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CHARACTERISATION OF THREE PROTEOLYTIC ENZYMES FROM FRENCH BEANS

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SUMMARY

1. Three enzymes with proteolytic activity towards casein at pH 6.0 have been isolated from the sap of French bean leaves.

2. Only one of the enzymes, which has been named phaseolain, readily hydrolyses carboxypeptidase-like, N-substituted dipeptides of the type Z-Gly–Leu. Z-Phe–Leu is a particularly good substrate for phaseolain.

3. Of the other two enzymes, proteinase *a* is readily separated from phaseolain by DEAE-cellulose chromatography, whereas proteinase *b* chromatographs with phaseolain in this system. Partial resolution of phaseolain and proteinase *b* can be obtained by gel chromatography on Sephadex G-75.

4. The three proteolytic enzymes have been characterised by their behaviour on DEAE-cellulose, their relative activities towards casein and dipeptide substrates and their migration on acrylamide gels.

5. Estimates of the molecular weights of the enzymes were made from Sephadex gel chromatography studies. Proteinase *a* behaved anomalously on Sephadex, but molecular weight values of 45 500 for proteinase *b* and 120 000 for phaseolain were obtained.

INTRODUCTION

N-substituted dipeptide substrates, such as Z-Gly–Leu have been used in a previous study[1] to demonstrate the presence of a proteolytic enzyme, with some features of a carboxypeptidase, in the sap of French beans (*Phaseolus vulgaris*). The activity of this partially purified enzyme towards peptides of known amino acid sequence, indicated that the preparation may have contained endopeptidase activity[1].

Exopeptidase activity cannot be ascribed to a peptidase solely on the basis of its activity towards N-substituted dipeptides commonly used for carboxypeptidase assays[2,3]. The endopeptidase activity noted in the preparation from French beans may have been an intrinsic property of one enzyme or alternatively it may have been due to the presence of other proteolytic enzymes[1].

Abbreviation: Z-, carbobenzoxy-.

Using casein as substrate to measure general protease activity, and N-substituted dipeptides to measure carboxypeptidase-like activity, the presence of three proteolytic enzymes in extracts from French bean leaves has been demonstrated. The activity of one of the these enzymes is most readily assayed with N-substituted dipeptide substrates of the type Z-Gly–Leu and the name phaseolain has been proposed for this enzyme[4]. Most attention has been given to the study of phaseolain. The other two proteolytic enzymes, proteinase *a* and proteinase *b*, both hydrolyse casein and, in addition, proteinase *a* has limited activity towards N-substituted dipeptide substrates.

In a previous communication[1] the recovery of units of enzyme activity and the specific activities of phaseolain preparations at each stage of purification were given. Here, similar purification procedures were used and it is the purpose of this investigation to emphasise the existence and some characteristic properties of three proteolytic enzymes in French bean extracts.

MATERIALS AND METHODS

Resins. DEAE-cellulose (Whatman DE50) was supplied by W. and R. Balston, England. Sephadex resins, all bead form (40–120 μ) were products of Pharmacia, Uppsala, Sweden.

Enzyme substrates. N-substituted dipeptides were supplied by Cyclo Chemical Corp., Los Angeles, Calif., U.S.A. Casein (light, white, soluble) was obtained from British Drug Houses, Poole, England.

Preparation of extracts with proteolytic activity. Acetone powders of a protein fraction were prepared as described earlier[1] and these preparations with activity towards Z-Gly–Leu were purified as before, except that the calcium phosphate gel steps were omitted[1]. In this investigation, 600 g of acetone powder were used as starting material.

Column chromatography. Chromatography of enzyme preparations was carried out at 4°. Elution from DEAE-cellulose was either stepwise with 0.05 M and 0.5 M sodium acetate buffers (pH 6.0), or with a linear gradient using these buffers as previously described[1].

Gel chromatography. (*a*) *For purification procedures.* In general, the methods described by WHITAKER[5] were used. Columns were 2.5 cm × 75 cm or 1 cm × 130 cm and the flow rate was 25 ml/h. The buffer used routinely was 0.05 M acetate (pH 6.0). (*b*) *For molecular weight studies: calibrating proteins.* The following re-crystallised preparations were used at a concentration of either 5 mg/ml or 2 mg/ml: from Sigma Chemical Co., St. Louis, Mo., U.S.A., cytochrome *c*, horse-heart, type 6; soybean trypsin inhibitor, type 1-S; pepsin, bovine serum albumin, fraction 5; from Mann Research Labs., New York, U.S.A., bovine pancreatic ribonuclease and ovalbumin; and from the Commonwealth Serum Labs., Melbourne, Australia, human γ-globulin, Cohn fraction 2.

The following molecular weight values were used: cytochrome *c*, 13 000; ribonuclease, 13 700; trypsin inhibitor, 21 500; pepsin, 35 000; ovalbumin, 45 000; bovine serum albumin, 68 000; γ-globulin, 160 000.

Column procedures. All protein solutions were loaded in a volume of 1 ml and a flow rate of 20 ml/h through the column was maintained by a peristaltic pump; experiments were carried out at 4°. For chromatography on columns 2.5 cm × 75 cm, the

eluting buffer was 0.05 M sodium acetate (pH 6.0), calibrating proteins were loaded individually at a concentration of 5 mg/ml and detected in the effluent at 280 mμ, and the fraction size was 4.0 ml. For the 1 cm × 130 cm columns, the buffer was 0.1 M sodium acetate (pH 6.0), containing 0.4 M NaCl, proteins were loaded at a concentration of 2 mg/ml and detected at 235 mμ and 2.5-ml fractions were collected.

In each case the void volume (V_0) was determined with blue dextran (mol.wt. $2 \cdot 10^6$) and the elution volumes of the calibrating proteins and of the proteolytic enzymes were determined by extrapolation of the sides of the elution profiles as described by WHITAKER[5].

Concentration of enzyme solutions. For large volumes this was achieved by dialysis against a 30% (w/v) solution of polyethylene glycol (carbowax 6 M, Shell Co., Australia) in 0.05 M sodium acetate (pH 6.0). With volumes of enzyme solutions of the order of 100 ml ultrafiltration was used[1].

Protein determinations. Protein determinations were carried out with the Folin–Ciocalteu reagent[6], or by alkaline hydrolysis followed by quantitative ninhydrin estimations[7].

Enzyme assays. (a) With N-substituted dipeptide substrates. The appropriate amount of enzyme was added to 1.0-ml samples of substrate (Z-Gly–Leu or Z-Phe–Leu, $2 \cdot 10^{-3}$ M in 0.05 M sodium acetate (pH 6.0)). Reactions (30°) were stopped by boiling the reaction mixture for 5 min at the appropriate time intervals, and the degree of hydrolysis of the substrate was estimated with ninhydrin[8].

(b) With casein as substrate. Portions of enzyme (0.1 to 0.25 ml) were added to 1 ml of 1% (w/v) casein in 0.05 M acetate (pH 6.0) and incubated at 30° for 1 or 2 h. Reactions were stopped with 3 ml of HAGIHARA's reagent[9], and the tubes left at 0° for 1 h before centrifuging (4000 × *g*, 30 min) and the $A_{280\ m\mu}$ (1 cm) of the supernatants read. In view of the recent warning about the use of this method for the assay of proteolytic activity[10], the $A_{260\ m\mu}$ was also measured in each sample. There was no indication that residual ribonuclease activity in the casein substrate contributed to the absorptions observed at 280 mμ.

Disc electrophoresis. The method of ORNSTEIN AND DAVIS[11], was used except that samples (0.05 or 0.1 ml) were loaded in 40% (w/v) sucrose solution. The standard gel (7%, pH 9.5) was used routinely. Protein bands were stained with 0.5% (w/v) amido black in 7% (v/v) acetic acid (1 h) and de-staining with 7% acetic acid was done electrolytically or by washing with gentle agitation for 48 h.

Elution of enzymes from acrylamide gels. Gels were cut into sections and these were homogenised with 1 ml of 0.05 M acetate buffer (pH 6.0). The gel was removed by centrifugation and the supernatant solutions were run onto DEAE-cellulose columns (0.5 cm × 5 cm) equilibrated with the same buffer. The columns were washed with 10 ml of 0.05 M acetate (pH 6.0) to remove glycine present in the gel buffer system, and fractions with enzymic activity were then eluted from DEAE-cellulose with 0.5 M acetate, pH 6.0.

RESULTS

Separation of proteinase a from phaseolain. When French bean extracts are purified as described earlier[1], proteinase *a* accompanies phaseolain up to the stage of DEAE-cellulose chromatography. A complete separation of proteinase *a* from partially

purified phaseolain preparations has been effected by stepwise elution of the mixture of enzymes from DEAE-cellulose.

The preparation called proteinase *a* therefore refers to the fraction with proteolytic activity which is not adsorbed to DEAE-cellulose in the presence of 0.05 M acetate (pH 6.0).

Further purification of extracts containing phaseolain. Proteinase *a* was removed from phaseolain preparations by DEAE-cellulose chromatography in 0.05 M acetate (pH 6.0). The column was then eluted with 0.5 M acetate (pH 6.0) and fractions with activity towards Z-Gly–Leu were collected, concentrated and equilibrated with 0.05 M acetate (pH 6.0) prior to gradient elution chromatography on DEAE-cellulose.

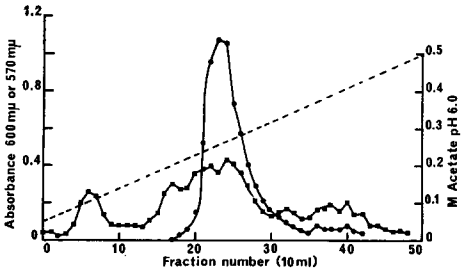
Fractions (10 ml) eluted from the DEAE-cellulose column (2 cm × 30 cm) with a linear gradient (500 ml) of acetate buffer were assayed for protein content[6] and for activity towards Z-Gly–Leu. The results of these determinations are shown in Fig. 1.

It can be seen from the results in Fig. 1 that the main protein peak corresponds only in part to the activity profile, and further purification procedures were therefore applied.

Partial resolution of phaseolain and proteinase b. For all subsequent purification steps fractions were assayed both with a carboxypeptidase substrate (Z-Gly–Leu or Z-Phe–Leu) and with an endopeptidase substrate (casein). This approach led to the recognition of proteinase *b*, an endopeptidase in phaseolain preparations.

The active fractions eluted from DEAE-cellulose (21–28, Fig. 1) were concentrated and run through a column of Sephadex G-75 (2.5 cm × 75 cm), equilibrated with 0.05 M acetate buffer (pH 6.0). Fractions (4 ml) were collected and samples from each were assayed with Z-Gly–Leu and casein respectively. The activity of fractions towards these two substrates is shown in Fig. 2.

It is clear that gel chromatography on Sephadex G-75 partially resolves two distinct proteolytic activities. Both fractions hydrolyse casein, but only one of them,



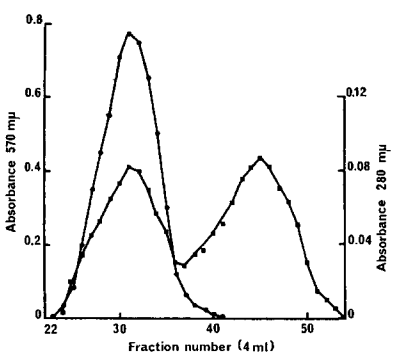


Fig. 1. Chromatography of preparation containing phaseolain on DEAE-cellulose. The linear gradient (500 ml) was 0.05 M to 0.5 M acetate pH 6.0. ■—■, Protein; ●—●, activity towards Z-Gly–Leu.

Fig. 2. Gel chromatography on Sephadex G-75 of preparation containing phaseolain from the DEAE-cellulose step (Fig. 1). Column 2.5 cm × 75 cm, flow rate, 25 ml/h. Assays for proteolytic activity with casein substrate (■—■) show that phaseolain preparations (●—●), assayed with Z-Gly–Leu, contain another endopeptidase, proteinase *b* which is retarded to a greater extent by the gel.

TABLE I

RELATIVE ACTIVITY OF PROTEINASE *a* AND OF PHASEOLAIN TOWARDS THREE SUBSTRATES

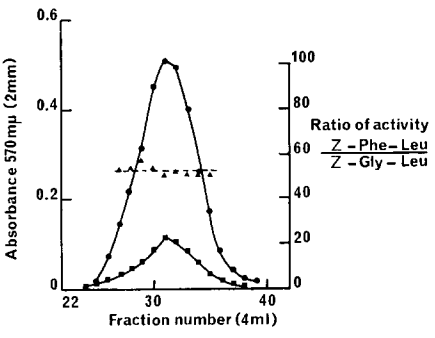
The values shown represent the relative activities of proteinase *a* and of phaseolain towards the three substrates. An arbitrary value of 1 is set for casein as substrate for both enzymes.

Enzyme	*Relative activity in standard assay procedure*		
	Casein	*Z-Gly–Leu*	*Z-Phe–Leu*
Proteinase *a*	1	0.67	50
Phaseolain	1	87	4614

phaseolain, hydrolyses the carboxypeptidase substrate Z-Gly–Leu. The proteolytic enzyme which is retarded to a greater extent by Sephadex G-75 (Fig. 2) is designated proteinase *b*.

Portions from each fraction (Fig. 2) were diluted and assayed for enzymic activity, with Z-Gly–Leu and with Z-Phe–Leu. The results of these assays (Fig. 3) show that for each fraction the ratio of activity towards the two substrates is the same, suggesting that at this stage of purification of the extract from French beans, only one enzyme, phaseolain, contributes to the hydrolysis of the two above-named N-substituted dipeptide substrates.

Fractions with phaseolain activity (Fig. 2, Fractions 24–35) were combined as were those with proteinase *b* activity (Fig. 2, Fractions 40–52).



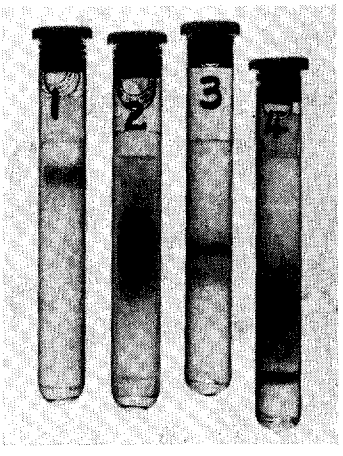


Fig. 3. Relative activity of fractions from gel chromatography on Sephadex G-75 to two dipeptide substrates. Samples from fractions shown in Fig. 2 were diluted to contain the equivalent of 0.0047 ml and 0.00043 ml respectively (in a total volume of 0.1 ml) for assay with Z-Gly–Leu (■—■) and Z-Phe-Leu (●—●). The ratio of activity towards the two substrates (▲—▲) was the same for all fractions.

Fig. 4. Acrylamide gel electrophoresis of preparations with proteolytic activity. Gel 1, sample of proteinase *a*. Gels 2 and 3 contained samples which had activity towards Z-Gly–Leu. Gel 2 contained a sample of specific activity 2.6 eluted from DEAE-cellulose with 0.5 M acetate (pH 6.0), and gel 3 was a sample (0.1 ml) of a fraction from the centre of the phaseolain activity peak from Sephadex G-75 (Fig. 2, Fraction 31) of specific activity 67.1. Gel 4 contained a sample from the centre of the proteinase *b* activity peak of Sephadex G-75 (Fig. 2, concentrated Fraction 45).

Relative activity of proteinase a and phaseolain towards different substrates. The activities of proteinase *a* and phaseolain (taken from the above Sephadex G-75 step) to casein, Z-Gly–Leu and Z-Phe–Leu are shown in Table I. Proteinase *b* does not hydrolyse either of the N-substituted dipeptides.

The values in Table I are calculated on the basis of absorption at 280 mμ or at 570 mμ in standard assay systems involving either casein or N-substituted dipeptides as substrates, for equivalent amounts of enzyme. The value for activity towards casein as substrate is set at unity in each case.

Whereas proteinase *a* hydrolyses casein and Z-Gly–Leu at comparable rates, the relative activity of phaseolain towards these two substrates is markedly different. The ratio of the rate of hydrolysis of Z-Phe–Leu to Z-Gly–Leu is 75 for proteinase *a* and 53 for phaseolain (Table I).

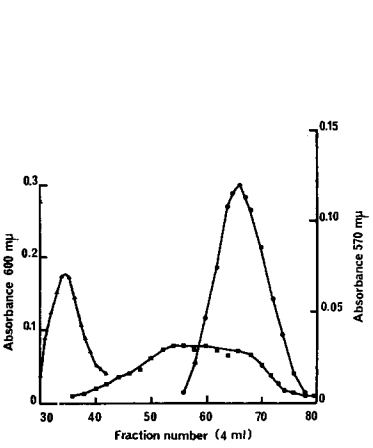
Acrylamide gel electrophoresis of proteinase a, phaseolain and proteinase b. Acrylamide gel electrophoresis patterns of proteinase *a*, phaseolain (at two stages of purification) and of proteinase *b*, are shown in Fig. 4.

The proteinase *a* sample is shown in gel 1. The gels labelled 2 and 3 (Fig. 4) were loaded with samples containing phaseolein at two stages of purification. Gel 2 contains a portion of phaseolain eluted step-wise from DEAE-cellulose with 0.5 M sodium acetate (pH 6.0) (*i.e.* prior to gradient elution on DEAE-cellulose). The specific activity of this preparation (see ref. 1) with Z-Phe–Leu as substrate was 2.6. The sample in gel 3 was taken from the centre of the phaseolain activity peak eluted from Sephadex G-75 (Fig. 2, Fraction 31). The specific activity of this sample was 67.1. These two gels show one major protein band near the centre of the gel which probably corresponds to phaseolain since it remains as the dominant feature of the protein pattern of the highly purified enzyme (gel 3).

The fourth gel was loaded with a sample of proteinase *b*. A 2-ml portion of Fraction 45 from the run in which phaseolain and proteinase *b* were partially resolved on Sephadex G-75 (Fig. 2) was concentrated 20-fold and loaded on the gel. The rapidly migrating anodic protein band was barely detectable without prior concentration of the sample.

Location of enzymic activity from acrylamide gels. Sections of the gels were eluted as described in the MATERIALS AND METHODS section. Gels loaded with samples of phaseolain (corresponding to gel 3, Fig. 4) and of proteinase *b* (gel 4, Fig. 4) were electrophoresed and then cut into three equal segments, corresponding to areas of proteinase *a* (upper segment), phaseolain (middle segment) and proteinase *b* (lower segment). The eluate from each segment was tested for activity, against Z-Phe–Leu and casein. The middle segment of the equivalent of gel 3 (Fig. 4) gave a strong positive assay with Z-Phe–Leu, and the lower segment of gel 4 showed activity towards casein. The middle segment of gel 4 also showed slight activity towards Z-Phe–Leu as might be expected since the sample applied was a concentrated fraction (Fig. 2, Fraction 45) of proteinase *b* which is not completely resolved from phaseolain by gel chromatography.

Estimates of the molecular weights of the three proteolytic enzymes. The use of the linear relationship between the ratio of the elution volume (V_e) to void volume (V_0) and the logarithm of the molecular weight of a series of well characterised proteins[5,12] has been used widely as a means of calibrating Sephadex columns for the determination of molecular weights of other proteins (see for example, ref. 13).



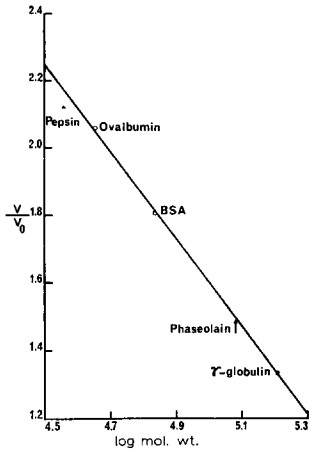


Fig. 5. Chromatography of proteinase *a* and phaseolain on Sephadex G-200. The column (2.5 cm × 75 cm) was eluted with 0.05 M acetate (pH 6.0). The void volume was determined with blue dextran (▲—▲; 600 mμ) and the elution profiles of proteinase *a* (■—■) and phaseolain (●—●) were located by enzymic assay (570 mμ).

Fig. 6. Molecular weight determination of phaseolain with Sephadex G-150. The column (2.5 cm × 75 cm) was eluted with 0.05 M acetate (pH 6.0). ▲, Pepsin; ○, ovalbumin; □, bovine serum albumin (BSA); ■, γ-globulin. Mol. wt. estimate for phaseolain 120 000.

Although it is necessary to be aware of possible concentration dependence effects[14] or interactions[15], the method is particularly useful for enzymes since their elution volumes can be detected by enzymic activity at very low protein concentrations.

Chromatography of proteinase a on Sephadex. Proteinase *a* is completely excluded from Sephadex G-75. Chromatography of this enzyme through a 2.5 cm × 75 cm column of Sephadex G-200 was attempted, but the elution profile (as shown by measurement of enzymic activity) never resembled a well-defined zone, but was more like a plot expected from frontal analysis. The relative positions of blue dextran, proteinase *a* and phaseolain chromatographed individually through the column are shown in Fig. 5. The proteinase *a* profile can be seen to spread from a position close to total exclusion (blue dextran) to one overlapping that of phaseolain. No attempt was made to estimate the molecular weight of proteinase *a*.

The molecular weight of phaseolain. A number of estimates of the molecular weight of phaseolain by gel chromatography have been made. Despite differences in the purity and concentration of preparations used for these studies there has been an over-all consistency in the results obtained (see Table II).

The elution volume to void volume ratio of a 1.1-mg sample of phaseolain chromatographed through a column (2.5 cm × 75 cm) of Sephadex G-150 as related to these ratios for pepsin, ovalbumin, bovine serum albumin and γ-globulin is shown in Fig. 6. The estimated molecular weight for phaseolain from this data and from similar chromatographic runs using enzyme preparations of different concentrations and purity (Table II) was 120 000.

The molecular weight of proteinase b. Proteinase *b* preparations were chromatographed through calibrated columns of Sephadex G-75 of dimensions 2.5 cm × 75 cm

TABLE II

ESTIMATES OF THE MOLECULAR WEIGHTS OF PHASEOLAIN AND PROTEINASE *b* BY GEL CHROMATOGRAPHY

Enzyme	*Concn. (mg/ml)*	*Sephadex*	*Column dimensions (cm)*	*Buffer (pH 6.0)*	*Mol. wt.*
Phaseolain	34.0	G-200	2.5 × 75	0.05 M acetate	122 000
	1.1	G-150	2.5 × 75	0.05 M acetate	120 000
	11.0	G-150	1 × 130	0.1 M acetate + 0.4 M NaCl	120 000
Proteinase *b*	7.8	G-75	2.5 × 75	0.05 M acetate	45 200
	6.0	G-75	1 × 130	0.1 M acetate + 0.4 M NaCl	45 500

and 1.0 cm × 130 cm. The relationship of the elution volumes obtained for proteinase *b* in these systems compared with the calibrating proteins is shown in Fig. 7. The molecular weight for proteinase *b* was estimated as 45 500.

In the chromatographic procedures summarised in Fig. 7B, phaseolain was present only as a minor contaminant of proteinase *b*, (it was not detectable at 235 mμ) yet its elution volume could be determined because of the sensitivity of enzymic assay with Z-Phe–Leu. Even at very low concentrations phaseolain is almost completely excluded from Sephadex G-75 (Fig. 7B), suggesting that if phaseolain is made up of sub-units as would be predicted for a protein of molecular weight 120 000, these sub-units do not apparently dissociate at very low enzyme concentrations.

A summary of the estimates of the molecular weights of proteinase *b* and phaseolain under the various conditions cited in the text is given in Table II.

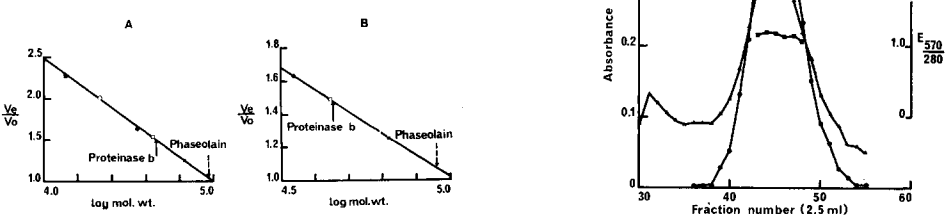


Fig. 7. Molecular weight determination of proteinase *b* with Sephadex G-75. The proteins used were ■, ribonuclease; ○, trypsin inhibitor; ●, pepsin; □, ovalbumin; ▲, bovine serum albumin. A. Column 2.5 cm × 75 cm eluted with 0.05 M acetate (pH 6.0). B. Column 1 cm × 130 cm eluted with 0.1 M acetate (pH 6.0), containing 0.4 M NaCl. The V_e/V_o values for proteinase *b* and phaseolain are indicated. Mol. wt. estimates for proteinase *b* were 45 200 (A) and 45 500 (B).

Fig. 8. Chromatography of phaseolain through Sephadex G-150. The enzyme sample (1.0 ml, 11 mg) was chromatographed through a column of Sephadex G-150 (1 cm × 130 cm) equilibrated with 0.1 M sodium acetate (pH 6.0) containing 0.4 M NaCl. The activity towards Z-Phe–Leu (●—●) and the absorbance at 280 mμ (▲—▲) of each fraction was measured. The ratio of these parameters across the peak is also shown (■—■).

Present status of the purity of phaseolain. Of the three plant proteolytic enzymes referred to here, phaseolain is of chief interest because it has some characteristics of a carboxypeptidase. The specificity of phaseolain can only be determined when a pure preparation of the enzyme is available.

Because the acrylamide gel pattern of the purest sample of phaseolain (Fig. 2, Fraction 31) showed apparent heterogeneity, further purification with Sephadex G-150 was attempted. Chromatography of phaseolain through a column (1 cm × 130 cm) of Sephadex G-150 is shown in Fig. 8.

There is a close correspondence between the protein elution profile (280 mμ) and the activity profile (Z-Phe–Leu as substrate), reflecting a relatively constant value of specific activity across the eluted peak and thus indicating a high degree of purity of the enzyme. Fractions 42–50 (Fig. 8) were combined and concentrated and portions were used for assays of specific activity and for further acrylamide gel electrophoresis. The specific activity with Z-Phe–Leu as substrate was found to be 61.8 (compare value of 67.1 for Fraction 31, Fig. 2) and the acrylamide gel pattern at pH 9.5 was identical to that of Fraction 31 cited above (Fig. 4, gel 3). Studies are currently in progress to determine whether the heterogeneity of these phaseolain samples seen on acrylamide gels at pH 9.5 represents an artifact of the electrophoresis system, a reversible dissociation of the enzyme, or a true heterogeneity.

DISCUSSION

The first report of the partial purification of a proteolytic enzyme from French bean sap indicated that the fraction which hydrolysed the carboxypeptidase substrates, such as Z-Gly–Leu, may also contain endopeptidase activity[1]. The work reported here shows that there are at least three enzymes with proteolytic activity in French bean sap. These have been characterised both by their relative activity towards casein and N-substituted dipeptide substrates (Table I) by their behaviour on DEAE-cellulose and Sephadex (Figs. 1, 2, 5, 8) and their mobility on acrylamide gel electrophoresis (Fig. 4). Only one of these enzymes readily hydrolyses carboxypeptidase dipeptide substrates and has been given the trivial name, phaseolain.

Proteinase *a* could not have been present in phaseolain preparations used for earlier specificity studies[1], but proteinase *b* would have been present since this enzyme chromatographs with phaseolain on DEAE-cellulose.

The complete separation of phaseolain from proteinase *b* has proved to be difficult. Of the preparative systems tried (see RESULTS), only gel chromatography on Sephadex G-75 resulted in partial resolution of the two enzymes (Fig. 2).

On the basis of the results presented here it is apparent that preparative acrylamide gel electrophoresis may well offer the best method for the complete separation of phaseolain from proteinase *b*. In the standard acrylamide system used (7%, pH 9.5), proteinase *b* migrates rapidly (near the bromophenol blue tracker dye), whereas phaseolain is located near the centre of the gel at the end of the run (Fig. 4). In fact, proteinase *b* is much less stable to handling than phaseolain, and it is clear from acrylamide gel patterns and sensitive proteolytic assays of portions of gels loaded with samples of phaseolain from the Sephadex G-150 step (to be reported elsewhere) that this final preparation of phaseolain is free from proteinase *b* activity. As such it

should be suitable for use in investigations of its specificity towards substrates of high and low molecular weight.

Gel chromatography studies for molecular weight estimations were confined to those conditions in which the plant proteolytic enzymes are normally manipulated—that is at 4° and pH 6.0. Detailed studies by NICHOL AND ROY[14] on sulphatase A have emphasised the possibility of large variations in the ratio V_e/V_0 due to changes in physical or chemical parameters. However, the tendency for proteins to associate is usually negligible in very dilute solutions (see for example ref. 14, sulphatase A at pH 5), and this situation can be applied to molecular weight determinations of enzymes by gel chromatography since the elution volume at very low protein concentrations can be determined by enzyme activity.

The molecular weight value for proteinase *b* of 45 500 is of the same order of magnitude as the molecular weights for a number of other proteolytic enzymes, for example, pepsin (35 000) (ref. 16), ficin (26 000) (ref. 17), and carboxypeptidases A and B (34 300) (ref. 18). Trypsin, α-chymotrypsin and papain also fall into this molecular weight range.

On the other hand the molecular weight value of 120 000 for phaseolain exceeds the values for other proteolytic enzymes except for hog kidney aminopeptidases for which a value of 280 000 is quoted[19].

The well characterised proteolytic enzymes readily undergo reversible association reactions[20], and although there has been a consistency in results obtained here for the molecular weights of proteinase *b* and phaseolain under different gel chromatography conditions (Table II), frontal analysis studies with sufficient quantities of the purified enzymes may yield more information about their macromolecular structure.

The properties of phaseolain reported here and elsewhere[1,4] indicate that this plant enzyme is very similar to a peptidase (peptidase β) recently isolated from an autolysate of brewers' yeast[3]. However, phaseolain is quite different from the pancreatic carboxypeptidases, as firstly, it is not inhibited by metal chelating reagents[1] and secondly, it is inhibited by DFP (ref. 4).

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