The Structure and Mechanism of Stem Bromelain

EVALUATION OF THE HOMOGENEITY OF PURIFIED STEM BROMELAIN, DETERMINATION OF THE MOLECULAR WEIGHT AND KINETIC ANALYSIS OF THE BROMELAIN-CATALYSED HYDROLYSIS OF *N*-BENZYLOXYCARBONYL- L-PHENYLALANYL-L-SERINE METHYL ESTER

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1. Purified stem bromelain (EC 3.4.22.4) was eluted from Sephadex G-100 as a single peak. The specific activity across the elution peak was approximately constant towards *p*-nitrophenyl hippurate but increased with elution volume with *N*²-benzoyl-L-arginine ethyl ester as substrate. 2. The apparent molecular weight, determined by elution analysis on Sephadex G-100, is $22\,500 \pm 1\,500$, an anomalously low value. 3. Purified stem bromelain was eluted from CM-cellulose CM-32 as a single peak and behaved as a single species during column electrophoresis on Sephadex G-100. 4. Purified stem bromelain migrates as a single band during polyacrylamide-gel electrophoresis under a wide variety of conditions. 5. The molecular weight determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate is $28\,500 \pm 1\,000$. 6. Sedimentation-velocity and equilibrium-ultracentrifugation experiments, under a variety of conditions, indicate that bromelain is an apparently homogeneous single peptide chain of mol.wt. $28\,400 \pm 1\,400$. 7. The *N*-terminal amino acid composition is 0.64 ± 0.04 mol of valine and 0.36 ± 0.04 mol of alanine per mol of enzyme of mol.wt. 28 500. (The amino acid recovery of the cyanate *N*-terminal amino acid analysis was standardized by inclusion of carbamoyl-norleucine at the cyclization stage.) 8. The pH-dependence of the Michaelis parameters of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester was determined. 9. The magnitude and pH-dependence of the Michaelis parameters have been interpreted in terms of the mechanism of the enzyme. 10. The enzyme is able to bind *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester relatively strongly but seems unable to make use of the binding energy to promote catalysis.

Stem bromelain (EC 3.4.22.4), the pineapple thiol-group protease, has been purified from the acetone-dried powder of stem juice by a number of workers (Murachi & Neurath, 1960; Ota *et al.*, 1964; Murachi *et al.*, 1964; Bobb, 1972). These authors have examined the molecular homogeneity of their respective purified enzyme preparations and have concluded that the samples of purified stem bromelain obtained by these methods are essentially homogeneous. However, El-Gharbawi & Whitaker (1963) reported the separation of five proteolytically active components by using cation-exchange chromatography and column electrophoresis in Sephadex G-100. Murachi *et al.* (1964) determined the molecular weight of stem bromelain by sedimentation-diffusion ultracentrifugation and obtained a value of 33 000. Ota *et al.* (1964) obtained a value of 35 000 computed from amino acid analysis and Bobb (1972) found a value of 23 000 by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulphate. Feinstein & Whitaker (1964) determined the molecular weights

of the five components of stem bromelain obtained by El-Gharbawi & Whitaker (1963). The estimates from amino acid analysis, column chromatography on Sephadex G-100 and for component II by ultracentrifugation by using the Archibald method (Schachman, 1957) gave values ranging from 18 000 to 25 000. Takahashi *et al.* (1973) have demonstrated that the apparently homogeneous preparation of Murachi *et al.* (1964) may be further fractionated into two very similar components by means of chromatography on SP-Sephadex. Kierstan (1973) has reported that bromelain, purified by covalent chromatography, has a molecular weight of about 28 000 on the basis of the stoichiometry of the reaction with 2,2'-dipyridyl disulphide.

The catalytic activity of stem bromelain has been little studied. Inagami & Murachi (1963) studied the bromelain-catalysed hydrolyses of *N*²-benzoyl-L-arginine ethyl ester and *N*²-benzoyl-L-arginine amide. These results have been analysed in terms of strong non-productive binding for the bromelain-catalysed

hydrolysis of *N*²-benzoyl-L-arginine amide (Brocklehurst *et al.*, 1967). Neither of these substrates can be regarded as kinetically specific since the apparent K_m for the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine ethyl ester is near 0.1 M, approximately 100-fold greater than that for the amide, and the apparent $k_{cat.}$ for the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine amide is 0.0035 s^{-1} , approximately one-hundredth of the value for the ester. Berger & Schechter (1970) have examined the papain-catalysed hydrolysis of a large number of peptide substrates. They have demonstrated that papain is able to interact with up to seven sequential amino acids in a peptide substrate and that interaction at these seven sites contributes to the kinetic specificity. These studies indicated that compounds of the type

where the arrow represents the point of cleavage, X and Y are L-amino acids, X being hydrophobic in nature, and R' is a suitable leaving group (alcohol, amine, amino acid or peptide), are good substrates. In particular, substrates having phenylalanine at position X were found to be most effective. Lowe & Yuthavong (1971a) have examined the papain-catalysed hydrolysis of a number of substrates incorporating these features and have been able to propose a detailed mechanism for papain-catalysed hydrolyses.

Little is known about the mechanism of bromelain-catalysed hydrolyses other than that a thiol group is required in the reduced form for activity (Murachi & Neurath, 1960). The bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine ethyl ester and amide have been interpreted in terms of an acyl-enzyme mechanism (Inagami & Murachi, 1963; Brocklehurst *et al.*, 1967).

where

$$K_m = \left(\frac{k_3}{k_2 + k_3} \right) \left(\frac{k_{-1} + k_2}{k_{+1}} \right)$$

and

$$k_{cat.} = \frac{k_2 k_3}{k_2 + k_3}$$

In this mechanism E represents free enzyme, ES the Michaelis complex, ES' the acyl-enzyme, P₁ the leaving group and P₂ the portion of the substrate that contains the acyl group. Brocklehurst *et al.* (1967) reported the observation of an absorption band having a maximal intensity at 316 nm after admixture of bromelain and methyl thionohippurate. This observation represents evidence for the formation of thionohippuryl-bromelain and hence suggests that

bromelain-catalysed hydrolyses may proceed via an acyl-enzyme mechanism. Observation of a decrease in the intensity of this absorption band, consequent on the bromelain-catalysed hydrolysis of methyl thionohippurate was, however, not reported.

The object of the studies reported in the present paper has been the clarification of some of the ambiguous results reported on the structure and catalytic activity of stem bromelain. Examination of the homogeneity of purified stem bromelain is of particular importance in view of the observation of apparent substrate activation in the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-serine methyl ester (Wharton *et al.*, 1974). This form of kinetic mechanism is equivalent to a system in which two enzymes are present, both of which may catalyse the hydrolysis of the substrate.

Materials and Methods

Materials

Bromelain. Crude bromelain powder was purified essentially as described by Wharton *et al.* (1968). A single batch of starting material (Miles-Seravac Ltd., Moneyrow Green, Holyport, Maidenhead, Berks., U.K., batch no. E.V. 1a, food-grade) was used for all of the studies reported here. The crude powder (10 g) was suspended in 50 ml of 0.2 M-potassium phosphate buffer, pH 6.05, and 100 mg of sodium tetrathionate added with stirring. This suspension was then subjected to purification as described by Wharton *et al.* (1968) except that all procedures, except precipitation with (NH₄)₂SO₄ were carried out at room temperature (21°C) and all buffers contained 1 mM-sodium tetrathionate. During the final dialysis, against 0.01 M-KCl containing 0.001 M-EDTA, a precipitate formed which was removed by centrifugation and discarded. The precipitated material differs from material present in the supernatant by being soluble only in strong salt solutions (at least 1 M). The nature of this precipitate has not been investigated. The precipitate constitutes about 15–25% of the total protein obtained as a result of (NH₄)₂SO₄ fractionation. The enzyme was finally precipitated by addition of (NH₄)₂SO₄ to 50% saturation and stored at 4°C in this form. The homogeneity of the purified enzyme was evaluated as a routine by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulphate as described by Weber & Osborn (1969) with modifications as described below. Most batches yielded a single band on staining with Coomassie Blue R but some batches contained some lower-molecular-weight material. Experiments using proteins of known molecular weight indicated that this contaminating material had a molecular weight of less than 10000. The main band, however, always migrated to a position identical

with that found for batches which did not contain material of molecular weight less than 10000. Batches free from low-molecular-weight material were used for the present studies. The catalytic activity towards *N*²-benzoyl-L-arginine ethyl ester of crude tetrathionate-treated enzyme was determined in the presence and absence of 5mM-cysteine, no activity being detectable in the absence of cysteine. This indicates that autolysis would be unlikely to occur to any significant extent during the purification procedure. Bromelain, purified by covalent chromatography on Sepharose-glutathione-2-thiopyridine (Brocklehurst *et al.*, 1973), was a gift from Dr. K. Brocklehurst, St. Bartholomew's Hospital Medical College, London E.C.1, U.K.

Sodium tetrathionate. This was prepared by the method of Gilman *et al.* (1946) and stored at 4°C.

N-Benzoyloxycarbonyl-L-phenylalanyl-L-serine methyl ester. *N-Benzoyloxycarbonyl-L-phenylalanine* was prepared as described by Greenstein & Winitz (1961) and had m.p. 88–90°C. *N-Benzoyloxycarbonyl-L-phenylalanine-N-hydroxysuccinimide ester* was prepared by the method of Anderson *et al.* (1964). The reaction was carried out in ethyl acetate-acetonitrile and the product, which was recrystallized from light petroleum (b.p. 60–80°C)-chloroform, had m.p. 136–137°C (lit. m.p. 140–140.5°C). The melting point did not alter as a result of further recrystallizations from light petroleum-chloroform. *L-Serine methyl ester hydrochloride* (0.4g) and triethylamine (0.4ml) were added to 20ml of acetonitrile at room temperature. *N-Benzoyloxycarbonyl-L-phenylalanine-N-hydroxysuccinimide ester* (1.0g) was added with stirring and the clear solution stirred for 2h. The solvent was removed *in vacuo*, the residue was dissolved in chloroform (40ml) and this was extracted with 50ml samples of 0.1M-NaHCO₃, 0.1M-HCl and water. The chloroform layer was dried over Na₂SO₄ and the chloroform removed *in vacuo*. The residue was recrystallized twice from propan-2-ol-water, m.p. 121–122°C [lit. m.p. 122–123°C; Susuki *et al.* (1969)], yield 0.36g. This material was examined by t.l.c. with chloroform-propan-2-ol (95:5, v/v) as solvent and found to migrate as a single spot. *N-Benzoyloxycarbonyl-L-phenylalanyl-L-serine methyl ester* was used for kinetic experiments as a 10 or 25mM solution in 50% acetonitrile.

N-Benzoyloxycarboxyl-L-phenylalanyl-glycine methyl ester. This was prepared as described for *N-Benzoyloxycarbonyl-L-phenylalanyl-L-serine methyl ester* with a yield of 55% and m.p. 121–122°C [lit. m.p. 122–123°C; Vogler *et al.* (1962)]. A stock solution was prepared as for *N-Benzoyloxycarbonyl-L-phenylalanyl-L-serine methyl ester*.

N²-Benzoyl-L-arginine ethyl ester. This was the product of BDH Chemicals Ltd., Poole, Dorset, U.K.; it was used as an 0.5M stock solution in water and stored at 4°C.

All other materials were of the highest grade of purity obtainable commercially and were used without further purification.

Methods

Gel filtration of bromelain on Sephadex G-100. A column [2.5cm×92cm (1in×40in)] of Sephadex G-100 was equilibrated at room temperature with 0.1M-sodium acetate buffer, pH5.2, containing 0.1M-NaCl. Constant flow was maintained by using a constant-head bottle to maintain a 50cm hydrostatic head. Fractions of volume 5ml were collected. The flow rate was approx. 20ml/h.

Chromatography of purified stem bromelain on CM-cellulose CM-32. Whatman CM-32 (BDH Chemicals Ltd.) was pre-cycled with 0.5M-NaOH and 0.5M-HCl, the fines were removed and the CM-32 was poured into a column (30cm×1.5cm). The column was equilibrated with 0.5M-sodium phosphate buffer, pH5.5, at room temperature. A sample of enzyme (5–10mg) extensively dialysed against the above buffer was applied to the column. The column was washed with approx. 100ml of the above buffer after which elution with a logarithmic gradient was commenced. The upper vessel contained 2M-NaCl in 0.05M-sodium phosphate buffer, pH5.5, and the lower vessel 150ml of 0.05M-sodium phosphate buffer, pH5.5.

Polyacrylamide disc gel electrophoresis. This was performed essentially as described by Davis (1964). The buffers used were acetate at pH4.0, phosphate at pH7.0, Tris at pH9.0 and bicarbonate at pH9.5, all at 0.05M. The gels were stained with Amido Black. Electrophoresis in the presence of 0.1% sodium dodecyl sulphate was performed by the method of Weber & Osborn (1969).

Column electrophoresis on Sephadex G-75. This was performed in an LKB Uniphor apparatus (LKB Produkter AB, S-16125 Brommer, Sweden) at room temperature. The column (2.5cm×40cm) of Sephadex G-75 was equilibrated with 0.05M-sodium acetate buffer, pH5.2, containing 0.1M-NaCl. The sample was loaded on to the column and run approximately half-way down the column. Current (100mA) was passed for 12h after which the column was eluted with the above buffer, the absorbance of the effluent being monitored by an LKB Uvicord u.v. column monitor.

Analytical ultracentrifugation studies. The centrifuge used for the sedimentation-velocity experiment was a Beckman model E instrument (Beckman Instruments Ltd., Glenrothes, Fife, U.K.) equipped with schlieren optics. The run was made at 20°C and 59780rev./min, the solvent being 0.1M-NaCl. The equilibrium experiments were performed on a similar instrument equipped with Rayleigh interference

optics. The experimental runs were performed at 25°C by using the long-column meniscus-depletion method described by Chervenka (1970). The enzyme samples were prepared by extensive dialysis of a sample of $(\text{NH}_4)_2\text{SO}_4$ -precipitated enzyme against water, followed by dialysis against the solvent, which contained, in addition to the appropriate salt, 0.01 M-sodium phosphate buffer, pH 6.5. Dialysis was performed at 4°C and for a minimum of 3 days in the case of dialysis against 6 M-guanidine hydrochloride. Runs were performed overnight, usually for about 14 h, and a photograph was taken at this stage. This photograph was compared with a similar photograph taken at 16 h to ensure that equilibrium had been attained. The plates were measured on a Nikon micro-comparator and plotted in the usual fashion (Yphantis, 1964). Straight lines resulted in all cases except in some of those where 6 M-guanidine hydrochloride was present.

N-Terminal amino acid estimation. This was performed as described by Stark & Smythe (1963). The procedure was standardized by means of carbamoyl-norleucine and this was incorporated into the samples of carbamoylated and untreated enzyme. The results of the procedure were somewhat variable quantitatively although the qualitative results were consistent. Accordingly the procedure was repeated several times.

Kinetics

Estimation of active enzyme concentration. The enzyme concentration used to calculate k_{cat} values for the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs_2). A dialysed sample of enzyme was incubated at room temperature with 5 mM-L-cysteine, pH 6.5, for 10 min and applied to a column (1 cm × 13 cm) of Sephadex G-25. The enzyme was eluted with 0.01 M-KCl containing 3 mM-EDTA that had been boiled and cooled under a stream of O_2 -free N_2 . The reactive thiol content of the enzyme was measured by the extent of reaction with Nbs_2 at pH 8.0 in 0.1 M-Tris-HCl buffer, the absorbance change at 412 nm being used together with a value of $\epsilon_{412} = 1.36 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Ellman, 1959).

Spectrophotometric method for measurement of the bromelain-catalysed hydrolysis of *p*-nitrophenyl hippurate. Samples of enzyme (0.01–0.1 ml) were incubated with 0.25 ml of 0.1 M-sodium phosphate buffer, pH 7.6, and 0.05 ml of neutralized 0.25 M-L-cysteine for 3 min. Phosphate buffer (pH 6.0; 1.0 ml of 0.03 M), water to 2.96 ml and 0.01 M-*p*-nitrophenyl hippurate in acetonitrile (0.05 ml) were added and the change in absorbance was followed at 320 nm. As the recorded

trace was usually slightly curved the rate was measured as a tangent after 20 s. Cysteine-mediated hydrolysis of *p*-nitrophenyl hippurate contributes to the rate and this was eliminated from the assay values, together with the rate of spontaneous hydrolysis, by subtracting the rate of a reaction, in which no enzyme was present, from each assay value.

Titrimetric assay of the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine ethyl ester. This was carried out as described by Wharton *et al.* (1968).

Titrimetric assay of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester. Since this substrate is soluble in water to the extent of approx. 1 mM the 10 mM and 25 mM stock solutions were prepared in 50% acetonitrile. The acetonitrile concentration in the assay mixture was maintained at 5%. The reaction volume was 5 ml comprising 2 mM-dithiothreitol, 1 mM-buffer, 1 mM-EDTA, 5% acetonitrile, 0.1 M-KCl and substrate of various concentrations between 0.1 and 2.5 mM. The enzyme was activated before each run by preincubation for 5 min at 25°C, pH 6.5, in the presence of 2.2 mM-dithiothreitol before addition of the buffer, adjustment to the pH of the assay and finally addition of the substrate and adjustment of the acetonitrile to 5%. The buffers were sodium acetate at pH 4.5–5.0, sodium phosphate at pH 5.0–8.0 and NaHCO_3 at pH 8.0–10.0. The buffers were overlapped at pH 5.0 and 8.0 and did not affect the measured initial velocities. The pH of the assay mixture was adjusted by means of an Aglar syringe containing either 0.1 M-NaOH or 0.1 M-HCl. The initial velocity was measured with a Radiometer (Copenhagen) titrator 11 equipped with a micro-titration apparatus (TTA 31) having a thermostatically controlled titration vessel maintained at 25°C. The titrant was 10 mM-NaOH prepared in CO_2 -free glass-distilled water delivered by an 0.25 ml burette. A stream of O_2 -free N_2 was passed over the surface of the reaction mixture.

Product analysis of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester. A sample of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester was saponified with 1.1 equivalents of 1.0 M-NaOH yielding *N*-benzyloxycarbonyl-L-phenylalanine, m.p. 147–148°C (lit. m.p. 147–149°C). A sample of enzyme was activated at pH 6.5 with 12.5 mM-cysteine and passed through Sephadex G-25 to remove the activator. The bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester was followed in a pH-stat at pH 7.0. The reaction mixture comprised enzyme solution (1.0 ml), substrate (10 mg) in 1.0 ml of acetonitrile, 0.5 M-KCl (1.0 ml) and 2.0 ml of water. The titrant was 0.1 M-NaOH. After approx. 75% reaction, the acetonitrile was removed by rotary evaporation, the solution was acidified and the remaining substrate and product were extracted into ethyl acetate. The ethyl acetate was removed *in vacuo*

and a sample of this material, *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine, and of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester were applied to a t.l.c. plate and eluted with chloroform-propan-2-ol (95:5, v/v). The bromelain-hydrolysed sample was eluted as two spots as shown on staining with iodine and dichlorofluorescein. These two spots coincided exactly with those of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine and the methyl ester. The R_F values were *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine, 0.49, *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester, 0.23, *N*-benzyloxycarbonyl-L-phenylalanine, 0.68. There is no evidence for the presence of *N*-benzyloxycarbonyl-L-phenylalanine in the bromelain-catalysed hydrolysate. Similar experiments were carried out in which 5mM-cysteine and 2mM-dithiothreitol were included in the reaction mixture. When cysteine was included a number of spots which were strongly stained by iodine were apparent on the t.l.c. plate. These are presumed to represent sulphur-containing compounds resulting from transpeptidation. Inclusion of dithiothreitol leads to some strongly iodine-staining material on the t.l.c. plate but very much less than was found for the reaction that involved cysteine. The procedure not involving cysteine or dithiothreitol was repeated at pH values of 5.0 and 8.0, the results being identical with those at pH 7.0.

Results

Gel filtration of stem bromelain on Sephadex G-100

Stem bromelain, purified as described in the Materials and Methods section, was applied to a column of Sephadex G-100 and was eluted as a single peak (Fig. 1). The apparent molecular weight was determined by measuring the elution volume of bromelain together with the elution volumes of a number of other proteins of established molecular weight. It was calculated from a number of plots of log (molecular weight) against elution volume and found to be 22500 ± 1500 , the error quoted being the maximum deviation from the average value of six determinations. Lysozyme was eluted later than expected on the basis of its molecular weight and therefore was not used as a standard. Standards that gave reproducible straight-line plots of log (molecular weight) against elution volume were bovine serum albumin, ovalbumin, chymotrypsinogen and myoglobin. Bromelain samples from a number of separate purifications of common starting material were analysed as above and behaved in a highly reproducible fashion. Increasing the NaCl content of the buffer to 1.0M did not affect the elution behaviour of the proteins. In Fig. 1 the specific activity with respect to benzoyl-L-arginine ethyl ester and *p*-nitrophenyl hippurate are presented. It is notable that the specific activity with

Fig. 1. Elution of purified stem bromelain from Sephadex G-100

●, E_{280} ; □, specific activity towards N^2 -benzoyl-L-arginine ethyl ester ($[S_0] = 0.05M$); ▲, specific activity towards *p*-nitrophenyl hippurate ($[S_0] = 0.17mM$). The column (2.5cm × 92cm) was eluted with 0.1M-sodium acetate buffer, pH 5.2, at a flow rate of 20ml/h. Fractions of volume 5ml were collected.

respect to benzoyl-L-arginine ethyl ester is higher on the low-molecular-weight (high-volume) side of the elution peak. The specific activity towards *p*-nitrophenyl hippurate is approximately constant across most of the peak, falling somewhat at high elution volume.

Chromatography of purified stem bromelain on CM-cellulose CM-32

Purified stem bromelain applied to a column of CM-cellulose CM-32 in buffers ranging in pH value from 5.0 to 6.1 was eluted with a salt gradient as a single peak in all cases.

Polyacrylamide disc gel electrophoresis

The homogeneity of purified stem bromelain has been studied, under a variety of conditions, by polyacrylamide-gel electrophoresis. At pH values of 4.0, 7.0, 9.0 and 9.5 bromelain migrates as a single band. Migration was towards the cathode at pH 4.0, 7.0 and 9.0 and slightly towards the anode at pH 9.5. This indicates that the enzyme is homogeneous by the criterion of electrophoretic migration in this system and that the isoelectric point is near to pH 9.5. Gross overloading of the gels produced a broad main band together with several much less dense bands, but these represented only a very small percentage of the total protein present.

Electrophoresis of purified stem bromelain on polyacrylamide discs in the presence of sodium dodecyl sulphate gave a single band in the presence and

absence of 1% 2-mercaptoethanol provided pre-incubation was carried out at 100°C with 1% 2-mercaptoethanol (Bobb, 1972). The apparent molecular weight of stem bromelain has been estimated by comparison of the electrophoretic mobilities of stem bromelain, bovine serum albumin, ovalbumin, pepsin, chymotrypsinogen and myoglobin in the presence of 0.1% sodium dodecyl sulphate. When standard values of the molecular weights of the reference proteins were used plots of log (molecular weight) against distance of migration were not linear. If, however, the 'best-fit' molecular weights of Dunker & Rueckert (1969), determined for 10% gels, were used, plots of log (molecular weight) against distance of migration were linear. The apparent molecular weight calculated from a number of experiments is $28\,500 \pm 1000$ (maximum deviation). Fig. 2 represents the results of a typical experiment (eight gels). A sample of bromelain prepared from different starting material by covalent chromatography on a column of Sepharose-glutathione-2-thiopyridine by the method of Brocklehurst *et al.* (1972) behaved identically with the enzyme prepared in this laboratory by the method described in the Materials and Methods section.

Analytical centrifugation studies

The homogeneity and molecular weight of purified stem bromelain has been studied by means of sedimentation velocity and equilibrium ultracentrifugation. The sedimentation-velocity experiment at 20°C

Fig. 2. Polyacrylamide-gel electrophoresis of bromelain and reference proteins in the presence of 0.1% sodium dodecyl sulphate

Electrophoresis was carried out as described by Weber & Osborn (1969). The reference proteins are bovine serum albumin (1) (mol.wt. 62000), ovalbumin (2) (mol.wt. 46400), pepsin (3) (mol.wt. 37000), chymotrypsinogen (5) (mol.wt. 26300) and myoglobin (6) (mol.wt. 17500). These molecular-weight values are from Dunker & Rueckert (1969) for 10% gels. The error bars represent maximum variation of migration. The apparent molecular weight of stem bromelain is $28\,500 \pm 1000$ (maximum deviation).

and 15mg/ml yielded an $s_{20,w}$ value of 2.94S. This value compares with that obtained by Murachi *et al.* (1964), of 2.73S when extrapolated to zero protein concentration and approx. 2.95S at 15mg/ml. The schlieren photographs demonstrated that purified stem bromelain apparently sediments in the ultracentrifuge as a single species. The sedimentation-velocity experiment has been regarded as a test of homogeneity rather than as a reliable method for the estimation of the molecular weight.

A large number of estimations of the molecular weight of purified stem bromelain were made under a variety of conditions, with the sedimentation-equilibrium technique (Table 1). This technique does not require knowledge of the diffusion constant of

Table 1. Molecular weight of stem bromelain determined by sedimentation-equilibrium ultracentrifugation

For details see the text.

Solvent	Mol.wt.	S.D.	Range of bromelain concn. (mg/ml)	No. of determinations
0.1M-NaCl	28400*	1400	1-10	9
0.01M-NaCl	34300†	—	2.25	1
1.0M-NaCl	28900*	600	1-5	4
6.0M-Guanidine hydrochloride	25800*	3700	1-3	8

* The rotor speed was varied from 21740 to 30410 rev./min.

† The plot of $\log(y_0 - y_r)$ versus r^2 was not linear in this case. The apparent molecular weight was computed from a tangent taken at a value of r^2 near to the cell base.

Fig. 3. Equilibrium ultracentrifugation of purified stem bromelain in 0.1M-NaCl and 6.0M-guanidine hydrochloride

●, 0.1M-NaCl, 20410 rev./min, 25°C, 1.25mg/ml, 14h apparent mol.wt. 28500. ○, 6.0M-guanidine hydrochloride, 25980 rev./min, 25°C, 2.25mg/ml, 18h, density 1.14, apparent mol.wt. 26200 ($\bar{v} = 0.743$). The salt solutions contained 0.01M-sodium phosphate buffer, pH6.5. Further details are given in the Materials and Methods section.

the enzyme and is thus expected to be less error-prone. Fig. 3 shows typical plots of data obtained in 0.1M-NaCl and 6.0M-guanidine hydrochloride. Variation of the protein concentration under any of the conditions quoted in Table 1 produced no observable change in the apparent molecular weight. Variation of the rotor speed had no effect on the value of the apparent molecular weight except in the experiments in 6.0M-guanidine hydrochloride where the apparent molecular weight increased slightly with the rotor speed. Aggregation of the protein apparently occurs in 0.01M-NaCl, but apparently not in 0.1M-NaCl, as increasing the salt concentration to 1.0M-NaCl causes no significant change in the molecular weight. The experiments in 6.0M-guanidine hydrochloride yielded rather less satisfactory results than did the other experiments. The plots of $\log(y_0 - y_r)$ against r^2 were not always entirely linear, the linearity improving as the rotor speed was increased. This is taken to suggest the presence of some lower-molecular-weight material which, at high rotor speeds, equilibrated near the bottom of the cell in the region of most of the intact protein.

Determination of the N-terminal amino acid residue of purified stem bromelain

The cyanate method of *N*-terminal amino acid determination has been reported as being both sensitive and quantitative (Stark & Smythe, 1963). Owing to the relative complexity of the procedure, errors are easily introduced and it has proved necessary to repeat the procedure several times to obtain results of satisfactory precision. As a result of these studies it was found that after subtracting relevant blank values the recovery of *N*-terminal amino acid from the procedure required a molecular weight for bromelain of 40000 or above. Thus it seemed that either partial recovery of *N*-terminal amino acid was being achieved or that some of the *N*-terminal amino groups were blocked. To ascertain whether complete recovery of the available *N*-terminal amino acid was possible a standard amino acid derivative, carbamoyl-norleucine, was added at the cyclization stage. As a result of this standardization the recovery of the norleucine varied from 40 to 80% from one experiment to another. The recovery of *N*-terminal amino acids was corrected to 100% on the basis of the fractional recovery of norleucine. When this correction was made, a consistent relative composition was apparent and gave the following mol of *N*-terminal amino acid/mol of enzyme ($\pm$ s.e.m. with the number of determinations in parentheses): 0.64 $\pm$ 0.04(6) of valine, 0.36 $\pm$ 0.04(6) of alanine and a small variable contribution from glycine and glutamic acid. The molecular weight calculated from the corrected recoveries of amino acids is 28 500 assuming 1 mol of *N*-terminal amino acid/mol of enzyme. It has not proved possible, by any of the methods used in the

present study, to achieve a separation of enzyme possessing a valine *N*-terminal amino acid from enzyme possessing an alanine *N*-terminal amino acid. If the forms arose by proteolytic scission, they might not differ enough (e.g. by a charged residue or by a number of neutral ones) to allow separation.

Column electrophoresis on Sephadex G-100

A number of experiments using the LKB Uniphor column electrophoresis apparatus were performed to examine homogeneity of the enzyme. The buffers used were 0.05M-sodium acetate, pH 5.2, and the same buffer including 0.1M-NaCl; 0.1% sodium dodecyl sulphate was included in one run. All experiments involved electrophoresis followed by elution with the electrophoresis buffer. In all cases the enzyme was eluted as a single peak and showed no sign of heterogeneity.

pH-dependence of the bromelain-catalysed hydrolysis of N-benzoyloxycarbonyl-L-phenylalanyl-L-serine methyl ester

Plots of $[S_0]/v_i$ against $[S_0]$ for the bromelain-catalysed hydrolysis of *N*-benzoyloxycarbonyl-L-phenylalanyl-L-serine methyl ester were linear at all pH values studied, the initial substrate concentration being varied between 0.1 and 2.5 mM. The pH-dependence of the Michaelis parameters of the bromelain-catalysed hydrolysis of *N*-benzoyloxycarbonyl-L-phenylalanyl-L-serine methyl ester has been determined between pH values of 4.5 and 10.0. Figs. 4 and 5 show the pH-dependence of $k_{cat.}$ and K_m respectively. The standard errors of the parameters, as indicated in Figs. 4 and 5, are rather larger than desirable and are due to the fact that these experiments have been carried out under conditions where the pH-stat assay technique is approaching the limit of sensitivity and reproducibility. The solid line in Fig. 4 is calculated by using eqn. (1):

$$k_{cat.}(\text{obs.}) = \frac{k_{cat.}}{1 + K_1/[H^+] + K_1 \cdot K_2/[H^+]^2} + \frac{k'_{cat.}}{1 + [H^+]/K_1 + K_2/[H^+]} \quad (1)$$

where K_1 is the ionization constant representative of the first proton to be lost from the system and $k_{cat.}$ the pH-independent $k_{cat.}$ at low pH; K_2 is the ionization constant representative of the second proton to be lost from the system and $k'_{cat.}$ the $k_{cat.}$ of the system which has lost one proton only. The Michaelis parameters were determined at pH 4.0 but were subject to large standard errors, accordingly the estimation at pH 4.0 was repeated several times and the mean values of $k_{cat.}$, after correction for partial protonation of the product, were always lower than the value of $k_{cat.}$ at pH 4.5. This indicates that the value of $k_{cat.}$

Fig. 4. pH-dependence of $k_{\text{cat.}}$ of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester

The error bars represent standard errors resulting from regression of the data, at a given pH value, by the method of Wilkinson (1961). The solid line is calculated by using eqn. (1). The values of the parameters of eqn. (1) are given in Table 2.

Fig. 5. pH-dependence of the Michaelis constant of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester

The error bars represent standard errors as in Fig. 4. The solid line is calculated by using eqn. (2). The values of the parameters of eqn. (2) are given in Table 2.

decreases in the acid region as would be expected by comparison with the pH-activity profile of the deacylation of *trans*-cinnamoyl-bromelain, which shows a dependence on a basic group of $\text{p}K_a$ 3.65 (C. W. Wharton, unpublished work). In addition, comparison with a number of papain-catalysed reactions and consideration of the proposed mechanism of action of papain, which is assumed to bear some similarity to that of bromelain, led to the expectation of a decrease in the value of $k_{\text{cat.}}$ at pH values less than approx. 4.5. If, as is suggested above, the pH- $k_{\text{cat.}}$ profile is characterized by an acid limb with a

Table 2. Kinetic parameters of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester

The values in this Table result from the fitting of eqns. (1) and (2) to the data presented in Figs. 4 and 5.

Eqn. (1)	Eqn. (2)
$\text{p}K_1 = 5.8$	$\text{p}K = 6.14$
$\text{p}K_2 = 8.9$	$K_{m_0} = 0.60 \text{ mM}$
$k_{\text{cat.}} = 1.47 \text{ s}^{-1}$	$K_m = 1.43 \text{ mM}$
$k'_{\text{cat.}} = 0.67 \text{ s}^{-1}$	

$\text{p}K$ value less than 4 then the value of $k_{\text{cat.}}$ in eqn. (1) will be an underestimate of the value which would be obtained if the putative acid limb was included in the derivation of eqn. (1). Since the evidence for such a limb is, in the present instance, not of a quantitative nature, an acid limb has not been included in eqn. (1). The form of the pH-activity profile presented in Fig. 4 will be further considered in the Discussion section.

The pH-dependence of K_m as presented in Fig. 5 has been fitted to eqn. (2) and the calculated values of the observed K_m are represented by the solid line in Fig. 5.

$$K_m(\text{obs.}) = K_{m_0} + \frac{\Delta K_m}{1 + K/[\text{H}^+]}$$
 (2)

where K_{m_0} is the pH-independent value of the observed K_m above pH 8.0 and ΔK_m is the pH-independent increment in the apparent K_m resulting from addition of one proton to the system. The ionization constant characteristic of the pH-dependent change in the observed K_m is represented by K . It is notable that the standard errors of the $K_m(\text{obs.})$ are smaller around neutrality and increase under more acidic or basic conditions. The values of the various parameters resulting from the fitting of eqns. (1) and (2) to the data presented in Figs. 4 and 5 are collected in Table 2.

Determination of the Michaelis parameters of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-glycine methyl ester at pH 7.0 revealed that $K_m = 0.53 \text{ mM}$ and $k_{\text{cat.}} = 3.4 \text{ s}^{-1}$. The solubility of this substrate in water was similar to that of the serine analogue. Further comments on the interpretation of the form of Figs. 4 and 5 and the parameters in Table 2 are in the Discussion section.

Discussion

Chromatography of purified stem bromelain

That purified bromelain is eluted as a single peak from Sephadex G-100 is demonstrated in Fig. 1. The

specific activity across the peak is not constant for the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine ethyl ester, being higher in the lower-molecular-weight region of the elution diagram. This contrasts with the relatively constant specific activity of the bromelain-catalysed hydrolysis of *p*-nitrophenyl hippurate. These results suggest that either all fractions are contaminated with a relatively non-specific esterase or that the bromelain eluted at the relatively high-molecular-weight side of the peak has lost its ability to bind *N*²-benzoyl-L-arginine ethyl ester while retaining the ability to catalyse the hydrolysis of *p*-nitrophenyl hippurate. The enzyme eluted from Sephadex G-100 lost all activity towards *N*²-benzoyl-L-arginine ethyl ester and *p*-nitrophenyl hippurate when a twofold excess of HgCl₂ was present in the assay medium and when the enzyme which was blocked with tetrathionate before purification was subject to assay in the absence of cysteine. This indicates that if an enzyme other than bromelain is present it relies on a free thiol group for activity. Bromelain purified by affinity chromatography on Sepharose- ϵ -amino hexanoyl-D-tryptophan methyl ester had a variable specific activity towards casein in that at higher elution volumes the specific activity was higher than that at the start of the elution peak (Bobb, 1972).

The apparent molecular weight of purified bromelain of 22500 ± 1500 determined by chromatography on Sephadex G-100 is considerably lower than the value of 28000 determined by Ota *et al.* (1972) by chromatography on Sephadex G-75. It is usual to include salt at a concentration of at least 0.1M to suppress ion-exchange effects (Whitaker, 1963) when using Sephadex elution chromatography for the purpose of determining molecular weights (Andrews, 1964). Ota *et al.* (1972), who used 0.02M-sodium citrate, pH 5.5, as eluent, in contrast with the 0.05M-sodium acetate, pH 5.5, containing 0.1M-NaCl used in the present study, obtained a higher value for the apparent molecular weight. This suggests that Sephadex G-75 may be less susceptible to such retarding effects than is G-100 or that citrate buffer is particularly effective in diminishing such effects. Bromelain, with an isoelectric point near pH 9.5 (Murachi *et al.*, 1964), will be particularly susceptible to charge interaction with the column material. Papain has been shown to be eluted considerably later than expected from Sephadex G-100 yielding a molecular weight of 17600 (Feinstein & Whitaker, 1964). Maggio & Shafer (1973) have found the molecular weight of papain, determined by thin-layer gel filtration on Sephadex G-100 with 0.05M-sodium phosphate buffer, pH 7.6, as eluent, to be 9400 which rose to 19000–20000 when 6M-guanidine hydrochloride was included in the buffer. Alkylation of the reactive cysteine residues had no effect on the apparent molecular weight indicating that the strong interaction

that occurs between papain and Sephadex G-100 does not require the enzyme to be in a catalytically active state. Englund *et al.* (1968) found that ficin (mol.wt 25000) was eluted after ribonuclease (mol.wt. 13700) from Sephadex G-75, the eluent being 0.04M-Tris-HCl, pH 7.6.

The elution of bromelain from CM-cellulose CM-32 as a single peak suggests that if purified bromelain is heterogeneous then the form of the heterogeneity is not of the type found for the various species present in crude ficin and ficin purified by the method of Hammond & Gutfreund (1959). As compared with bromelain, ficin has a similar molecular weight (25000), isoelectric point (approx. pH 9.0) and catalytic specificity (Hammond & Gutfreund, 1959; Englund *et al.*, 1968).

Polyacrylamide disc gel electrophoresis of purified stem bromelain

Polyacrylamide disc gel electrophoresis performed at various pH values yielded no evidence that purified bromelain prepared from our starting material by the method described in the Materials and Methods section is heterogeneous. The isoelectric point is near to pH 9.5 since the direction of migration reverses near to this value. The apparent molecular weight of 28500 ± 1000 , determined by electrophoresis in the presence of sodium dodecyl sulphate, is very similar to the value of 28000 reported by Ota *et al.* (1972) and the value of 28300 reported for fraction 6 by Takahashi *et al.* (1973). The presence of 1% 2-mercaptoethanol in the electrophoresis procedure did not affect this migration of bromelain, in particular, it did not cause the protein to migrate as two distinct bands as noted by Bobb (1972).

Behaviour of purified stem bromelain in the analytical ultracentrifuge

Murachi *et al.* (1964) found that purified bromelain sedimented as a single band in the ultracentrifuge, as was found in the present study; the values of $s_{20,w}$ are comparable. Equilibrium-ultracentrifugation experiments indicate a molecular weight of 28400 ± 1400 in 0.1M-NaCl, this value being close to that obtained from electrophoresis in the presence of 0.1% sodium dodecyl sulphate. Thus it is proposed that the value of 25800 obtained in the presence of 6.0M-guanidine hydrochloride (see Table 1) coupled with the rather large standard error of 3700 does not indicate association in 0.1M-NaCl since the value of 28400 obtained in this solvent is within the standard error of the estimate of the value obtained in 6.0M-guanidine hydrochloride. The linearity of the plots of $\log(v_0 - v_r)$ versus r^2 as presented in Fig. 3, represents another

criterion by which stem bromelain, purified as described above, is apparently homogeneous.

Quantitative N-terminal amino acid analysis of purified stem bromelain

The results of the quantitative *N*-terminal amino acid analysis, indicates that bromelain, prepared as described in the Materials and Methods section, is heterogeneous in terms of the *N*-terminal amino acid. Ota *et al.* (1964) found that stem bromelain was much less heterogeneous in this respect, although the principal *N*-terminal amino acid (valine) and contaminating *N*-terminal amino acid (alanine) were the same as was found here. The two very similar fractions resolved by Takahashi *et al.* (1973) have identical *N*-terminal tripeptide sequences (Val-Pro-Gln) and thus the rather high alanine *N*-terminal content reported here cannot be explained on the basis that the preparation is an unresolved mixture of these two components. Thus the conclusion from the results of the *N*-terminal analysis is that the bromelain preparation described here is probably very similar to component I-1 of Ota *et al.* (1972).

Autolysis of stem bromelain during the purification procedure

Attempts to achieve separation of any heterogeneous components present in purified stem bromelain by column electrophoresis on Sephadex G-100 as described by El-Gharbawi & Whitaker (1963) always yielded a single peak on subsequent elution of the enzyme from the column. It must be assumed that the samples of bromelain studied by El-Gharbawi & Whitaker (1963) had been subjected to autodigestion at some stage of the preparation as the molecular weights determined by these authors are considerably lower than those reported in the present and other papers (Ota *et al.*, 1972; Takahashi *et al.*, 1973). During the extraction of bromelain, the pineapple-stem juice is treated with H_2S at a pH between 3.5 and 5.5 in order to suppress oxidative processes (Heinicke, 1958). The pH-dependence of the crude bromelain-catalysed hydrolysis of *N*-benzoyl-L-arginine ethyl ester is characterized by a bell-shaped pH profile ($[S_0] = 0.1M$) with pK values of 5.70 and 8.40 (Wharton, 1969). The pH values given by Heinicke (1958) cover a considerable range and it is possible that different batches of enzyme, incubated with H_2S at various pH values between 3.5 and 5.5, may vary in the degree of autodigestion that has occurred, since the rate of autodigestion will increase with increasing pH in this range. Autodigestion may also occur during the purification procedure even at 2–4°C if the enzyme is not reversibly blocked. Thus some of the variability, found by various workers, in the properties of stem bromelain may arise from autodigestion, which may occur at two different stages

of the preparation, only one of which may be eliminated unless the manufacturing process can be altered to incorporate reversible blocking of the enzyme during extraction from the stem and stem juice.

Kinetic specificity of purified stem bromelain

The kinetics of bromelain-catalysed reactions have been little studied. Inagami & Murachi (1963) have studied the pH-dependence of the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine ethyl ester and amide. The pH-dependence of the value of K_m for the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine ethyl ester is anomalous in that the profile is bell-shaped having the highest values of K_m near neutrality. The pH- K_m profile is characterized also by an apparent additional ionization, required in the base form for lowering K_m , producing a 'hump' in the profile in the region of pH 4.0–5.0. Thus although the ionization in the alkaline region is fitted reasonably well by a single ionization, the acid region is complex. The associated k_{cat} -pH profile is a bell-shaped curve having an apparently simple ionization in the acid region. That *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester is a much more specific substrate for bromelain than is *N*²-benzoyl-L-arginine ethyl ester is apparent from a comparison of the k_{cat}/K_m values at pH 7.0. The former has a value of approx. $870M^{-1}\cdot s^{-1}$ and the latter of $3.0M^{-1}\cdot s^{-1}$, a 290-fold difference. This specificity for *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester results almost entirely from the large difference between the values of K_m for the two substrates. Indeed, the value of 0.17M for the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine ethyl ester is so large as to suggest that this substrate should be regarded as essentially non-specific. The K_m value found for the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-glycine methyl ester is similar to that found for the serine analogue but surprisingly k_{cat} is 4.85 times as large. This difference in k_{cat} values may be due to steric interference of the serine hydroxymethyl group or to the alkane chain length being insufficiently long to place the serine hydroxyl group in a suitably hydrophilic environment. The second alternative is preferred since peptide inhibitors having leucine on the carboxyl side of phenylalanine are much more effective in inhibiting papain than are those having glycine in this position (Berger & Schechter, 1970). The expression for k_{cat} in the acyl-enzyme mechanism involves k_2 and k_3 and it is important to consider the contribution of each of these rate constants to the observed value of k_{cat} . The bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-glycine nitrophenylester (20% dimethylformamide, pH 7.45) is not characterized by an initial burst of nitrophenol, which indicates that $k_2 \gg k_3$ for this substrate (C. W. Wharton, unpublished work).

If $k_2 \gg k_3$ for *N*-benzyloxycarbonyl-L-phenylalanyl-glycine *p*-nitrophenyl ester then it is most unlikely that $k_2 \gg k_3$ for the methyl ester.

Accordingly it seems reasonable to assume that the $k_{\text{cat.}}$ -pH profile for the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester represents the pH-dependence of k_2 or a rate-limiting conformational change occurring before acylation. Brocklehurst *et al.* (1967) have analysed the data of Inagami & Murachi (1963) for the bromelain-catalysed hydrolysis of *N*²-benzoyl-L-arginine ethyl ester and amide and found that $k_2 \approx k_3$ for the ester. It is surprising that k_2 is apparently not larger than k_3 in the case of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester and the glycine analogue since in the similar papain-catalysed hydrolyses it is apparent that the kinetic specificity is enhanced as a result of the very effective acylation of substrates of this type (Lowe & Yuthavong, 1971a). It is possible that the benzyloxycarbonyl group, which resembles phenylalanine, may bind to the site normally expected to accommodate the L-phenylalanine side chain. This would have the result of placing the L-phenylalanine side chain in the position normally occupied by L-serine. This binding mode, if it occurs, would be non-productive, since *N*-benzyloxycarbonyl-L-phenylalanine has not been found in product-analysis experiments. No initial burst of *p*-nitrophenol is apparent in the bromelain-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-glycine *p*-nitrophenyl ester (pH 7.0, 3.3% acetonitrile) indicating that $k_2 \gg k_3$ (C. W. Wharton, unpublished work). Thus it is unlikely that the relatively low value of $k_{\text{cat.}}$ (k_2) can be ascribed to non-productive binding due to incorrect binding of the benzyloxycarbonyl group.

If $k_2 \ll k_3$, then K_m may be interpreted as K_s assuming $k_{-1} \gg k_2$. In these circumstances it is apparent that *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester is able to bind to bromelain much more effectively than *N*²-benzoyl-L-arginine ethyl ester, but that the increased binding energy cannot be used to facilitate acylation. The above considerations all involve the assumption that bromelain-catalysed hydrolyses proceed via an acyl-enzyme mechanism. Brocklehurst *et al.* (1967) reported the formation of an absorption band at 316nm on admixture of methyl thionohippurate and bromelain. The decay of the absorption band, indicative of deacylation, was very slow and precipitation usually intervened before a significant decrease in the intensity of this band had occurred (Wharton, 1969). At lower pH (4.9), experiments have been more successful in that a 30% decrease in the steady-state concentration of the intermediate was observed before the solution became cloudy (C. W. Wharton, unpublished work). It is thus apparent that the bromelain-catalysed hydrolysis of methyl thionohippurate involves acyl-enzyme formation. It remains a possibility that the adduct absorbing at

316nm represents a dead-end complex. The properties of *trans*-cinnamoyl-bromelain, which has $\lambda_{\text{max.}} = 323\text{nm}$ are in most respects similar to those of *trans*-cinnamoyl-papain (Brubacher & Bender, 1966; C. W. Wharton, unpublished work). These observations suggest that bromelain-catalysed reactions involve the formation of acyl-enzyme intermediates.

pH-dependence of the bromelain-catalysed hydrolysis of N-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester

The pH-dependence of both $k_{\text{cat.}}$ and K_m of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester are of an unusual form. The most marked feature of both pH-dependencies is the ionization seen in the region of pH 6.0. Deprotonation in this pH region leads to better apparent binding and to a decrease in the value of the catalytic rate constant. The value of $k_{\text{cat.}}/K_m$ remains nearly constant, in this region, owing to the compensating effects of $k_{\text{cat.}}$ and K_m . The fall in the magnitude of $k_{\text{cat.}}$ (k_2) at high pH may be ascribed to the lack of the proton necessary for the acid-catalysed decomposition of the tetrahedral intermediate of the acylation reaction. If such an intermediate is involved then a change in the rate-limiting step of acylation is implicated. Thus formation of the tetrahedral intermediate will be rate-limiting at low pH and decomposition of the intermediate will be rate-limiting at high pH. That a decrease in the value of $k_{\text{cat.}}$ was not observed at pH 4.5 indicates that the *pK* of the putative acid limb is less than 4.0 (experiments at pH 4.0, although characterized by very large standard errors, indicate a decrease in the value of $k_{\text{cat.}}$ at this pH). Brocklehurst *et al.* (1972) have studied the pH-dependence of the reaction of bromelain with 2,2'-dipyridyl disulphide. The pH dependence of the reaction of purified (T) bromelain with 2,2'-dipyridyl disulphide is characterized by a number of ionizations, one of which has a *pK* value of 6.4. These authors tentatively suggest that protonation of this group (which is not the active-site histidine group) causes a structural change which results in enhancement of the reactivity of the histidine-cysteine system at the active site. Since only one histidine residue is present in bromelain (Ota *et al.*, 1972; Takahashi *et al.*, 1973) it is reasonable to suggest that this other residue with a *pK* near to 6.0 may be a carboxyl group. Shinitzky & Goldman (1967) have reported that the fluorescent emission intensity of bromelain is influenced by an ionizing group characterized by a *pK* of 6.2. However, by analogy with similar studies on papain it seems likely that this ionization is characteristic of non-activatable bromelain (Sluyterman & De Graaf, 1970). In the case of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester it is proposed that the group with the *pK* of about 6.0, seen

in the pH-dependence of both K_m (K_s) and k_{cat} (k_2), is the same as that observed in the reaction of T-bromelain with 2,2'-dipyridyl disulphide since the enzyme preparation used in the present studies is the 'T' form. Murachi & Yamazaki (1970) have examined the pH-dependence of the optical-rotary dispersion, viscosity and sedimentation coefficient from pH 7 upwards. No evidence of a conformation change was apparent in the neutral pH region although the studies did not cover the pH range (4.0–7.0) of particular interest here. No direct comparison with similar papain-catalysed reactions is possible since the pH-dependence of such papain-catalysed ester hydrolyses have not been reported.

Lowe & Yuthavong (1971b) have determined the pH-dependence of the Michaelis parameters of the papain-catalysed hydrolysis of *N*-acetyl-L-phenylalanyl-glycine *p*-nitroanilide. In this reaction, where $k_{cat} = k_2$ and $K_m = K_s$, the pH-dependence of k_2 is bell-shaped having pK values of 4.2 and 8.2, and K_s is pH-independent between pH 3.7 and 9.3. No kinetic evidence is apparent in the literature which suggests that an ionization near to pH 6.0 affects the Michaelis parameters of the papain-catalysed hydrolysis of neutral ester substrates.

On the presently available evidence it seems that the ionization near pH 6.0 seen in the pH-dependence of the Michaelis parameters of the bromelain-catalysed hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-serine methyl ester is a unique feature of this enzyme amongst the thiol-group proteases.

References

- Anderson, G. W., Zimmerman, J. E. & Callahan, F. M. (1964) *J. Amer. Chem. Soc.* **86**, 1839–1842
- Andrews, P. (1964) *Biochem. J.* **91**, 222–233
- Berger, A. & Schecter, I. (1970) *Phil. Trans. Roy. Soc. London Ser. B* **257**, 249–264
- Bobb, D. (1972) *Prep. Biochem.* **2**, 347–354
- Brocklehurst, K., Crook, E. M. & Wharton, C. W. (1967) *Chem. Commun.* 1185–1187
- Brocklehurst, K., Crook, E. M. & Kierstan, M. J. (1972) *Biochem. J.* **128**, 979–982
- Brocklehurst, K., Carlsson, J., Kierstan, M. J. & Crook, E. M. (1973) *Biochem. J.* **133**, 573–584
- Brubacher, L. J. & Bender, M. L. (1966) *J. Amer. Chem. Soc.* **88**, 5871–5880
- Chervenka, C. H. (1970) *Anal. Biochem.* **34**, 24–29
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dunker, A. K. & Rueckert, R. R. (1969) *J. Biol. Chem.* **244**, 5074–5080
- El-Gharbawi, M. & Whitaker, J. R. (1963) *Biochemistry* **2**, 476–481
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Englund, P. T., King, T. P., Craig, L. C. & Walti, A. (1968) *Biochemistry* **7**, 163–175
- Feinstein, G. & Whitaker, J. R. (1964) *Biochemistry* **3**, 1050–1054
- Gilman, A., Philips, F. S., Kolle, E. S., Allen, R. F. & St. John, E. (1946) *Amer. J. Physiol.* **147**, 115–126
- Greenstein, J. P. & Winitz, M. (1961) *Chemistry of the Amino Acids*, vol. 2, p. 895, Wiley, New York and London
- Hammond, B. R. & Gutfreund, H. (1959) *Biochem. J.* **72**, 349–357
- Heinicke, R. M. (1958) *U.S. Patent* 3002891; *Chem. Abstr.* **56**, 3814
- Inagami, T. & Murachi, T. (1963) *Biochemistry* **2**, 1439–1444
- Kierstan, M. J. (1973) Ph.D. Thesis, University of London
- Lowe, G. & Yuthavong, Y. (1971a) *Biochem. J.* **124**, 107–115
- Lowe, G. & Yuthavong, Y. (1971b) *Biochem. J.* **124**, 117–122
- Maggio, E. T. & Shafer, J. A. (1973) *Anal. Biochem.* **54**, 616–618
- Murachi, T. & Neurath, H. (1960) *J. Biol. Chem.* **235**, 99–107
- Murachi, T. & Yamazaki, M. (1970) *Biochemistry* **9**, 1935–1942
- Murachi, T., Yasui, M. & Yasuda, Y. (1964) *Biochemistry* **3**, 48–55
- Ota, S., Moore, S. & Stein, W. H. (1964) *Biochemistry* **2**, 180–185
- Ota, S., Horie, K., Hagino, F., Hashimoto, C. & Date, H. (1972) *J. Biochem. (Tokyo)* **71**, 817–830
- Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32–95
- Shinitzky, M. & Goldman, R. (1967) *Eur. J. Biochem.* **3**, 139–144
- Sluyterman, L. A. E. & De Graaf, M. J. M. (1970) *Biochim. Biophys. Acta* **200**, 595–597
- Stark, G. R. & Smythe, D. G. (1963) *J. Biol. Chem.* **238**, 214–226
- Susuki, K., Abiko, T. & Eudo, N. (1969) *Chem. Pharm. Bull.* **17**, 1671–1678
- Takahashi, N., Yasuda, Y., Goto, K., Miyak, J. & Murachi, T. (1973) *J. Biochem. (Tokyo)* **74**, 355–373
- Vogler, K., Lanz, P. & Lergier, W. (1962) *Helv. Chim. Acta* **45**, 561–568
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wharton, C. W. (1969) Ph.D. Thesis, University of London
- Wharton, C. W., Crook, E. M. & Brocklehurst, K. (1968) *Eur. J. Biochem.* **6**, 565–571
- Wharton, C. W., Cornish-Bowden, A., Brocklehurst, K. & Crook, E. M. (1974) *Biochem. J.* **141**, 365–381
- Whitaker, J. R. (1963) *Anal. Chem.* **35**, 1950–1953
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317