AN ANTIBODY-SPECIFIC METHOD FOR THE REMOVAL OF AN ENDOPEPTIDASE FROM THE PLANT CARBOXYPEPTIDASE, PHASEOLAIN

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SUMMARY

Extensive fractionation by conventional procedures only allows partial purification of phaseolain, a carboxypeptidase-like enzyme from French beans. Such preparations still contain an endopeptidase contaminant (proteinase "c"). However, proteinase "c" can be purified by methods including ion-exchange chromatography, and insoluble antibody to proteinase "c" has been used to remove all traces of this endopeptidase from partially purified phaseolain preparations. Phaseolain now appears to be a true carboxypeptidase.

INTRODUCTION

In 1965 Wells (1) reported an extensive purification procedure for a carboxypeptidase-like enzyme from French bean leaves. This enzyme, named phaseolain, is of considerable interest because of its ability to cleave proline from the carboxyl-termini of substrates (1). However, even after the removal of two endopeptidases (proteinases "a" and "b", ref. 2) the presence of contaminating proteolytic activity in phaseolain extracts still remained a possibility, since both casein and the carboxypeptidase substrate, CBZ-L-phe-L-leu[a], were hydrolysed.

We have investigated the purity of phaseolain further with the specific aim of determining whether the highly purified extract (2) consisted of one proteinase having some properties of a

[a]Abbreviations used: CBZ = carbobenzoxy-; TPCK = L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; dansyl = 1-dimethylamino naphthalene-sulphonyl-.

carboxypeptidase but also containing endopeptidase activity inherent in the molecule, or whether separate endopeptidase and exopeptidase activities were present. We have now shown that the latter is the case. The endopeptidase has been named proteinase "c". We report here the successful elimination of proteinase "c" from phaseolain by use of specific insolubilised antibody to the endopeptidase.

EXPERIMENTAL

Enzyme assays: Phaseolain was prepared and assayed as described by Wells (1,2). Endopeptidase activity was assayed by measuring the release of radioactivity from ^{14}C-carbamylhaemoglobin (^{14}C-Hb) into CCl_3COOH-soluble material. The radioactive substrate was prepared by incubating $K^{14}CNO$ (0.1 μC, Radiochemical Centre, Amersham, England) with bovine haemoglobin (1.0 gm) in distilled water at 50° for 2 hr, followed by exhaustive dialysis against distilled water. The assay was performed by incubating ^{14}C-Hb (0.1 mg) in 0.1 M sodium citrate-sodium phosphate buffer, pH 3.6 with the enzyme sample (40°) for the required time. The reaction was stopped by adding 0.1 ml of dialysed bovine serum protein (20 mg/ml) and 0.2 ml of 50% (w/v) CCl_3COOH. After standing at 4° for 1 hr, the assays were centrifuged (3,000 g, 30 min), 0.1 ml of the supernatant dried on Whatman GF/C glass fibre papers and the radioactivity measured in a Packard Liquid Scintillation Spectrometer. A unit of activity is defined as 100 cpm released/min/ml.

Preparation of insolubilised antibody to proteinase "c": Purified proteinase "c" (2.0 mg, see Results and Discussion) was injected into a rabbit at multiple sub-cutaneous sites in complete Freund's adjuvant (3). The rabbit was bled from a marginal ear vein (30 - 40 ml) 7 days later and on alternate days thereafter.

The γ-globulin fraction was prepared essentially as described by Goldstein et al. (4). Immunodiffusion tests on the antibody were done in agar gels (1.0% w/v in physiological saline). Insolubilisation of the partially purified γ-globulin was achieved by the method of Cuatrecasas et al. (5) which involved the coupling of the γ-globulin to Sepharose 4B beads after cyanogen bromide activation.

Detection of proteolytic activity after acrylamide gel electrophoresis: Acrylamide gels were prepared and electrophoresis carried out as described by Davis (6) using the high pH formulation. The distribution of enzymic activity on the gels after electrophoresis was determined as described previously (2).

RESULTS AND DISCUSSION

Figure 1 shows a densitometer tracing and distribution of phaseolain and proteinase "c" after acrylamide gel electro-

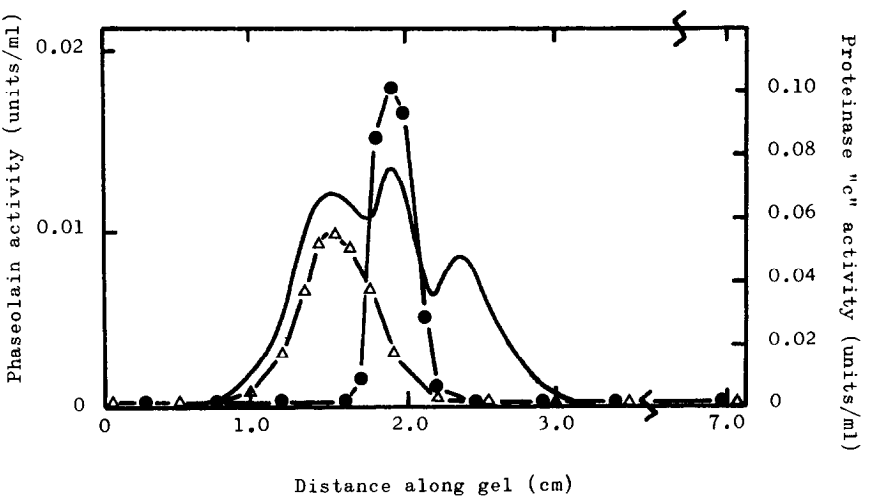

Figure 1. Densitometer tracing (solid line) of an acrylamide gel of phaseolain prepared as described by Wells (2), prior to removal of proteinase "c". Gels were stained with 0.1% (w/v) Amido Black in 7.0% (v/v) acetic acid, and destained by washing with 7.0% acetic acid (24 hr). Gels were sliced into 2 mm portions and eluted for assay for proteinase "c" (Δ——— Δ) and phaseolain (●——— ●) activities.

phoresis. These results show that the highly purified phaseolain contains a contaminating endopeptidase which is partially resolved by this technique. That there are two distinct proteolytic activities present in phaseolain preparations is further substantiated by the fact that the endopeptidase activity has a pH optimum for the digestion of ^{14}C-Hb at pH 3.6 - 3.8, whereas the optimum for phaseolain is at pH 5.7 when CBZ-L-phe-L-leu is used as the substrate.

Before any specificity studies could be attempted with phaseolain, proteinase "c" activity had to be eliminated. Three approaches were tried. The first of these was a combination of recognised protein fractionation procedures including low pH treatment, $(NH_4)_2SO_4$ fractionation, extensive anion and cation exchange chromatography, repeated gel filtration, affinity chromatography (5), isoelectric focussing (7), and preparative acrylamide gel electrophoresis. Repeated Sephadex G-200 chromatography did not achieve any separation of the two activities. The more recent technique of isoelectric focussing and preparative gel electrophoresis also were of little use. In both cases, low recoveries of phaseolain were obtained. Phaseolain is inhibited by low salt concentrations (1) and the salt concentrations required for isoelectric focussing (less than 0.004 M) probably account for the large losses. The high pH for an extended period during preparative acrylamide gel electrophoresis was also responsible for such losses. Because of these low yields (less than 10%) another approach, that of selective inhibition, was sought for the removal of proteinase "c" activity from phaseolain. Although a number of protein modifying reagents and proteinase inhibitors were tried, this second approach also failed.

It was eventually found that chromatography of partially

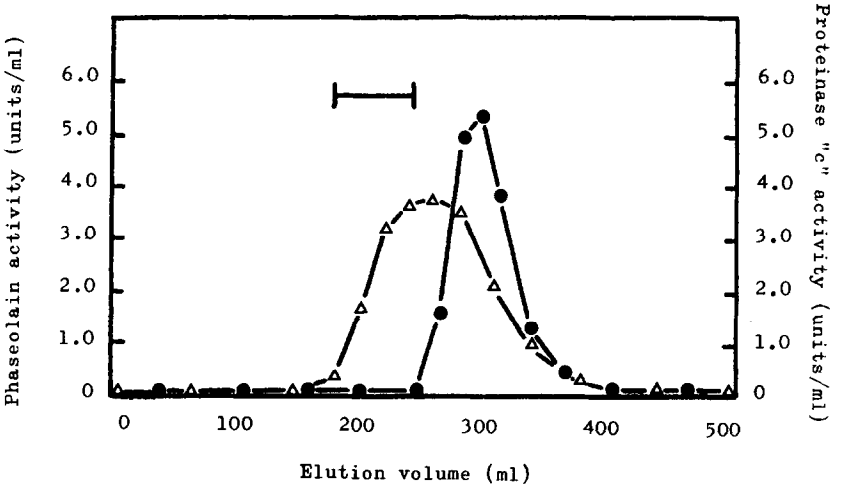

Figure 2. Chromatography of partially purified phaseolain on DEAE-cellulose column (1.0 cm x 20 cm) equilibrated with 0.05 M sodium borate buffer, pH 8.5. A linear gradient (500 ml) up to 0.5 M NaCl in the starting buffer was applied to the column and fractions (4.0 ml) were collected at a flow rate of 0.2 ml/min into tubes containing sufficient 4.0 M sodium acetate, pH 5.7 to bring the pH of the eluted fractions back to pH 5.7. Phaseolain (● ——— ●); proteinase "c" (Δ ——— Δ).

purified phaseolain on DEAE-cellulose at pH 8.5 (which is the upper pH limit for stability of phaseolain) did at least give partial separation of the two enzymes (Fig. 2). Unfortunately, proteinase "c" always tailed badly, making it impossible to obtain pure phaseolain by this method, but it did allow preparation of pure proteinase "c" (fractions pooled are indicated by the bar in Fig. 2). This enzyme was completely homogeneous when examined by acrylamide gel electrophoresis, and more importantly, contained no phaseolain activity. Rechromatography of phaseolain under identical conditions to those in Figure 2 still did not remove proteinase "c" completely.

The purification of proteinase "c" enabled the successful application of a third approach, that of an antibody-specific method, for the removal of this enzyme from partially purified

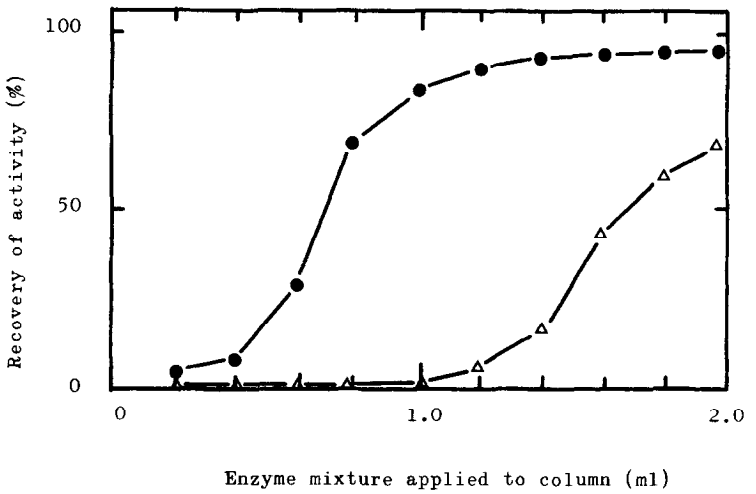

Figure 3. Recovery of enzymic activity after chromatography of aliquots (0.2 ml containing 100 μg of protein) of partially purified phaseolain through a column (V_t = 1.7 ml) of Sepharose-proteinase "c" antibody. The eluting buffer was 0.0175 M sodium phosphate, pH 7.2 and chromatography was done at room temperature (about 17°). Phaseolain (● ——— ●); proteinase "c" (Δ ——— Δ).

phaseolain. The γ-globulin fraction of the serum (Experimental), shown by immunodiffusion to contain antibody to proteinase "c", was covalently cross-linked to Sepharose 4B gel. When this immobilised antibody was tested for its ability specifically to remove proteinase "c" from partially purified phaseolain, some cross-reaction of the antibody with phaseolain was observed. Figure 3 shows the results of chromatographing small aliquots of the solution containing the proteinases through a column of the Sepharose-antibody complex. When small amounts of the solution were loaded onto the column, both activities were absorbed. However, when larger amounts were chromatographed, phaseolain eluted in preference to proteinase "c". Thus by careful choice of the ratio

$$\frac{\text{enzyme mixture loaded (mg)}}{\text{total column volume } (V_t, \text{ ml})}$$

pure phaseolain activity could be obtained. This ratio was cal-

culated to be 270 μg of protein per ml of column volume (which contained 2.0 mg of γ-globulin). Under these conditions, 60-65% of the total phaseolain loaded was recovered from the column with no detectable proteinase "c" activity.

Two points of general interest can be emphasised. First, recognition of endopeptidase activity may often be missed in the absence of a sensitive assay. In this work, the use of the ^{14}C-Hb substrate provided the sensitivity enabling detection of proteinase "c" distinct from phaseolain on acrylamide gels. The second and more important aspect is that the strategy of using insolubilised antibody for the removal of a contaminant protein (itself obtainable in pure form) can be very useful where other procedures are not satisfactory. Certainly phaseolain-enriched fractions could be obtained by chromatographic methods, but the specific antibody procedure was the only one which removed all traces of endopeptidase activity. This point has been checked with an extremely sensitive microtechnique involving the detection in peptide substrates of newly exposed NH_2-terminal residues as their dansyl derivatives[b]. Using this assay, we have been able to detect chymotryptic contamination in trypsin preparations which have been either purified by CM-cellulose chromatography (8) or treated with TPCK (9), but we have not detected any endopeptidase activity in our final phaseolain preparation, which therefore appears to be a true carboxypeptidase.

The success of the insoluble antibody approach for the removal of traces of contaminating proteins which are not wholly fractionated by other means may also find application in such problems as the absolute removal of chymotrypsin from trypsin or of ribonuclease from deoxyribonuclease.

[b]Wells, J.R.E. and Milne, P.R., manuscript in preparation.

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