotrypsin activity of the trypsin preparation which had not been treated with TPCK. There is ample precedence for the fact that peptide cleavages characteristic of chymotrypsin are frequently noted with crystalline preparations of trypsin (Kostka and Carpenter, 1964; Travis and Liener, 1965; Heller and Smith, 1966). This would not be unexpected in the experiment reported here since the chymotrypsin content of the untreated trypsin was appreciable (0.4% and the latter was employed at a rather high ratio of enzyme to substrate (1:10).

The sequence of amino acids surrounding the reactive thiol group (Cys*) of four plant proteases is now known:

Papain:	Cys-Gly-Ser-Cys*-Trp	(Light et al., 1964)
Ficin:	Cys-Gly-Ser-Cys*	(Wong and Liener, 1964)
Stem bromelain:	Cys-Gly-Ala-Cys*-Trp	(This work)
Chymopapain B:	Ser-Gly-Glu-Cys*-Tyr	(Tsunoda and Yasunobu, 1966)

It is apparent that the sequence of stem bromelain is very similar to that of papain and ficin, the only difference being that in bromelain serine is replaced by alanine in the residue adjacent to cysteine. The reason for the difference in the sequence of chymopapain B from that of the other plant proteases is not known although Tsunoda and Yasunobu (1966) have speculated upon this point. Lowe (1966) has recently pointed out that, allowing for reversal and permitted interchanges of amino acids, the peptide sequence around the cysteine residue of papain and ficin is in fact strikingly similar to the sequence around the active serine residue of the animal proteases. Our results with stem bromelain reinforce the evolutionary and functional significance which this structural similarity no doubt implies.

References

Brigham, M. P., Stein, W. H., and Moore, S., J. Clin. Invest. 39, 1633 (1960).
Easley, C. W., Biochim. Biophys. Acta 107, 386 (1965).
Ellman, G. L., Arch. Biochem. Biophys. 82, 70 (1959).
Heller, J. and Smith, E. L., J. Biol. Chem. 241, 3165 (1966).
Konigsberg, W. and Hill, R. J., J. Biol. Chem. 237, 2547 (1962).
Kostka, V. and Carpenter, F. H., J. Biol. Chem. 239, 1799 (1964).
Light, A., Frater, R., Kimmel, J. R., and Smith, E. L., Proc. Nat. Acad. Sci. U.S. 52, 1276 (1964).
Lowe, G., Nature 212, 1263 (1966).
Lowe, G. and Williams, A., Biochem. J. 96, 189 (1965).
Miake, K., Mizuno, M., and Murachi, T., 18th Symposium of Enzyme Chemistry, Aug. 24-27, 1966, Sapporo, Japan, p. 343.
Murachi, T., Yasui, M., and Yasuda, Y., Biochemistry 3, 48 (1964).
Ota, S., Moore, S., and Stein, W. H., Biochemistry 3, 180 (1964).
Raftery, M. A. and Cole, R. D., J. Biol. Chem. 241, 3457 (1966).
Reisfeld, R. A., Lewis, U. J., and Williams, D. E., Nature 195, 281 (1962).
Smith, E. L. and Kimmel, J. R., in The Enzymes, ed. by Boyer, P. D., Lardy, H., and Myrback, K., Vol. 4, p. 133, Academic Press, N.Y. (1960).
Smyth, D. G., Blumenfeld, O. O., and Konigsberg, W., Biochem. J. 91, 589 (1964).

Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem. 30, 1190 (1958).
Spies, J. R. and Chambers, D. C., Anal. Chem. 21, 1249 (1949).
Travis, J. and Liener, I. E., J. Biol. Chem. 240, 1967 (1965).
Tsunoda, J. N. and Yasunobu, K. T., J. Biol. Chem. 241, 4610 (1966).
Wong, R. C. and Liener, I. E., Biochem. Biophys. Res. Commun. 17, 470 (1964).