a labeling technique might not discern between models A and B, C and F, C and G, E and F, etc. Since no qualitative differences were found between 5- and 180-min digestion or between soluble and solid-phase enzyme digestion, and because of the distinctive membrane asymmetry clearly indicated by the data, we infer that the enzyme probes revealed rather than obscured intrinsic membrane organizational features.

References

Albrink, M. J. (1959), *J. Lipid Res. 1*, 53.

Berg, H. C. (1969), *Biochim. Biophys. Acta 183*, 65.

Cook, G. M. W., and Eylar, E. H. (1965), *Biochim. Biophys. Acta 101*, 57.

Ernster, L., Zetterström, R., and Lindberg, D. (1950), *Acta Chem. Scand. 4*, 942.

Eylar, E. H., Madoff, M. A., Brody, O. V., and Oncley, J. L. (1962), *J. Biol. Chem. 237*, 1992.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10*, 2606.

Furthmayr, H. and Timpl, R. (1970), *Eur. J. Biochem. 15*, 301.

Ibrahim, S. A., and Thompson, R. H. S. (1965), *Biochim. Biophys. Acta 99*, 331.

Ito, A., and Sato, R. (1969), *J. Cell. Biol. 40*, 179.

Leffler, H. H., and McDougald, C. H. (1963), *Am. J. Clin. Pathol. 39*, 311.

Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., and Farr, A. L. (1954), *J. Biol. Chem. 207*, 1.

Marchesi, V. T., Steers, E., Tillack, T. W., and Marchesi, S. L. (1969), *in* Red Cell Membrane, Structure and Function, Jamieson, G. A., and Greenwalt, T. J., Ed., Philadelphia, Pa., J. B. Lippincott Co., p 117.

Mitchell, C. D., and Hanahan, D. J. (1966), *Biochemistry 5*, 51.

Phillips, D. R., and Morrison, M. (1970), *Biochem. Biophys. Res. Commun. 40*, 284.

Pinto da Silva, P., Douglas, S. D., and Branton, D. (1970), *J. Cell Biol. 47*, 159a.

Rega, A. F., Weed, R. I., Reed, C. F., Berg, C. G., and Rothstein, A. (1967), *Biochim. Biophys. Acta 147*, 297.

Rosenberg, S. A., and Guidotti, G. (1969), *in* Red Cell Membrane, Structure and Function, Jamieson, G. A., and Greenwalt, T. J., Ed., Philadelphia, Pa., J. B. Lippincott Co., p 91.

Steck, T. L., Weinstein, R. S., Straus, J. H., and Wallach, D. F. H. (1970), *Science 168*, 255.

Sweeley, C. C., and Dawson, G. (1969), *in* Red Cell Membrane, Structure and Function, Jamieson, G. A., and Greenwalt, T. J., Ed., Philadelphia, Pa., J. B. Lippincott Co., p 172.

Tillack, T. W., Scott, R. E., and Marchesi, V. T. (1970), *J. Cell Biol. 47*, 213a.

Warren, L. (1959), *J. Biol. Chem. 234*, 1971.

Ways, P. O., and Hanahan, D. J. (1964), *J. Lipid Res. 5*, 318.

Weinstein, R. S., and Koo, V. M. (1968), *Proc. Soc. Exp. Biol. Med. 128*, 353.

Winzler, R. J. (1969), *in* Cellular Recognition, Smith, T. R., and Good, R. A., Ed., New York, N. Y., Appleton-Century-Crofts, p 11.

Periodate Oxidation of Carbohydrate Moiety of Stem Bromelain without Much Alteration in Enzymatic Activity*

Yoko Yasuda, Noriko Takahashi, and Takashi Murachi†

ABSTRACT: A possible noninvolvement of the carbohydrate moiety of stem bromelain in the mechanism of catalysis was examined by oxidizing neutral sugar residues of the glycoenzyme with periodate. In 0.1 M sodium metaperiodate stem bromelain consumed 26–27 moles of the oxidant after 3–5 hr at 0° in the dark. The reactive SH group of the enzyme was found to be intact, because this group has been protected with tetrathionate before and during the oxidation with periodate. Total neutral sugars remaining after 1-, 3-, and 5-hr oxidation were 20, 5, and 2%, respectively, while the enzymatic activity on casein was lost only partially, remaining 83, 72, and 64%, respectively. The activity on α-*N*-benzoyl-L-arginine ethyl ester and *N*-benzoylglycine ethyl ester and the milk clotting activity also decreased only to similar extents.

These results suggest that neutral sugars of stem bromelain may not be essential participants in catalysis. The amino acid composition of periodate-oxidized stem bromelain did not show appreciable change from that of the unoxidized enzyme, except that some of methionine residues were oxidized to form methionine sulfoxide. No significant conformational change was detected by the measurements of optical rotatory dispersion and circular dichroism spectra after the periodate oxidation. The true mechanism for the observed partial inactivation of stem bromelain by periodate is still undetermined.

A pineapple thiol protease, stem bromelain, is a glycoenzyme with a molecular weight of 33,000 (Murachi *et al.*, 1964), which contains a single heterooligosaccharide unit per molecule. Being a glycoprotein is one of the marked differences in the structure of stem bromelain and papain (Murachi and Takahashi, 1970). The composition and structure of the carbohydrate moiety were studied using

* From the Department of Biochemistry, Nagoya City University School of Medicine, Nagoya, Japan. *Received February 8, 1971.* This work was supported in part by a grant from the Ministry of Education, Japan. A part of this work was presented at the 8th International Congress of Biochemistry, Sept 3–9, 1970, Interlaken, Switzerland (by N. T. and T. M.).

† To whom to address correspondence.

glycopeptides isolated from proteolytic digests of the parent enzyme (Scocca and Lee, 1969; Yasuda *et al.*, 1970). We proposed a probable structure to be: α-D-Man-(1→2)-α-D-Man-(1→2 or 6)-[α-L-Fuc-(1→6 or 2)]-α-D-Man-(β-D-Xyl)-β-D-GlcNAc-(1→3 or 4)-β-D-GlcNAc-(1→β-NH_2-N of Asn)-peptide (Yasuda *et al.*, 1970). We were then interested in examining functions of this carbohydrate moiety in stem bromelain.

The biological significance of carbohydrate moiety of a glycoprotein has been a matter of much speculation. On interaction with other macromolecules some of the nonreducing sugar residues are well known as specific determinants, as in blood-group substances, phytohemagglutinin receptor site of erythrocytes (Kornfeld and Kornfeld, 1970), etc. The speculation was made by Eylar (1965) that in secreted mammalian glycoproteins glycosylation is somehow involved in the mechanism of secretion. Removal of sialic acid from glycoenzymes was shown not to affect enzymatic activities (Margolis and Feigelson, 1964; Svensmark and Heilbronn, 1964; Szewczuk and Connell, 1964; Schoenmakers *et al.*, 1965). A comparison between bovine pancreatic ribonucleases A and B (Plummer and Hirs, 1963, 1964) and modification experiments on chloroperoxidase from *Caldariomyces fumago* (Lee and Hager, 1970) and glucoamylase I from *Aspergillus niger* (Pazur *et al.*, 1970) indicated that carbohydrate moieties did not appear to play any major role in enzymatic activity. Nothing was known, however, as to whether the carbohydrate moiety of stem bromelain is involved in the catalytic mechanism.

In the present investigation, attempts were first made to obtain a carbohydrate-free stem bromelain by removing the sugar residues either by means of appropriate glycosidases or by chemical modification. Only the latter approach was found to be successful in yielding a modified but enzymatically active species.

Materials and Methods

Tetrathionate-Blocked Stem Bromelain. Stem bromelain[1] (fraction 6) prepared by the method of Murachi *et al.* (1964) was further purified by gel filtration on Sephadex G-50 in 0.05 M sodium acetate buffer (pH 5.2). After dialysis in 0.05 M Tris-HCl buffer (pH 9.2) for 3 hr, the enzyme was activated with tenfold molar excess of L-cysteine at 0° for 10 min. Sodium tetrathionate (20- to 50-fold molar excess), dissolved in small portion of water, was added to block the activated SH group. The excess reagents were removed by gel filtration on Sephadex G-25. The inactivated enzyme thus obtained had no caseinolytic activity (see below) and no milk clotting activity (see below) even after 20-min incubation, indicating a practically complete blocking. Protein concentration of tetrathionate-blocked stem bromelain was calculated by using molar absorptivity of the untreated stem bromelain, *i.e.*, $\epsilon_{280\ m\mu}$ 6.68 × 10^4 (Murachi *et al.*, 1965), since the absorbance at 280 mμ of *S*-sulfosulfenyl group was negligible.

Reagents. Sodium tetrathionate was purchased from K & K Laboratories. Casein according to Hammarsten was a product of Merck AG. α-*N*-Benzoyl-L-arginine ethyl ester hydrochloride and trifluoroacetic acid were obtained from the Institute for Protein Research, Osaka University. *N*-Benzoylglycine ethyl ester was purchased from Mann Research Laboratories, DTNB[2] from Calbiochem, silicone SFHF emulsion from Toray Co., egg-white lysozyme (six-times crystallized) from Seikagaku Kogyo, and trypsin from Worthington Biochemical Corp. Guanidine hydrochloride was recrystallized three times from methanol. Skim milk powder, Creap, from Morinaga Milk Co. was employed for the milk clotting assay.

Determination of Periodate. Periodate concentration in protein-free solutions was determined by measuring the absorption at 290 mμ (Ikenaka, 1963). The consumption of periodate in the course of oxidation was followed by the microtitrimetric method of Fleury and Lange (1933).

Preparation of Periodate-Oxidized Stem Bromelain. Tetrathionate-blocked stem bromelain (0.4 g) was oxidized in 0.1 M sodium metaperiodate in a total volume of 30 ml. No buffer was used; pH of the reaction mixture was 5.3. Reaction was continued with stirring at 0° in the dark for 1, 3, and 5 hr. The oxidation reaction was stopped by the addition of 6 ml of 50% ethylene glycol and the mixture was allowed to stand for 30 min at room temperature. The oxidized protein was recovered by gel filtration on a 200-ml column of Sephadex G-25 in 0.05 M sodium acetate buffer (pH 5.2) followed by dialysis against water at 5° for 2 hr. Some turbidity occurred in the latter phase of the oxidation, but it disappeared upon the addition of ethylene glycol. Control enzyme preparation was obtained in the same manner without periodate. The molar absorptivity at 280 mμ of the periodate-oxidized stem bromelain was assumed to be identical with that of the untreated stem bromelain, *i.e.*, $\epsilon_{280\ m\mu}$ 6.68 × 10^4, since tyrosine and tryptophan residues were found not to be oxidized under the conditions used (see Table IV).

Enzymatic Activity. Caseinolytic activity was measured at 30° as described by Murachi (1970) in the presence of 0.005 M L-cysteine used as an activator. A linear proportionality was observed between activity and amount of the enzyme in a range from 1 × 10^{-3} to 3 × 10^{-3} μmole of enzyme per assay.

The hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester and *N*-benzoylglycine ethyl ester was measured by using a pH-Stat (Radiometer Model SBR2/SBU1/TTT1) at pH 6.0, according to the method of Inagami and Murachi (1963). The reaction mixture contained 0.005 M L-cysteine, 0.1 M KCl, 0.01–0.04 M substrate, and the enzyme (0.1–0.4 μmole) in a total volume of 10 ml. The reaction was performed at 25° in a jacketed beaker under stirring with a small amount of silicone defoaming emulsion. The titrant was 0.01–0.25 N NaOH. The overall rate of hydrolysis, v_0, was calculated as millimolar substrate decomposed per minute. The concentration of α-*N*-benzoyl-L-arginine ethyl ester was determined by the amount of a standard alkali required to neutralize the acid liberated on complete hydrolysis by trypsin at pH 8.0.

Milk clotting activity was measured at 37° in 5% milk solution in 0.066 M sodium acetate buffer (pH 5.3) with 1 × 10^{-3} to 10 × 10^{-3} μmole of enzyme. The enzyme solution had been preincubated for 10 min at 0° in 0.075 M L-cysteine.

Amino Acid Analysis. Amino acid analysis was performed with a Hitachi Model KLA-3B amino acid analyzer according to the method of Spackman *et al.* (1953). Acid hydrolysis was carried out in an evacuated sealed tube in constant-

[1] We are indebted to Dr. Ralph M. Heinicke, Dole Corp., Honolulu, Hawaii, for a generous supply of crude bromelain preparation (lot UX-11-1).

[2] Abbreviation used is: DTNB, 5,5′-dithiobis(nitrobenzoic acid).

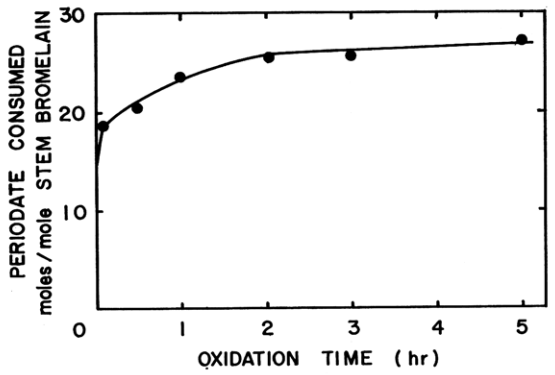

FIGURE 1: Periodate oxidation of stem bromelain in 0.1 M sodium metaperiodate at 0° in the dark. A 200-fold molar excess of the oxidant was used. The active site SH group was previously blocked with $Na_2S_4O_6$.

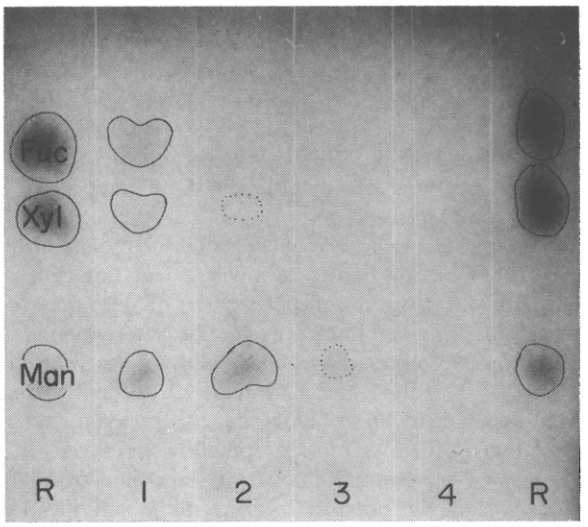

FIGURE 2: Neutral sugars remaining in periodate-oxidized stem bromelain. Fuc, Xyl, and Man stand for fucose, xylose, and mannose, respectively. R is for the standard marker substance. Acid hydrolysates of control (1), 1-hr oxidized (2), 3-hr oxidized (3), and 5-hr oxidized (4) stem bromelain were passed through Amberlite IR-120 (H^+ form) resin, and then developed by descending paper chromatography (acetic acid–ethyl acetate–water, 1:3:3, v/v). The paper was stained with alkaline $AgNO_3$. The amount of the sample used for the control was one-half of that used for the oxidized samples.

boiling HCl (twice redistilled) at 110° for 20 hr. Alkaline hydrolysis was done in an evacuated sealed Pyrex tube with 5 N KOH (N_2 bubbled) at 110° for 20 hr. The analysis was carried out in duplicate or triplicate, and the data obtained were averaged.

Determination of SH Group. Free SH group was determined by the method of Ellman (1959). After reactivation of blocked SH group of the enzyme with 10-fold molar excess of L-cysteine at 0° for 10 min, the enzyme solution was applied on a Sephadex G-25 column which had been washed with N_2-bubbled 0.05 M Tris-HCl buffer (pH 8.1). The effluent was collected in a N_2 atmosphere. To a portion of the effluent that contained protein was immediately added 0.01 M DTNB, the mixture was allowed to stand for 30 min at pH 8.0, and the absorbance at 412 mμ was measured.

Spectrophotometric Determination of Tryptophan and Tyrosine Residues. Tryptophan was determined by measuring the absorption at 280 and 288 mμ in 6 M guanidine hydrochloride (Edelhoch, 1967). The content of tyrosine was determined from the alkaline difference spectra at 295 and 300 mμ (Edelhoch, 1967), and at 244 mμ (Tachibana and Murachi, 1966). Egg-white lysozyme was used as a reference substance.

Determination of Sugars. The sample protein was hydrolyzed in 2.5 N trifluoroacetic acid at 100° for 8 hr (Yasuda *et al.*, 1970), and the hydrolysate was passed through an Amberlite IR-120 (H^+ form) resin. The unadsorbed neutral sugars were separated by paper chromatography using solvent system, acetic acid–ethyl acetate–water (1:3:3, upper phase, v/v). After elution from the paper, mannose and fucose were determined by the methods of cysteine–H_2SO_4 for hexose and methylpentose, respectively, and xylose by the orcinol–HCl method (Ashwell, 1957). Sugars on the guide strip of the paper chromatogram were located with silver nitrate–acetone reagent. Glucosamine was determined by the method of modified Morgan–Elson reaction (Reissig *et al.*, 1955) in hydrolysate of the sample protein with 2.5 N trifluoroacetic acid at 100° for 20.5 hr.

Optical Rotatory Dispersion and Circular Dichroism. A Jasco Model ORD/UV-5 spectropolarimeter with circular dichroism attachment was used. For optical rotatory dispersion measurement in 350- to 650-mμ region a 5-cm cylindrical cell with quartz windows was used with 0.5–0.6% enzyme solution, a Millipore filtrate. For 220- to 290-mμ region 0.5-cm and 0.2-mm quartz cells were used with 0.015 and 0.3–0.8% enzyme, respectively. For circular dichroism measurements in 240- to 300-mμ region a 0.5-cm quartz cell and for those in 200- to 240-mμ region a 0.2-mm cell were used with 0.14% enzyme. The mean residue rotation, $[m']$, in units of deg, and mean residue ellipticity, $[\theta]$, in units of (deg cm²) per dmole, were calculated in the manner described in our previous communications (Murachi and Yamazaki, 1970; Sakai *et al.*, 1970). Optical rotatory dispersion parameters, a_0 and b_0 (Moffit and Yang, 1956) and λ_c (Yang and Doty, 1957), were also calculated from the data in the visible wavelength region. Egg-white lysozyme was used as a reference substance.

Results

Periodate Consumption of Stem Bromelain. When stem bromelain was oxidized in 0.1 M sodium metaperiodate at 0° in the dark, a rapid consumption of the oxidant occurred. As shown in Figure 1, more than 15 moles of periodate were consumed within 5 min, and after 3–5 hr the consumption reached to a level of 26–27 moles/mole of protein. Earlier experiments using the glycopeptide portion in 0.025 M periodate showed that approximately 8 moles of periodate was required to oxidize all the neutral sugar residues (Yasuda *et al.*, 1970).

Sugars of Periodate-Oxidized Stem Bromelain. Figure 2 is a photographic representation of the paper chromatograms for component neutral sugars in stem bromelain before and after the oxidation in 0.1 M periodate. In Table I are summarized the contents of sugars remaining after periodate oxidation. After 1-hr oxidation in 0.1 M periodate fucose and xylose were almost decomposed while only a portion of mannose remained, and after 3- and 5-hr oxidation mannose also disappeared. The content of glucosamine was found to be unchanged. The value for total neutral sugars in Table I was calculated as a sum of the values for individual sugars.

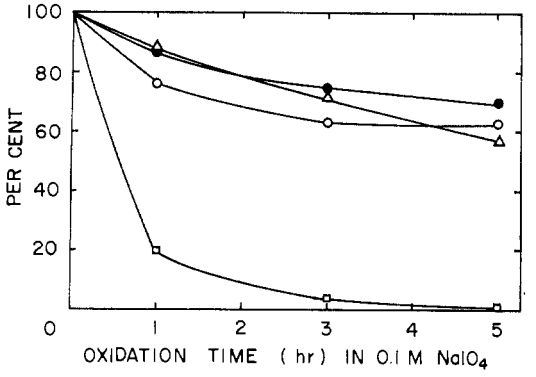

FIGURE 3: Change in content of neutral sugars and alteration in enzymatic activity by periodate oxidation of stem bromelain. Relative rate of hydrolysis of casein (●); relative values of k_0/K_m for α-*N*-benzoyl-L-arginine ethyl ester (○) and for *N*-benzoylglycine ethyl ester (△); neutral sugars remaining (□).

The change in total neutral sugar content with time is illustrated in Figure 3 for comparison to the change in enzymatic activity (see below).

Alteration in Enzymatic Activity of Periodate-Oxidized Stem Bromelain. The relative rates of hydrolyses of casein and synthetic substrates were compared between the control and oxidized enzymes. The results obtained are shown in Figure 3. The caseinolytic activity is expressed in per cent, taking the specific activity of the control enzyme preparation as 100%. The control enzyme did not show any loss of its activity during 5-hr incubation without periodate at 0° in 0.1 M sodium acetate buffer at pH 5.0. For synthetic substrates relative $k_0/K_{m,app}$ values, in per cent of the control, are plotted. Milk clotting times observed were in a range from 16 to 100 sec. Although milk clotting activity as expressed in reciprocals of clotting time was known not to be linearly proportional to the enzyme concentration, the calculation for the present experiments gave values of 60, 60, and 50% activity remaining after 1-, 3-, and 5-hr oxidation, respectively. Thus in all cases partial inactivation was observed.

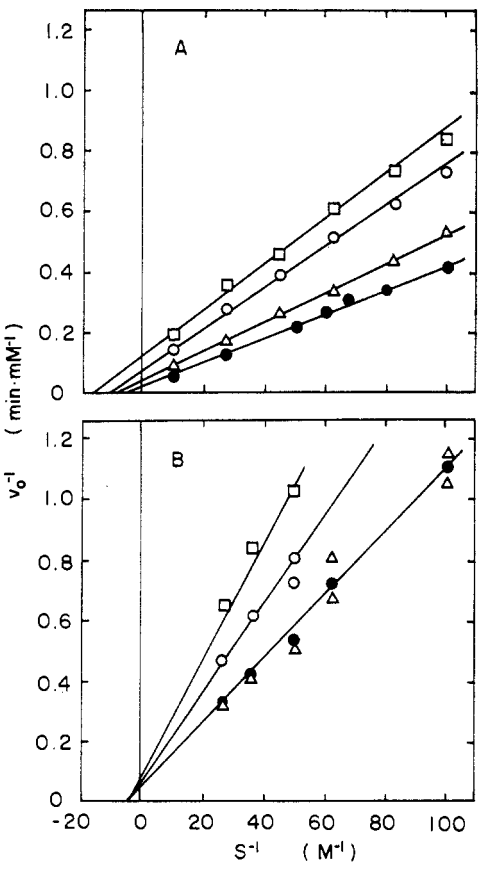

FIGURE 4: Lineweaver–Burk plot for periodate-oxidized stem bromelain. Substrates are α-*N*-benzoyl-L-arginine ethyl ester in A, and *N*-benzoylglycine ethyl ester in B. Control enzyme (●); 1-hr oxidized enzyme (△); 3-hr oxidized enzyme (○); 5-hr oxidized enzyme (□). v_0, millimolar substrate hydrolyzed per minute; *S*, molar concentration of substrate.

Change in Kinetic Parameters. To examine the nature of partial inactivation of the periodate-oxidized enzyme preparation kinetic experiments were made with α-*N*-benzoyl-L-arginine ethyl ester and *N*-benzoylglycine ethyl ester as substrates. Lineweaver–Burk plots are shown in Figure 4. Overall rate constant, k_0, and apparent Michaelis constant,

TABLE I: Determination of Sugars in Periodate-Oxidized Stem Bromelain.

Enzyme Preparation	Moles per Mole of Protein				
	Glucosamine[a]	Neutral Sugar[b]			
		Mannose	Fucose	Xylose	Total (%)
Control	1.82	2.44	1.34	0.98	4.76 (100)
1-hr oxidation	1.95	0.62	0.10	0.23	0.95 (20)
3-hr oxidation	1.98	0.22			0.22 (5)
5-hr oxidation	2.02	<0.1			<0.1 (<2)

[a] Glucosamine was determined by the method of modified Morgan–Elson reaction (Reissig *et al.*, 1955) in hydrolysate of sample protein with 2.5 N trifluoroacetic acid at 100° for 20.5 hr. [b] Neutral sugar was separated by paper chromatography (see Figure 2). After elution from the paper, mannose and fucose were determined by the method of cysteine–H_2SO_4 for hexose and methylpentose, respectively, and xylose by the orcinol–HCl method (Ashwell, 1957). The value for total is a sum of the values for individual sugars; per cent in parentheses.

TABLE II: Kinetic Parameters in Hydrolysis of α-*N*-Benzoyl-L-arginine Ethyl Ester and *N*-Benzoylglycine Ethyl Ester Catalyzed by Periodate-Oxidized Stem Bromelain, at pH 6.0, 25°.

Substrate	Enzyme Preparation	k_0 (min^{-1})	$K_{m,app}$ (M)
α-*N*-Benzoyl-L-arginine ethyl ester	Control	44.4	0.20
	1-hr oxidation	23.5	0.14
	3-hr oxidation	12.4	0.09
	5-hr oxidation	8.4	0.06
N-Benzoylglycine ethyl ester	Control	23.3	0.21
	1-hr oxidation	20.4	0.21
	3-hr oxidation	18.5	0.22
	5-hr oxidation	13.6	0.25

TABLE III: Amino Acid Composition of Periodate-Oxidized Stem Bromelain.[a]

Amino Acid	Enzyme Preparation							
	Control		1-hr Oxidation		3-hr Oxidation		5-hr Oxidation	
	Acid Hydrolysis	Alkaline Hydrolysis	Acid Hydrolysis	Alkaline Hydrolysis	Acid Hydrolysis	Alkaline Hydrolysis	Acid Hydrolysis	Alkaline Hydrolysis
Methionine sulfoxide	0	0	0	1.4	0	1.4	0	1.3
Aspartic acid	27	27	27.4	26.3	27.4	28.0	27.3	27.6
Threonine	12		13.3		12.6		12.6	
Serine	24		24.9		23.4		24.2	
Glutamic acid	20	20	21.1	20.9	21.3	19.9	21.3	20.1
Proline	13	13	12.7	12.4	12.2	12.2	13.3	12.7
Glycine	29	29	30.7	27.4	27.8	28.0	29.1	29.3
Alanine	30		29.0		30.1		29.5	
Half-cystine	11		11.3		11.0		11.3	
Valine	19	19	19.4	20.1	19.6	19.3	19.7	19.7
Methionine	4	4	4.3	2.9	4.0	2.6	4.0	2.3
Isoleucine	20	20	19.8	20.5	20.6	20.0	21.1	20.1
Leucine	9	9	9.5	8.5	9.3	8.8	9.4	8.8
Tyrosine	19	19	19.3	18.2	18.0	18.3	18.0	17.7
Phenylalanine	9	9	8.9	9.0	9.1	9.0	9.0	9.0
Lysine	20	20	19.5	20.1	18.6	19.4	19.6	20.1
Histidine	1		0.94		0.80		0.92	
Arginine	10	0	10.4	0	10.1	0	10.0	0
Tryptophan								
Ornithine		10		9.8		9.9		9.7
Glucosamine	2		2.1		2.1		2.3	

[a] Molar ratio. Correction factors for the individual amino acids were calculated so that the experimental data for the control enzyme fit the values reported previously (Murachi, 1964). The same correction factors were also applied to the data for periodate-oxidized enzymes. With alkaline hydrolysate the data for the following amino acids were omitted, because threonine, serine, half-cystine, and histidine were decomposed partially or completely; alanine gave higher yield, which might be formed from other amino acids, *e.g.*, serine and cystine; tryptophan was found only in trace amounts either before or after periodate oxidation. The value for isoleucine in alkaline hydrolysate included *allo*-isoleucine, which was eluted just before isoleucine. Ornithine of the control preparation was assumed to be 10 moles representing derivation from arginine.

$K_{m,app}$, are calculated from the data in Figure 3, and the values obtained are summarized in Table II. The $K_{m,app}$ value for α-*N*-benzoyl-L-arginine ethyl ester decreased as the oxidation time was prolonged; affinity of the enzyme for this substrate apparently increased. By contrast, in the case of *N*-benzoylglycine ethyl ester practically no change was observed on $K_{m,app}$ values. k_0 values after periodate oxidation decreased in both cases, but the decrease was larger with the arginine substrate than with the glycine substrate. The mechanisms of these changes will be discussed later.

Change in SH Group. Stem bromelain has one reactive SH group per molecule of mol wt 33,000 (Murachi and Yasui, 1965). In the present experiments, this SH group was reversibly blocked with tetrathionate during the periodate oxidation. After removal of the blocking under anaerobic condition, the content of SH group was found to be 0.97 mole/mole in control enzyme; 0.92, 0.90, and 1.00 mole per mole in 1-, 3-, and 5-hr oxidized preparations, respectively. These results indicate that the reactive SH group was not oxidized by periodate, and hence that the partial inactivation observed (Figure 3) did not arise from the partial loss of the active site SH group.

Amino Acid Analysis. In Table III are summarized the results of amino acid analysis of the control and periodate-oxidized stem bromelain. With acid hydrolysis no significant differences could be detected between the oxidized and control enzyme preparations. With alkaline hydrolysis, however, methionine sulfoxide was found in periodate-oxidized preparations, accompanying decrease in content of methionine residue. Methionine sulfone, which could also be present in the alkaline hydrolysate, was not determined because its elution position overlapped with that of aspartic acid in the amino acid analysis system employed. Oxidized derivatives of methionine are known to be able to revert to methionine under the condition of acid hydrolysis of protein (Ray and Koshland, 1962; Floyd *et al.*, 1963).

Spectrophotometric Determination of Tryptophan and Tyrosine. The contents of tryptophan and tyrosine in the control and oxidized enzyme preparations were compared by several different methods. The results obtained are summarized in Table IV. The content of tryptophan did not change before and after the periodate oxidation. The content of tyrosine varied depending on the methods employed, but for each one of the methods, the results obtained showed

TABLE IV: Spectrophotometric Determination of Tryptophan and Tyrosine of Periodate-Oxidized Stem Bromelain.

Amino Acid	Wavelength and Molar Absorptivity Used for Calculation		Moles/Mole of Enzyme Preparation: Control	1-hr Oxidation	3-hr Oxidation	5-hr Oxidation
Tryptophan	$\epsilon_{288m\mu}$[a]	4,815[b]	8.0	7.9	8.4	8.2
	$\epsilon_{280m\mu}$[a]	5,690[b]				
Tryosine	$\Delta\epsilon_{295m\mu}$[c]	2,480[b]	16.9	16.6	16.6	16.6
	$\Delta\epsilon_{300m\mu}$[c]	2,270[b]	16.9	16.6	16.9	17.5
	$\Delta\epsilon_{244m\mu}$[d]	11,000[e]	18.4	18.4	18.3	18.4

[a] At neutral pH in 6 M guanidine hydrochloride the following ϵ values (Edelhoch, 1967) were also used for calculation: 385 and 1280 for tyrosine at 288 and 280 mμ; 70 and 145 for cystine at 288 and 280 mμ, respectively. [b] From Edelhoch (1967). [c] Difference spectra at pH 12 and at pH 5.4 in 6 M guanidine hydrochloride. [d] Difference spectra at pH 13 and at pH 5.4 after 3 hr at 25°. Effect of SH group was not considered. [e] From Tachibana and Murachi (1966).

TABLE V: Optical Rotatory Dispersion and Circular Dichroism Parameters of Periodate-Oxidized Stem Bromelain.

Method	Parameter	Enzyme Preparation: Control	3-hr Oxidation	5-hr Oxidation
Optical rotatory dispersion	$[m']_{233m\mu}$ (deg)	−3450	−3350	−3350
	a_0 (deg)	−225	−230	−260
	b_0 (deg)	−65	−64	−43
	λ_c (mμ)	235	235	224
Circular dichroism	$[\theta]_{222m\mu}$ ((deg cm^2) dmole^{-1})	−5100	−5450	−5200
	$[\theta]_{280m\mu}$ ((deg cm^2) dmole^{-1})	210	210	190

no significant differences between the control and oxidized samples.

Conformational Change of Periodate-Oxidized Stem Bromelain. The optical rotatory dispersion and circular dichroism spectra in 200- to 300-mμ region of periodate-oxidized stem bromelain were indistinguishable from those of the control enzyme previously reported (Murachi and Yamazaki, 1970; Sakai *et al.*, 1970). Representative data of the calculated rotation and ellipticity values are summarized in Table V. The table also contains data of dispersion parameters obtained from measurements in 350- to 650-mμ region. It is apparent from Table V that no significant conformational change can be detected after the periodate oxidation as far as these optical criteria are concerned. The 5-hr-oxidized enzyme gave some distinguishable changes in parameters, particularly in those for the visible wavelength region. These changes may or may not be correlated to the fact that this enzyme preparation was faintly yellowish.

Discussion

The theoretically best approach to investigating the role of carbohydrate moiety in a glycoenzyme must be to obtain a carbohydrate-free enzyme and compare its function with the function of the original glycoenzyme. A complete removal of the carbohydrate moiety with slightest modification of amino acid residues is desirable. It was first hoped that specific glycosidases may cleave off the carbohydrate residues while the peptide part remains intact. In the preceding paper (Yasuda *et al.*, 1970), we reported the usefulness of several glycosidases in elucidating the structure of the carbohydrate moiety of a glycopeptide isolated from stem bromelain. Attempt was then made to allow these glycosidases react directly with stem bromelain. The enzymes tested included α-D-mannosidase, α-L-fucosidase, β-D-xylosidase, and β-D-*N*-acetylglucosaminidase from mollusc. It was found, however, that an incubation as long as at least 2 days at 37° in 1 M NaCl at pH 4.5 was required for the action of these glycosidases, and that such a prolonged incubation in an acidic medium resulted in disappearance of the enzymatic activity of the substrate glycoprotein before any detectable cleavage of sugar residues occurred. Besides, the fact that the substrate glycoprotein is a proteolytic enzyme made the problem more complicated. The attempt to use glycosidases was thus discontinued.

Our second method of choice was to use periodate which was also shown to be useful in elucidating the structure of stem bromelain glycopeptide (Yasuda *et al.*, 1970). Since stem bromelain is an SH protease, we had to know how to protect the SH group from the possible oxidation by periodate. Tetrathionate was found to fulfill the requirement, because it binds the essential SH group to protect this from oxidation, while it can be readily removed by an excess of cysteine, restoring a full enzyme activity. Thus, stem bromelain was first converted into tetrathionate-blocked enzyme protein, and then subjected to an oxidation with 0.1 M or approximately 200-fold molar excess of sodium metaperiodate for relatively short periods of time. The disappearance of neutral sugars by the oxidation was found to be fairly rapid to reach close to a complete removal after 5 hr, whereas the enzymatic activities, as determined with three different kinds of the substrate, were only partially decreased (Figure 2 and Table III). Time courses for sugars and activities shown in Figure 2 suggest that the destruction of the sugars and the decrease in activity represent only indirectly related phenomena. It may be concluded that the neutral sugar residues of stem bromelain are not directly involved in the catalytic mechanism.

The number of moles of periodate consumed by 1 mole of stem bromelain reached 26–27 after 3- to 5-hr oxidation (Figure 1). This may indicate an overoxidation of sugar residues, since the proposed structure for the carbohydrate moiety requires only 8 moles of periodate to oxidize all the susceptible linkages to produce dialdehydes and formic acid (Yasuda *et al.*, 1970). Under the present experimental condition, an overoxidation may have yielded further oxidized products from sugar residues with some oxidation also occurring on the amino acid residues, since periodate is known to be reactive with some amino acid residues (Clamp

and Hough, 1965). For example, methionine was oxidized by periodate in α-chymotrypsin (Knowles, 1965), cysteine and tryptophan in ovalbumin (Maekawa and Kushibe, 1954), and tryptophan in lysozyme (Maekawa and Kushibe, 1955) and in myoglobin (Atassi, 1967). Such possibilities were examined by performing amino acid analysis on the periodate-oxidized stem bromelain. The contents of tryptophan and tyrosine were also determined by several different kinds of spectrophotometric methods. It was found, as shown in Tables III and IV, that practically no change has occurred in the amino acid composition except methionine which was partially oxidized to form methionine sulfoxide.

The conditions used for the carbohydrate oxidation are very close to those described by Atassi (1967) for a tryptophan oxidation. Therefore, on chemical grounds one could expect an oxidation of tryptophan that might account for the measurable decrease in activity of the enzyme. However, availability of tryptophyl residues to the oxidizing agent must be different from one protein to the other, and in fact, there was no indication of a tryptophan oxidation as far as the spectrophotometric determinations on periodate-oxidized stem bromelain were concerned (Table IV). Tyrosine is also an amino acid that is likely to be altered during oxidation. As shown in Table III, the 3- and 5-hr oxidized proteins gave 18 instead of 19 tyrosines. However, this fact may not be very significant, because tyrosine is difficult to determine with perfect precision in acid or alkaline hydrolysates. Again, spectrophotometric data (Table IV) indicate no large alteration in tyrosine chromophore. Nevertheless, in view of the difficulty associated with interpretation of spectrophotometric results, it may still be open whether strictly no change in aromatic amino acid side-chain groups has occurred.

There has been no experimental evidence for or against the involvement of a methionine residue in stem bromelain catalysis. Papain contains no methionine (Drenth *et al.*, 1968). Chymotrypsin is 55% active if the methionine-192 that is three residues from the active site serine is oxidized to methionine sulfoxide (Knowles, 1965) and in trypsin the residue is replaced with glutamine (Walsh and Neurath, 1964).

The decrease in $K_{m,app}$ value observed with α-*N*-benzoyl-L-arginine ethyl ester (Table II and Figure 3A) may be attributed to the probable introduction of some acidic group or groups to the enzyme protein as a result of periodate oxidation, particularly due to an overoxidation (see above). In fact, the affinity of the enzyme to *N*-benzoylglycine ethyl ester, which has no basic side-chain group, remained practically unchanged before and after the oxidation (Table II and Figure 3B). On the other hand, no reasonable explanation seems to be possible for the larger decrease in k_0 value with α-*N*-benzoyl-L-arginine ethyl ester than with *N*-benzoylglycine ethyl ester (Table II). Thus, we cannot completely understand at present how the partial loss of enzymatic activity has been brought forth by periodate oxidation.

References

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

Atassi, M. Z. (1967), *Arch. Biochem. Biophys. 120*, 56.

Clamp, J. R., and Hough, L. (1965), *Biochem. J. 94*, 17.

Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., and Wolthers, B. G. (1968), *Nature (London) 218*, 929.

Edelhoch, H. (1967), *Biochemistry 6*, 1948.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Eylar, E. H. (1965), *J. Theoret. Biol. 10*, 89.

Fleury, P., and Lange, J. (1933), *J. Pharm. Chim. (8), 17*, 107.

Floyd, N. F., Cammaroti, M. S., and Lavine, T. F. (1963), *Arch. Biochem. Biophys. 102*, 343.

Ikenaka, T. (1963), *J. Biochem. (Tokyo) 54*, 328.

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

Knowles, J. R. (1965), *Biochem. J. 95*, 180.

Kornfeld, R., and Kornfeld, S. (1970), *J. Biol. Chem. 245*, 2536.

Lee, T., and Hager, L. P. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 599Abs.

Maekawa, K., and Kushibe, M. (1954), *Bull. Chem. Soc. Jap. 27*, 277.

Maekawa, K., and Kushibe, M. (1955), *Bull. Agr. Chem. Soc. Jap. 19*, 28.

Margolis, F., and Feigelson, P. (1964), *Biochim. Biophys. Acta 89*, 357.

Moffit, W., and Yang, J. T. (1956), *Proc. Nat. Acad. Sci. U. S. 42*, 596.

Murachi, T. (1964), *Biochemistry 3*, 932.

Murachi, T. (1970), *Methods Enzymol. 19*, 273.

Murachi, T., Inagami, T., and Yasui, M. (1965), *Biochemistry 4*, 2815.

Murachi, T., and Takahashi, N. (1970), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., Copenhagen, Munksgaard, p 298.

Murachi, T., and Yamazaki, M. (1970), *Biochemistry 9*, 1935.

Murachi, T., and Yasui, M. (1965), *Biochemistry 4*, 2275.

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

Pazur, J. T., Knull, H. R., and Simpson, D. L. (1970), *Biochem. Biophys. Research Commun. 40*, 110.

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

Plummer, T. H., Jr., and Hirs, C. H. W. (1964), *J. Biol. Chem. 239*, 2530.

Ray, W. J., and Koshland, D. E. (1962), *J. Biol. Chem. 237*, 2493.

Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem. 217*, 959.

Sakai, T., Ikeda, K., Hamaguchi, K., and Murachi, T. (1970), *Biochemistry 9*, 1939.

Schoenmakers, J. G. G., Matze, R., Haanen, C., and Zieliken, F. (1965), *Biochim. Biophys. Acta 101*, 166.

Scocca, J., and Lee, Y. C. (1969), *J. Biol. Chem. 244*, 4852.

Spackman, D. H., Stein, W. H., and Moore, S. (1953), *Anal. Chem. 30*, 1190.

Svensmark, O., and Heilbronn, E. (1964), *Biochim. Biophys. Acta 92*, 400.

Szewczuk, A., and Connell, G. E. (1964), *Biochim. Biophys. Acta 83*, 218.

Tachibana, A., and Murachi, T. (1966), *Biochemistry 5*, 2756.

Walsh, K. A., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.

Yang, J. T., and Doty, P. (1957), *J. Amer. Chem. Soc. 79*, 761.

Yasuda, Y., Takahashi, N., and Murachi, T. (1970), *Biochemistry 9*, 25.