Phytochemistry, Vol. 23, No. 5, pp. 963–964, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF HEVAIN, A SERINE PROTEASE FROM *HEVEA BRASILIENSIS**

K. R. LYNN and N. A. CLEVETTE-RADFORD

Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6

(*Revised received* 11 *October* 1983)

Key Word Index—*Hevea brasiliensis*: Euphorbiaceae; hevain; serine protease.

Abstract—A serine protease of MW 69 000 has been isolated, in homogeneous form, from the latex of *Hevea brasiliensis*. The enzyme, named hevain, has only limited esterolytic and proteolytic abilities, a maximum activity in the pH range 6.5–7.5, and a pI of 4.3. Hevain has a notably high content of acidic amino acids, while the aromatic residues are present in relatively minor amounts.

INTRODUCTION

We have recently reported the isolation and some characteristics of the proteases found in two members of the family Euphorbiaceae, namely *Euphorbia lathyris* [1] and *E. pulcherrima* [2]. Each of these plants yielded a single, homogeneous proteolytic enzyme named, respectively, euphorbain *l* and *p*, and these were remarkably similar in amino acid composition. The two enzymes differed in MWs and in reactivities [1, 2]. They were, however, both inhibited by di-isopropyl fluorophosphate, and so probably belong to the serine-centred group of enzymes which includes trypsin.

The latex of another member of the Euphorbiaceae, *Hevea brasiliensis* is commercially available (as the source of natural rubber) and so offered material enabling us to extend the investigation described above to a different genus. While the presence of both a protease [3] and a protease inhibitor [4] in the latex of *Hevea brasiliensis* has been reported, the former has not, previously, been isolated and described. Following the common practice, we have given the enzyme the trivial name hevain, and have found it to be also a serine protease with a somewhat restricted range of substrates.

RESULTS AND DISCUSSION

The enzyme isolated was shown to be homogeneous both in anionic and cationic polyacrylamide disc gels, and by HPLC on a TSK column. This latter procedure was calibrated for MW determination with lysozyme, myoglobin, ovalbumen, bovine serum albumen and immunoglobulin G. The hevain was then shown to have a MW of 69 000, which was confirmed on a calibrated Biogel P100 column [5].

The amino acid composition of the hevain (Table 1) shows that the enzyme is rich in glutamic acid, has a very low content of aromatic amino acids, and only two cysteine residues per molecule. Furthermore, neither galactosamine nor glucosamine were found in the hevain following mild hydrolysis (4 M HCl for 6 hr). The composition of hevain is, then, completely different from those of euphorbains *l* [1] and *p* [2] and, while the latter two enzymes are similar to cocoonase [1], we have not found a protein compositionally related to hevain. On subjecting the enzyme to SDS polyacrylamide gel electrophoresis, five protein bands were observed, with MWs ranging from about 18 to 60 000, suggesting that hevain is composed of a number of chains. This was confirmed by determination of *N*-terminal residues using the Edman procedure, when the results showed that several such residues are present in the molecule. HPLC showed that detergents alone could dissociate the hevain into component peptides, and these are being further investigated. Using both chromatofocusing and isoelectric focusing methods, a single charged form of hevain was observed at pI = 4.3.

The enzyme was without measurable activity for

Table 1. Amino acid composition of hevain

Residue	No.
Cys	2
Asp	37
Thr	72
Ser	20
Glu	160
Pro	96
Gly	19
Ala	107
Val	34
Met	0
Ile	16
Leu	19
Tyr	2
Phe	2
His	7
Lys	55
Arg	6
Trp	0

*N.R.C.C. 23010.

azocoll, a protease substrate of general usefulness, nor did it show significant ability to attack either carbobenzoxylysine *p*-nitrophenyl ester or benzoylarginine naphthylamide. It was active with azocasein, this substrate being used throughout the purification reported here. With carbobenzoxyglycine *p*-nitrophenyl ester (CGN), a maximum of activity was observed, in a conventional flattened 'bell' shaped profile, at pH 6.5–7.5. The protease inhibitor recently reported as a constituent of the latex of *Hevea brasiliensis* by Archer [4] apparently functions in the alkaline range, and so may be effective against the hevain described here.

Hevain was immediately inactivated in the presence of a 1000-fold molar excess of diisopropyl fluorophosphate, which strongly suggests that it is, like the other proteases isolated from species of Euphorbiaceae [1, 2], a serine-centred enzyme, even though it was unaffected by phenylmethylsulphonyl fluoride [6] and soyabean, lima bean and ovomucoid trypsin inhibitors. Prolonged dialysis against *o*-phenanthroline was without effect on hevain, as was exposure to EDTA, suggesting that the enzyme does not have a requirement for metals. Similarly the presence of Ca^{2+}, Mg^{2+}, Mn^{2+} and Li^{+} ions did not affect the reactivity of hevain.

Woodward's reagent [7] and diazo-DL-norleucine methyl ester [8] in ten-fold molar excess, which are inhibitors of carboxyl-centred enzymes, were also without effect on the activity of hevain. The enzyme was not inhibited by *p*-chloromercuribenzoate, nor activated by reducing agents, and so is not reliant on the presence of sulphydryl groups, as are some other plant proteases (of the papain family, for example). Experiments with 1,2-cyclohexadione, which shows specificity for arginine residues [9], did not affect the activity of hevain, nor did *N*-α-tosyl-L-lysine chloromethylketone which is specific for histidine [10], or pyridoxal-5′-phosphate which is specific [11] for lysine residues. There was seen to be a loss of enzymic activity with only two reagents: *N*-bromosuccinimide effected a loss of 90% of the activity within two hr, and trinitrobenzene sulphonic acid caused a drop of 60% of the enzyme activity within 5 hr. The former of these two reagents attacks tryptophan residues, though without high specificity [11]; the latter is non-specific, modifying amino groups [12]. This pattern of response to specific and non-specific reagents was observed previously with euphorbain *l* [1] and suggests that it has a general relationship with hevain.

EXPERIMENTAL

Reagents. The latex used was purchased from General Latex and Chemicals, Ltd., Brampton, Ont. as delivered to them from their plantation supplier and contained, to prevent coagulation of the rubber during transport, a high concn of ammonia. The crude latex was dialysed against H_2O at 4°, and rubbery solid formed in this process removed manually from the aq. material which was then frozen. After slow thawing, manual separation of the rubber gave a yellow liquid containing the protease. The powder obtained on lyophilization of that liquid was stored at −10° and used as the source of the enzyme described here.

The Sephadex G25 and G100, Sephacryl S300, DEAE-Sepharose CL-6B, Polybuffer and PBE 94 were all obtained from Pharmacia. Sigma supplied the phenylmethylsulphonyl fluoride, diazo-DL-norleucine methyl ester, *p*-chloromercuribenzoate, *N*-α-tosyl-L-lysine-chloromethyl ketone, soya bean trypsin inhibitor, pyridoxal-5′-phosphate, CBZ-glycine-*p*-nitrophenyl ester, azocasein and di-isopropyl fluorophosphate. The lima bean and ovomucoid trypsin inhibitors were from P & L Biochemicals, and picryl sulphonic acid, Woodward's reagent (*N*-ethyl-5-phenylisoxolium 3-sulphonate) and 1,2-cyclohexadione from Aldrich. Pierce supplied methane sulphonic acid, while reagents for disc-gel electrophoresis, Bio-gel and Biolyte ampholytes were from Bio-Rad. *N*-bromosuccinimide was obtained from BDH, and all other reagents were of analytical grade.

Procedure. Assays—A 0.2% soln of azocasein in 1 ml of 200 mM Tris–HCl buffer, pH 7, was digested with enzyme at 37° for 30 min. The reaction was stopped with trichloroacetic acid (1 ml), clarified by centrifugation, and the $A_{410\,nm}$ of the supernatant recorded in a 1 cm cell. One unit of activity produced a change of $A_{410\,nm}$ of *1* in 1 min. The esterolytic assay used CBZ-glycine-*p*-nitrophenyl ester as has been described [1]. Amino acid analyses, polyacrylamide gel electrophoresis, determination of MW on HPLC, iso-electric focusing and chromatofocusing were performed as reported elsewhere [1].

Purification of hevain. The following steps were all performed at 4°; the buffer used throughout was NaOAc, 10 mM, pH 6. Latex powder (5 g) was dissolved in a small vol. of buffer and subjected to gel-filtration on Sephadex G-25 (2.5 × 25 cm) when pigments were removed, the enzyme eluting at V_0. That enzyme soln was applied to DEAE-Sepharose CL-6B (2 × 35 cm), which was then washed with buffer (200 ml) and eluted with a linear gradient from zero to 1 M NaCl in the NaOAc buffer. The enzymatically active peak, collected at *ca* 500 mM NaCl, was dialysed against H_2O and lyophilized. Two further gel-filtrations, first on Sephadex G100, followed, after dialysis *versus* H_2O and lyophilization again, by Sephacryl S300 (both columns 1.5 × 95 cm), then produced protease which was homogeneous on both anionic and cationic gel-electrophoresis. The sp. act. with azocasein, of the crude powder used as a source of hevain was 67 units/g, that of the purified enzyme, 12 000 units/g.

REFERENCES

1. Lynn, K. R. and Clevette-Radford, N. A. (1983) *Biochim. Biophys. Acta.* **746**, 154.
2. Lynn, K. R. and Clevette-Radford, N. A. (1984) *Phytochemistry* **23**, 682.
3. Pujarniscle, S. (1968) *Physiol. Veg.* **6**, 27.
4. Archer, B. L. (1983) *Phytochemistry* **22**, 633.
5. Andrews, P. (1970) *Methods Biochem. Analysis* **18**, 1.
6. Fahrney, D. E. and Gould, A. M. (1963) *J. Am. Chem. Soc.* **85**, 997.
7. Barke, A. J. and Weber, B. H. (1974) *J. Biol. Chem.* **249**, 5452.
8. Rajagapolan, T. G., Stein, W. H. and Moore, S. (1966) *J. Biol. Chem.* **241**, 4295.
9. Liu, W.-H., Feinstein, G., Osuga, D. T., Haynes, R. and Feeney, R. E. (1968) *Biochemistry* **7**, 2886.
10. Shaw, E., Mares-Guia, M. and Cohen, W. (1965) *Biochemistry* **4**, 2219.
11. Kapoor, M. and Maclean, S. (1976) *Int. J. Biochem.* **7**, 49.
12. Haynes, R., Osuga, D. T. and Feeney, R. E. (1967) *Biochemistry* **6**, 541.