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Purification and Properties of a Proteolytic Enzyme from French Beans

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1. A proteolytic enzyme with some features of a carboxypeptidase has been purified some 1180-fold from the sap of French beans (*Phaseolus vulgaris* var. Prince). A bright blue protein, plastocyanin, was separated from the enzyme by DEAE-cellulose chromatography. 2. Unlike carboxypeptidase A or B of animal origin, there is no evidence that the enzyme is a metalloprotein. There was no stimulation of activity by a number of metal ions, reducing agents or 2-mercaptoethanol. Neither EDTA nor 1,10-*o*-phenanthroline inhibited the enzyme. 3. The proteolytic enzyme from beans, readily soluble at neutral or slightly acidic pH values, has a pH optimum of pH 5·6 for the hydrolysis of leucine from benzyloxycarbonylglycyl-L-leucine. Solutions of the enzyme in 0·1 M-sodium acetate, pH 5·5, lose about 2% of their activity/week at 4°. Virtually no loss of activity results after prolonged storage at −15°. 4. Incubation of the bean enzyme with peptides indicates that the enzyme will release acidic, neutral and basic amino acid residues as well as proline, although adjacent acidic residues in a peptide appear to inhibit the enzyme. The possibility of endopeptidase activity in the purified preparation requires further examination.

From discrepancies in the values obtained for the *C*-terminal groups of virus preparations isolated from French beans (*Phaseolus vulgaris* var. Prince), and because of the loss of *C*-terminal threonine from tobacco mosaic virus after incubation with bean sap, M. W. Rees & M. N. Short (unpublished work) of this Laboratory concluded that bean sap contained an enzyme with carboxypeptidase-like activity.

The presented results confirm this suggestion and describe a 1180-fold purification from bean sap of a proteolytic enzyme with some of the properties of a carboxypeptidase. These properties are compared with those of bovine pancreatic carboxypeptidase A (Vallee, Rupley, Coombs & Neurath, 1960) and with carboxypeptidase C (Zuber, 1964), the latter being the only carboxypeptidase reported from plant tissues.

Since the objective was to ascertain whether bean sap contained an enzyme with carboxypeptidase activity, proteolytic activity was assayed at all stages of purification with substrates such as benzyloxycarbonylglycyl-L-leucine. Although the purified enzyme from the sap exhibits some of the properties of a carboxypeptidase, the possiblity of endopeptidase activity in this preparation also exists.

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MATERIALS

All organic reagents or organic solvents used were of analytical grade.

Buffers. Phosphate buffers were prepared by adjusting solutions of NaH_2PO_4 of appropriate molarity to the required pH with N-NaOH. Similarly, acetate buffers were prepared by adjusting solutions of acetic acid to the required pH with N-NaOH. A mixed phosphate–acetate buffer was used for the determination of the pH optimum of the bean enzyme. Solutions of 0·5 M-Na_2HPO_4 were adjusted to the required pH (from 7·0 to 4·14) with 2N-acetic acid and diluted with water to contain a final concentration of 0·25 M-phosphate.

Enzyme substrates. Benzyloxycarbonylglycyl-L-tryptophan, benzyloxycarbonylglycyl-L-leucine and benzyloxycarbonylglycyl-L-phenylalanine were products of Mann Research Laboratories Inc. (New York, N.Y., U.S.A.).

Peptides. The peptide Tyr-Thr-Pro-Lys-Ala (IP-3; Ambler, 1963) from the *C*-terminal end of the B chain of insulin was kindly supplied by Dr R. P. Ambler (Medical Research Council Laboratory of Molecular Biology, Cambridge). Two other peptides were isolated from the tryptic digests of TMV† protein. The first, having the

† Abbreviations: TMV, tobacco mosaic virus; BGL, benzyloxycarbonylglycyl-L-leucine.

sequence Asp-Glu-Leu-Arg (A-4), was obtained from a strain of TMV (M. W. Rees, unpublished work) and the second, T-12, comprised the 17 *C*-terminal amino acid residues of type strain TMV protein (Tsugita *et al.* 1960).

Resins. DEAE-cellulose (Eastman Kodak Co., Rochester, N.Y., U.S.A.) was prepared according to the method of Peterson & Sober (1956). Chelex-100 resin (100–200 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) was heated at 80° for 2hr. in aq. 3N-NH_3 soln., then washed with N-HCl, 0·5N-NaOH and water. A 10% (w/v) suspension of resin in 0·2M-Na_2CO_3 was used.

Calcium phosphate gel. The gel (13·1% of solids) was a product of Sigma Chemical Co., St. Louis, Mo., U.S.A.

Trypsin. The crystalline freeze-dried enzyme was a product of Worthington Biochemicals Corp., Freehold, N.J., U.S.A.

METHODS

Preparation of a stable extract for enzyme purification. French beans (*Phaseolus vulgaris* var. Prince) were harvested about 10 days after emergence (plants about 12in. high, showing third trifoliate leaves) and macerated in a large mechanical mincer in sufficient quantity to yield 2l. of sap. After screening through two layers of muslin, the sap was centrifuged at 4° in a Serval RC-2 refrigerated centrifuge at 10000rev./min. for 60min. All subsequent procedures were carried out at 4°. The supernatant was brought to 40% saturation of $(NH_4)_2SO_4$ by addition of the salt, and the mixture was stirred slowly for 1hr. after the solid had dissolved. The precipitate was removed by centrifuging at 10000rev./min. for 10min. and the amber supernatant was adjusted to 80% saturation with $(NH_4)_2SO_4$. The precipitate resulting from 40–80% saturation of bean sap with $(NH_4)_2SO_4$ was collected by centrifuging at 10000rev./min. for 10min., drained of excess of liquid, and the pellets were then homogenized and washed with acetone at −15° (Millerd, Morton & Wells, 1963); the dry powder was stored at −15°. Batch-wise preparations as described above yielded 200g. of acetone-dried powder preparation from 26l. of bean sap; the proteolytic activity towards BGL of this material stored at −15° did not decline over a period of several months.

Enzyme assay. (*a*) Initial extracts. Assays for proteolytic activity in initial plant extracts were carried out with benzyloxycarbonylglycyl-L-tryptophan as substrate. In general, reaction mixtures (1·5ml.) contained 5μmoles of this substrate and 20–100mg. of plant extract in 0·1M-NaH_2PO_4, pH6·0. Reactions were stopped at zero time and at appropriate time-intervals (4–16hr.) by boiling for 2min.; denatured protein was removed by centrifuging at 5000rev./min. for 5min. and the supernatants were passed through columns (8cm.×3mm.) of Dowex 50 (H^+ form). After washing the columns with water, adsorbed compounds were eluted with aq. N-NH_3 soln. (2·0ml.) and the eluates were dried with a stream of air at 40°. The dried residues were dissolved in 0·1ml. of water, and portions (usually 50μl.) were applied to Whatman 3MM paper. Amino acids were separated by chromatography with propan-1-ol–water (7:3, v/v) with which NH_3 is present in the vapour phase (Markham & Smith, 1952). Tryptophan, arising from enzymic hydrolysis of benzyloxycarbonylglycyl-L-tryptophan, was located by virtue of its fluorescence in ultraviolet light. Interference from ultraviolet-absorbing compounds, present in considerable quantities in initial plant extracts, was overcome by making fluorescence prints of the chromatograms (Smith & Markham, 1950). Since endogenous amino acids were also present in very large amounts in these extracts, it was found impossible to identify tryptophan, leucine or phenylalanine specifically hydrolysed from benzyloxycarbonylglycyl-L-tryptophan, BGL or benzyloxycarbonylglycyl-L-phenylalanine by the ninhydrin procedure. The fluorescence technique, with benzyloxycarbonylglycyl-L-tryptophan as substrate, although qualitative, provided a highly sensitive and specific assay for carboxypeptidase-like proteolytic activity.

(*b*) Purified fractions. When the bean proteolytic enzyme had been further purified, it was possible to assay its activity by a quantitative ninhydrin technique (Chibnall, Mangan & Rees, 1958). Reaction mixtures (1·5ml.) incubated at 30° contained 10μmoles of BGL and 0·5ml. of enzyme (5–60mg. of protein) and a final concentration of 0·05M-sodium acetate, pH5·6. At zero time and at 15min. intervals, 0·1ml. portions of the reaction mixtures were removed and the amount of leucine hydrolysed from BGL was calculated from a standard calibration graph of leucine assayed under the same conditions.

A semi-quantitative ninhydrin procedure was also used to scan enzyme activity eluted from DEAE-cellulose (see Table 1 and Fig. 1). Portions (0·05ml.) from each fraction were incubated with 0·2ml. of 0·01M-BGL. At zero time, 30min. and 60min. after the addition of enzyme to the substrate, 10μl. portions were removed from reaction mixtures and spotted and dried on Whatman 3MM paper. Enzymic activity was manifested by the intensity of the spots after dipping the paper in 0·5% (w/v) ninhydrin in acetone. It was possible to detect 0·5μg. of leucine by this method.

Protein determination. Protein was estimated with the Folin–Ciocalteu reagent as described by Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as a protein standard.

Ultrafiltration. Dilute enzyme solutions were concentrated by ultrafiltration essentially as described by Gibbins & Norris (1963). Frequent changes of equilibrating buffer in the evacuated vessel were made.

Unit of enzyme activity. One unit of activity is defined as the amount of enzyme required to hydrolyse 1μmole of leucine from BGL/min. in the above quantitative assay. The specific activity of the bean proteolytic enzyme is expressed as the number of μmoles of leucine hydrolysed from BGL/min./mg. of protein.

Preparation of calcium phosphate gel. The gel was made into a slurry with sufficient 0·25M-NaH_2PO_4 to reduce the pH of the mixture to pH5·5. After thorough mixing, the liquid was removed by centrifuging at 1000rev./min. for 5min. and the gel was washed four times with an equal volume of water. After two further washes with 0·02M-NaH_2PO_4, pH5·5, the gel was suspended in an equal volume of the same buffer and the mixture shaken mechanically for 3hr. to ensure complete dispersion of the gel. Liquid remaining above the solids after standing for 48hr. at 4° was removed by suction and the remaining fine white suspension was used for enzyme purification.

DEAE-cellulose chromatography. The washed resin was dispersed in water and poured into a glass column (1·5cm. diam.) fitted with a sintered-glass filter. The slurry was added in five successive stages and gradually increasing N_2 pressure from 1 to 5lb./in.^{2} was used to pack the column to

a height of 20 cm. The column was thoroughly equilibrated with 0·05 M-sodium acetate, pH 6·0, at 4° and chromatography of the concentrated blue protein fraction eluted from $Ca_3(PO_4)_2$ gel (see Table 1) was effected with a linear gradient (500 ml.) from 0·05 M- to 0·3 M-sodium acetate, pH 6·0; the flow rate was about 1 ml./min. The extinctions of the fractions (4 ml.) were determined at 278 mμ (1 cm. light-path) and 0·05 ml. portions of each fraction were removed for enzymic assay by a semi-quantitative ninhydrin technique with BGL as substrate.

Removal of bivalent cations from reaction mixtures. A number of bivalent cations interfere with ninhydrin assays. In those experiments in which the effects of Zn^{2+}, Co^{2+}, Cu^{2+} and Fe^{2+} on the bean enzyme activity were tested (see Table 2), the metals were removed from reaction mixtures, before the estimation of leucine, with Chelex-100 resin. The enzymic reactions were stopped by boiling and 1·0 ml. portions of a suspension of Chelex-100 resin (10%, w/v, in 0·2 M-Na_2CO_3) were added; the final pH was about 9·0. After thorough mixing (10 min.) the resin was precipitated by centrifuging at 3000 rev./min. at 5 min. and 2·0 ml. portions of the supernatants were removed into test tubes; 0·2 ml. of N-HCl was added to each to bring the pH to about pH 5·5. Control experiments showed that complete recovery of leucine could be obtained by this method whereas failure to remove traces of the bivalent cations before assay resulted in low and unreliable ninhydrin colour development.

Preparation of protein from TMV. Nucleic acid was dissociated from TMV with 67% (v/v) acetic acid (Fraenkel-Conrat, 1957). The protein solution was equilibrated with 8 vol. of acetone for 16 hr. at room temperature and the flocculated protein was collected, washed and dried (M. W. Rees & M. N. Short, unpublished work).

Digestion of TMV protein with trypsin. A weighed amount (100–200 mg.) of TMV protein was thoroughly dispersed in 5 ml. of 1% (w/v) NH_4HCO_3 with the aid of a glass homogenizer. To this white suspension was added a drop of octan-2-ol and a solution of trypsin (1–2 ml. in water) corresponding to 1% (w/w) of the substrate. The mixture was incubated at 37° for 3–4 hr. and any residue left undigested was removed by centrifuging at 2000 rev./min. for 10 min. The digest was freeze-dried and stored at −15°.

Isolation of peptides from tryptic digests of TMV protein. Peptides from tryptic digests of TMV protein were separated by paper electrophoresis in pyridine–acetate buffer, pH 6·5, on sheets of paper 63·5 cm. × 25·5 cm. (Michl, 1951). The load for Whatman 3MM paper was 0·4 mg. of peptides/cm. and for Whatman no. 17 paper, 1 mg./cm. Potentials of 31·5 v/cm. for 2·5 hr. or 15·7 v/cm. for 5 hr. were applied. Peptides were located on 1 cm. strips cut from either side of the preparative sheets by chlorination (Reindel & Hoppé, 1954); the appropriate peptide bands were eluted from paper with aq. 0·2 N-NH_3 soln., freeze-dried and residues were made up to 2·0 ml. in 0·1 M-sodium acetate, pH 5·6.

Analysis of peptides. Portions (0·25 ml.) of each peptide were hydrolysed by refluxing with 6·0 ml. of constant-boiling HCl for 24 hr. in round-bottomed flasks fitted with air condensers. The excess of acid was removed on a rotary evaporator at 40° and the residue dissolved in citrate buffer, pH 2·2 (2·5 ml.); 1·0 ml. portions were applied to the appropriate columns for the estimation of basic and acidic and neutral amino acids on a Beckman–Spinco automatic amino acid analyser.

Estimation of enzymic hydrolysis of peptides. Each peptide solution (1·0 ml.) was incubated with 0·25 ml. (40 μg. of protein) of purified bean proteolytic enzyme for 16 hr. at 30°. Portions (0·4–0·5 ml.) of the reaction mixtures were analysed for free amino acid content on an automatic amino acid analyser. The degree of enzymic hydrolysis was expressed as a percentage of the value for the particular amino acid as found by acid hydrolysis of the appropriate peptide. In those instances in which peptides interfered with identification or estimation of amino acids on the automatic amino acid analyser, further portions of the reaction mixture were desalted and freeze-dried. The residue was dissolved in water (20–50 μl.) and peptides were separated from amino acids by paper electrophoresis at pH 6·5. The amino acids eluted from the paper with aq. 0·2 N-NH_3 soln. were freeze-dried and the residues dissolved in citrate buffer (pH 2·2). Quantitative estimation of these amino acids was then carried out with the amino acid analyser. The recovery of amino acids after this treatment was about 85%. The appropriate peptide and enzyme blanks were also run; in no case was there any measurable content of free amino acids in these samples.

RESULTS

Purification of the proteolytic enzyme from bean sap

Extraction of acetone-dried powder at pH 4·0. All purification procedures were carried out at 4°. The acetone-dried powder from the 40–80% saturation of ammonium sulphate of bean sap (200 g.; see the Methods section) was thoroughly dispersed in 3200 ml. of 0·05 M-sodium acetate, pH 4·0, and extracted for 10 min. This treatment resulted in a massive precipitation of protein and material derived from nucleic acid normally soluble near neutrality. The precipitate was removed by centrifuging at 10000 rev./min. for 10 min. and the supernatant containing the enzyme was adjusted to pH 5·5 by the dropwise addition of 2 N-sodium hydroxide.

Ammonium sulphate fractionation. The brown solution of protein (3150 ml. containing 29·2 g. of protein) was brought to 60% saturation of ammonium sulphate by slow addition of 1268 g. of the solid salt over a period of 90 min. The final volume was adjusted to 4200 ml. with water and the mixture was stirred slowly for a further 60 min. The precipitate which formed was removed by centrifuging at 10000 rev./min. for 10 min. and discarded. The dark-brown supernatant (containing 2·6 g. of protein) was brought to 75% saturation with ammonium sulphate (480 g. of ammonium sulphate) and the final volume adjusted to 4600 ml. After further slow stirring (60 min.) the mixture was centrifuged and the supernatant discarded. The protein precipitate was dissolved in 1 l. of 0·02 M-sodium phosphate, pH 5·7, and the blue–black solution of protein was dialysed against two changes of 10 l. of the same buffer for 16 hr. The small amount of denatured protein present after dialysis was removed by centrifuging.

Adsorption of inert proteins on calcium phosphate gel. To the supernatant solution (1080ml.), 120ml. of calcium phosphate gel (see the Methods section) was added and the mixture was stirred for 15min. The gel adsorbed most of the coloured components from the solution and a considerable quantity of enzymically inactive protein. The gel was removed by centrifuging at 2000rev./min. for 5min. and discarded.

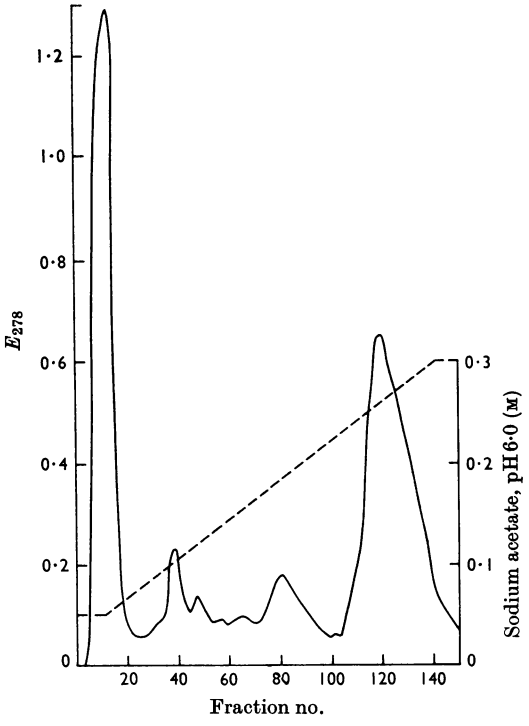

Fig. 1. The concentrated blue protein solution eluted from $Ca_3(PO_4)_2$ gel (30ml., 260mg. of protein; Table 1) was chromatographed on a column (20cm. × 1·5cm.) of DEAE-cellulose at pH6·0. Extinctions (1cm.) of fractions (4ml.) at 278mμ were measured (——) and 0·05ml. portions of each fraction were removed for assay of enzyme activity by a semi-quantitative ninhydrin assay (see the Methods section). A linear gradient of sodium acetate (0·05–0·3M, 500ml.; – – –) separated the fraction containing proteolytic activity (fractions 73–100) from the blue protein (fractions 105–145).

Adsorption and elution from calcium phosphate gel. A further 600ml. of calcium phosphate gel was added to the supernatant from the preceding step (1130ml.; about 1g. of protein) and the mixture was stirred for 30min. The gel containing the adsorbed bean proteolytic enzyme was collected by centrifuging at 2000rev./min for 5min. and was then washed with 2 × 600ml. of 0·02M-sodium phosphate, pH5·7, the supernatant solutions being discarded each time. The enzyme was extracted from the gel by three successive washes with 600ml. of 0·12M-sodium phosphate, pH5·7. On each occasion the gel was thoroughly dispersed in the buffer and stirred for 30min.; the supernatants were collected after low-speed centrifugation. The light-blue protein solution (1770ml.; about 460mg. of protein) was concentrated to 30ml. by ultrafiltration (see the Methods section) against frequent changes of 0·05M-sodium acetate, pH6·0. The concentrated solution of protein had one distinct absorption maximum at 597mμ in the visible region; the ratio $E_{278m\mu}/E_{597m\mu}$ was 3·1.

DEAE-cellulose chromatography at pH6·0. A column of DEAE-cellulose, 20cm. × 1·5cm., was thoroughly equilibrated with 0·05M-sodium acetate, pH6·0 (see the Methods section). A compact blue band, about 1cm. in depth on top of the column, marked the zone of protein adsorption. The linear gradient elution sequence which separated the blue protein from the fraction containing proteolytic activity is shown in Fig. 1. Fractions (4ml.) numbered 73–100 were combined and concentrated (12mg.). The separated blue protein had a ratio ($E_{278m\mu}/E_{597m\mu}$) 1·1.

DEAE-cellulose chromatography at pH5·0. Further purification of the enzyme could be obtained by similar chromatography with sodium acetate at pH5·0. However, good resolution of inert protein

Table 1. *Purification of the proteolytic enzyme from bean sap*

Experimental details are given in the text.

Stage of purification	Vol. of extract (ml.)	Total protein (mg.)	Total units	Sp. activity (units/mg. of protein)	Recovery (%)
Supernatant after extraction at pH4·0 of acetone-dried powder material	3240	29160	111·7	0·004	100
60–75% saturation with $(NH_4)_2SO_4$ and dialysis	1080	2614	96·1	0·026	86·0
Adsorption of inert proteins on $Ca_3(PO_4)_2$ gel	1130	944	81·3	0·086	72·8
Elution from $Ca_3(PO_4)_2$ gel with 0·12M-phosphate	1770	460	73·7	0·160	66·0
Ultrafiltration, removal of denatured protein	30	260	69·9	0·269	62·6
Elution from DEAE-cellulose, pH6·0	100	12	56·8	4·73	50·8

from the proteolytic enzyme at this lower pH value depended on a gradient elution starting at low ionic strength (0·01M) and equilibration of the enzyme with 0·01M-sodium acetate, pH 5·0, resulted in the loss of nearly 70% of the original activity. This procedure is therefore not included in the overall purification scheme shown in Table 1.

pH *and the hydrolysis of leucine from BGL by the bean proteolytic enzyme*

The activity of the bean enzyme was tested over a range pH 4·14–7·0 with BGL as substrate. Phosphate–acetate buffers (see the Methods section) of the following values were prepared: pH 4·14, 4·5, 4·8, 5·0, 5·2, 5·4, 5·7, 6·0, 6·5 and 7·0. Reaction mixtures incubated at 30° contained 1·4ml. of the appropriate buffer, 0·5ml. of 0·01M-BGL (in 0·02M-sodium acetate, pH 5·5) and 0·1ml. of purified enzyme (about 4μg. of protein). Reactions were stopped at zero time and after 60min. by boiling for 2min. and the degree of hydrolysis of leucine from BGL was determined in each case by quantitative ninhydrin assays. Previous experiments under similar conditions showed that the rate of hydrolysis of leucine was constant throughout the 1hr. incubation.

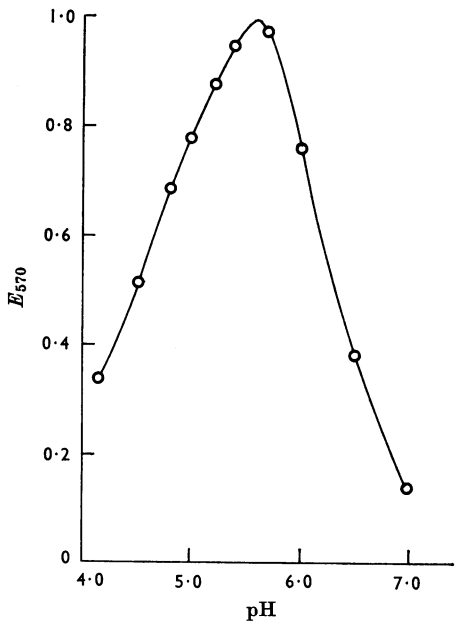

Fig. 2. Rate of hydrolysis of leucine from BGL by the proteolytic enzyme from beans as a function of pH was tested over the range pH 4·14–7·0. Reaction mixtures incubated at 30° contained 1·4ml. of the appropriate phosphate–acetate buffer (see the Methods section), 0·5ml. of 0·01M-BGl and 0·1ml. (4μg. of protein) of enzyme. Reactions were stopped at zero time and after 60min. by boiling (2min.) and extinctions at 570mμ were read after assay with ninhydrin.

The results (Fig. 2) indicate that bean proteolytic enzyme has an optimum at pH 5·6 for BGL and that enzymic activity decreases rapidly on either side of this value, particularly towards more alkaline pH values.

Although phosphate ions apparently inhibit carboxypeptidase C (Zuber, 1964) and carboxypeptidases of animal origin (Smith & Hanson, 1949) the activity of the bean enzyme towards the carboxypeptidase substrate (BGL) at pH 5·6 is some 20% higher with 0·25M-sodium phosphate than with 0·25M-sodium acetate.

Effects of some metal ions on the activity of bean proteolytic enzyme

Pancreatic carboxypeptidase A is a metalloprotein which contains 1g.atom of zinc/mole of enzyme protein (Vallee & Neurath, 1955). The metal component can be removed by dialysis at more acidic values than pH 5·5 or at neutral pH with 1,10-*o*-phenanthroline; in either case, the loss of enzymic activity is directly proportional to the amount of zinc removed (Vallee *et al.* 1960). Complete reactivation can be effected by the addition of Zn^{2+}. Further, other metal ions, notably those of the first transition series, will form complexes with the apoprotein of carboxypeptidase. Compared with the native enzyme these complexes exhibit decreased activity towards carboxypeptidase A substrates with the exception of cobalt carboxypeptidase which has about twice the activity of the zinc-containing enzyme. The copper complex is completely inactive (Valle *et al.* 1960).

Four metal ions, Zn^{2+}, Co^{2+}, Cu^{2+} and Fe^{2+}, were tested for their effects on purified bean proteolytic enzyme. The enzyme was diluted in 0·2M-sodium acetate, pH 5·6, to contain 40μg. of protein/ml. Portions (0·1ml.) were added to 0·1ml. of the appropriate solutions (2mM) of metal salts (all sulphates, except cobaltous chloride) and the mixtures were kept at 4° for 16hr. before the addition of substrate. The substrate solution contained the appropriate metal salt (mM), 10μmoles of BGL and a final concentration of 0·1M-sodium acetate, pH 5·6. The substrate–metal solution (1·5ml.) was added to the enzyme–metal solution (0·2ml.) and incubated at 30° for 1hr. The degree of hydrolysis of leucine from BGL in each case was determined with ninhydrin after the removal of metal ions from the reaction mixtures with Chelex-100 resin (see the Methods section).

None of the metal ions tested increased the activity of the bean enzyme and both Cu^{2+} and Fe^{2+} were inhibitory (see Table 2).

Effects of chelating agents on bean proteolytic enzyme

Solutions of EDTA (50mM), 1,10-*o*-phenanthroline (10mM) and 2-mercaptoethanol (50mM) were dissolved in 0·1M-sodium acetate and adjusted to pH7·0. Portions (0·1ml.) of these solutions were added to 0·1ml. of purified enzyme (diluted with 0·1M-sodium acetate, pH7·0, to contain 40μg. of protein/ml.) and the mixtures kept at 4° for 16hr. before the addition of substrate. A substrate–chelate–buffer mixture [5μmoles of BGL, EDTA (25mM) or 1,10-*o*-phenanthroline (5mM) or 2-mercaptoethanol (25mM) and 0·1M-sodium acetate, pH5·6] was prepared for each chelating agent and 1·0ml. portions were added to the enzyme–chelate mixture (0·2ml.). Incubations (30°) were stopped at zero time and after 60min. by boiling for 2min. Those reaction mixtures containing 2-mercaptoethanol were dried at 60° with a stream of air followed by three cycles of addition of 1·0ml. of water and drying. The residues, now free of 2-mercaptoethanol, were made up to a volume of 1·2ml. and assayed with the other samples with ninhydrin.

Although EDTA appeared to cause 30% inhibition of the enzyme, it was found that this discrepancy from the control value was probably due to a pH effect. Whereas all other reaction mixtures were at pH5·6, those containing EDTA were at pH6·1 and this difference in pH values would be sufficient to account for the apparent inhibition (see Fig. 2). The addition of 2-mercaptoethanol resulted in some 21% inhibition of the enzyme, suggesting that there is no requirement for SH groups; 1,10-*o*-phenanthroline had no effect on the enzymic activity (Table 2).

Dialysis of the enzyme against buffers of low ionic strength results in gradual loss in activity; complete inactivation occurs within 24hr. if the enzyme is dialysed against distilled water. Attempts to reactivate partially or completely inactivated preparations with Zn^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+} or Hg^{2+} with or without 2-mercaptoethanol or sodium dithionite were completely unsuccessful.

Table 2. *Effects of some metal ions and chelating agents on the activity of the proteolytic enzyme from beans*

Purified bean proteolytic enzyme (4μg. of protein in 0·1ml.) was equilibrated with an equal volume of each of the additives at the concentrations shown for 16hr. at 4°. The substrate (5 or 10μmoles) of BGL containing the same concentration of metal ions or chelating agent as shown in the Table and a final concentration of 0·1M-sodium acetate, pH5·6) was added, and incubations (30°) were stopped at zero time or after 60min. by boiling. The amount of leucine hydrolysed from BGL was estimated with ninhydrin and activities are expressed as percentages of the controls. Details of the removal of metal ions or 2-mercaptoethanol from reaction mixtures before the estimation of leucine are given in the text.

Addition to reaction mixtures	Concn. of additive (mM)	Activity (% of controls)
$ZnSO_4$	1	90·3
$CoCl_2$	1	100·0
$CuSO_4$	1	13·7
$FeSO_4$	1	11·3
EDTA	25	104·0*
1,10-*o*-Phenanthroline	5	99·0
2-Mercaptoethanol	25	79·0

* Value corrected to allow for final pH of reaction mixture (see the text).

Hydrolysis of peptides by bean proteolytic enzyme

The three peptides, IP-3, A-4 and T-12 (see the Materials section), were chosen as substrates for the bean enzyme chiefly to compare the specificity of this enzyme with carboxypeptidases A and B of animal origin and with carboxypeptidase C isolated from the peel of citrus fruit.

Incubation of peptide IP-3 (Tyr-Thr-Pro-Lys-Ala) with carboxypeptidases A and B would result in the liberation of only alanine, owing to the position of the proline residue. The presence of a basic amino acid, lysine, also provided information about the specificity of the proteolytic enzyme from beans. The second peptide substrate, A-4 (Asp-Glu-Leu-Arg), having a *C*-terminal arginine residue, further tested the ability of the bean enzyme to release basic residues, and as such indicated its possible use in the analysis of peptides from tryptic digests. The presence of adjacent acidic amino acids was also pertinent to the specificity requirements of the enzyme.

In view of the considerable changes in the physical properties of TMV after incubation of the virus with carboxypeptidase A *in vitro* (Harris & Knight, 1955), it was decided to determine whether the bean enzyme would release threonine from the carboxyl end of T-12, the *C*-terminal peptide of TMV protein.

(*a*) *Peptide IP-3.* This peptide (about 1·4μmoles/ml.) was incubated with the purified bean enzyme as described in the Methods section. The results from enzymic hydrolysis (Table 3) are reasonably consistent with the established sequence of the peptide: Tyr-Thr-Pro-Lys-Ala. It is apparent that alanine (100% hydrolysis) does not hinder the enzyme, whereas the next residue, lysine (24·5% hydrolysis), is probably rate-limiting. The recovery of proline (21·0% hydrolysis) and tyrosine (24·3% hydrolysis) implies that some 0·3μmole of threonine (see Table 3) should have been recovered. Although a very small amount of free threonine was

found in one analysis, its virtual absence in analyses of the reaction mixture is unexplained at present. Incubation of the proteolytic enzyme from beans with peptide IP-3 indicates that this enzyme will hydrolyse neutral amino acids (like carboxypeptidase A) and, more slowly, basic residues (like carboxypeptidase B). In common with carboxypeptidase C, the bean enzyme will release proline from peptides.

(*b*) *Peptide A*-4. Asp-Glu-Leu-Arg. Incubation of A-4 (about 0·4 μmole of peptide/ml.) with the bean enzyme confirmed that it is able to hydrolyse *C*-terminal basic amino acids, in this instance, arginine (Table 3). Somewhat unexpectedly, leucine was released very slowly. Since BGL is an excellent substrate for the enzyme, it is probable that it was the proximity of two adjacent acidic residues in the peptide (almost unhydrolysed) rather than leucine itself which prevented further hydrolysis of the peptide by the enzyme.

(*c*) *Peptide T*-12. The primary structure of the *C*-terminal tryptic peptide from TMV protein (Tsugita *et al.* 1960; Anderer, Uhlig, Weber & Schramm, 1960) is: Ser-Ser-Phe-Glu-Ser-Ser-Ser-Gly-Leu-Val-Trp-Thr-Ser-Gly-Pro-Ala-Thr. Considerable difficulty was experienced in obtaining good yields of the peptide and this was particularly so if the material was purified by paper chromatography after paper electrophoresis at pH 6·5. Contaminants of lysine, arginine and isoleucine (16–20% of a residue/mol.) were present.

The results of incubation of bean proteolytic enzyme with peptide T-12 (about 0·4 μmole/ml.) show that the enzyme will release *C*-terminal threonine from the peptide; 69% of the total threonine (2 residues/mol.) was recovered. Alanine (28·5%) and proline (12·6%) were also released.

Because of the non-kinetic nature of the experiment, it is difficult to assess the significance of the percentage enzymic hydrolysis of those amino acids present as more than 1 residue/mol. of the peptide. Although tryptophan was not estimated, valine (87·8%), leucine (52·1%), glutamic acid (32·9%) and phenylalanine (47·6%) were recovered in appreciable quantities. While it is conceivable that the bean enzyme has some endopeptidase activity, the possibility of more than one *C*-terminal residue being present in the substrate due to chymotryptic activity in the tryptic digest of TMV protein cannot be dismissed. Although the primary interest here was to determine whether bean carboxypeptidase would release the *C*-terminal threonine from the TMV sequence, the possibility of endopeptidase activity in the bean enzyme requires further clarification.

Table 3. *Enzymic hydrolysis of two peptides by the proteolytic enzyme from beans*

The peptides (1·0 ml.) were incubated with 0·25 ml. (40 μg. of protein) of purified bean enzyme for 16 hr. at 30°. Amino acids were assayed on an automatic amino acid analyser. The degree of enzymic hydrolysis of each amino acid is expressed as a percentage of the total amount of the appropriate amino acid present in the peptide as found by acid hydrolysis.

Peptide	Concn. (μmoles/ml.)	Sequence	Enzymic hydrolysis (%)
IP-3	1·4	Tyr-Thr-Pro-Lys-Ala	Ala (100), Lys (24·5), Pro (21·0), Thr?*, Tyr (24·3)
A-4	0·4	Asp-Glu-Leu-Arg	Arg (58·5), Leu (16·3), Glu (about 3), Asp (about 3)

* Absence of Thr unexplained (see the text).

DISCUSSION

The results presented here are primarily concerned with the purification and properties of a proteolytic enzyme with some features of a carboxypeptidase from bean sap. However, mention should also be made of the bright-blue protein also isolated from the sap which was finally separated from the enzyme by DEAE-cellulose chromatography (Fig. 1). There is every indication that the coloured protein is identical with plastocyanin isolated from spinach leaves (Katoh, Shiratori & Takamiya, 1962). The ultraviolet-absorption spectrum showed the same fine structure bands at 253, 259, 265 and 269 mμ attributable to phenylalanine, a main peak at 278 mμ and a shoulder at 284 mμ (tyrosine). These characteristics reflect a low content of tryptophan and tyrosine compared with phenylalanine. In the visible region, the most prominent band had an absorption maximum at 597 mμ; less well-defined bands occurred at 770 mμ and 460 mμ. Complete loss of colour could be effected by the addition of reducing agents (ascorbic acid, sodium dithionite) or chelating agents (potassium cyanide, 1,10-*o*-phenanthroline), whereas the fine structure bands in the ultraviolet region remained. A gradual decrease in the shoulder at 284 mμ accompanied prolonged dialysis and was probably a reflexion of partial denaturation of the copper-containing protein. The ratio ($E_{278m\mu}/E_{597m\mu}$) 1·1 of the plastocyanin from beans indicates that the purity of this preparation approached that of other workers (Katoh *et al.* 1962).

The proteolytic enzyme from bean sap differs from carboxypeptidase A or B isolated from animal tissues chiefly in that it does not appear to be a metallo-enzyme. There was no evidence for stimu-

lation of activity by a wide range of metal ions and, furthermore, treatment of the enzyme with EDTA or 1,10-*o*-phenanthroline resulted in negligible losses in enzymic activity. Unlike carboxypeptidase A, the bean enzyme is readily soluble in dilute buffers at neutral or slightly acidic pH values.

In terms of properties and specificities, the bean enzyme is like carboxypeptidase C of citrus peel (Zuber, 1964) in many respects, The pH optima, inhibition by Fe^{2+} and the stabilizing effect of salts are similar for both. Although the presence of two adjacent acidic residues appears to inhibit the action of the bean proteolytic enzyme (Table 3), both of the plant enzymes are capable of releasing single acidic residues as well as neutral and basic amino acids. Their ability to hydrolyse proline from peptides clearly distinguishes the plant enzymes from carboxypeptidases of animal origin.

Carboxypeptidase C differs in at least two respects from the bean enzyme. First, it is inhibited by phosphate ions, whereas the bean enzyme is not; secondly, purified carboxypeptidase C is unstable (Zuber, 1964). Equilibration of the proteolytic enzyme from beans with buffers of low ionic strength (0·02 M or less) or with distilled water does lead to inactivation, but the purified enzyme only loses about 2% of its activity per week when stored at 4° in 0·1 M-sodium acetate, pH 5·5. Of greater importance is the stability of the enzyme of freezing; solutions of purified enzyme have been stored at −15° for 4 months with virtually no loss in activity.

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