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IN VITRO PROTEOLYTIC PROCESSING OF PEA AND JACK BEAN STORAGE PROTEINS BY AN ENDOPEPTIDASE FROM LUPIN SEEDS

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Key Word Index—Legume seeds; storage proteins; proteolysis; lupin endopeptidase.

Abstract—The highly specific proteolytic breakdown observed upon prolonged treatment of pea legumin and pea and jack bean vicilin with a thiol endopeptidase purified from mature lupin seeds has been studied in detail. Proteolytic cleavage occurred in the acidic subunits of pea legumin, whereas the basic subunits were unaffected. Jack bean vicilin (*M*, 47 K) was cleaved near the middle of the polypeptide chain, whereas pea vicilin (*M*, 50 K) was cleaved into two fragments of *M*, 30 K and 20 K, respectively. The 30 K *M*, polypeptide chain contained covalently linked carbohydrate and had an *N*-terminal sequence suggesting that cleavage had taken place between the α and β region of the vicilin 50 K *M*, polypeptide as previously described *in vivo*. These results suggested that the cleavage specificity of lupin endopeptidase was in the proximity of paired arginine amino acid residues.

The changes in the vicilin polypeptides due to proteolytic cleavage by lupin enzyme and those occurring during germination of pea seeds are also reported and discussed.

INTRODUCTION

Several proteolytic enzymes act on legume storage proteins during seed development and germination [1]. In the latter event the breakdown of storage proteins is massive and a number of proteolytic enzymes with broad specificity is involved [2, 3]. In contrast only a limited proteolysis of storage proteins has been observed during seed development which may occur both co- and post-translationally [4–7], except for the removal of the signal peptide in the precursor protein molecules, the physiological role of proteolysis is unknown. Although several endo- and exopeptidases have been detected and purified in germinating legume seeds [8–10], little information is available on the proteinases which act during seed development. The difficulties of purifying these enzymes and of assaying their activities have prevented an accurate description of their properties and molecular characteristics. For example a protease which was partially purified from pea seeds failed to degrade proteins *in vitro* [11]. Recently a proteinase from mature lupin seeds has been partially purified and characterized [12, 13]. It is a thiol endopeptidase of *M*, around 60 K with an acidic isoelectric point, a neutral pH optimum and a specificity for lupin storage proteins; it was not active on various animal proteins [12]. In the present work this lupin protease has been used to proteolytically cleave some storage proteins from pea and jack bean seeds *in vitro*.

RESULTS

Mature pea seed legumin, which is essentially formed by a group of acidic subunits of *M*, around 40 K linked by disulphide bridges to basic subunits of *M*, around 20 K [7], underwent proteolytic cleavage upon prolonged incubation in the presence of lupin endopeptidase (Fig. 1). Cleavage of the acidic subunits was complete after 66 hr and no hydrolysis of legumin was observed in the absence of the endopeptidase (data not shown). Basic subunits were not affected by the enzyme (Fig. 1). After 48 hr two minor bands (C and E) increased following the disappearance of bands indicated as A, B and D in Fig. 1, and a number of minor polypeptide fragments were formed. All these polypeptides were further degraded within the next 12 hr leading to three major bands of M, 31 K, 30 K, and 29 K, respectively. A minor band of about 25 K *M*, already present in the unreacted legumin and identified as a 'small legumin' acidic subunit [14], was unaffected by the enzyme. Jack bean vicilin (*M*, 47 K) was cleaved near the middle of the polypeptide chain producing fragments which had *M*, about 25 K (Fig. 2B). No proteolytic degradation was observed in the protein incubated in the absence of lupin endopeptidase (Fig. 2A). The 50 K and 47 K polypeptides of pea vicilin were cleaved into two fragments of *M*, 30 K and 20 K, respectively, after 48 hr incubation with lupin endopeptidase (Fig. 3A). Proteolytic processing was complete in 72 hr. No autolysis was observed to take place over a similar time period (Fig. 3B). The 30 K *M*, fragment contained covalently linked carbohydrate (data not shown). *N*-Terminal amino acid analysis of the 30 K *M*, fragment separated by gel permeation chromatography under denaturing conditions gave the following amino acid sequence: Asp–Arg–Arg–Glu Gln, which corresponds to the *N*-terminal sequence of the β + γ fragment of the vicilin polypeptide (Fig. 4).

SDS-PAGE patterns of mature vicilin, of vicilin from germinating pea seeds and of 50 K *M*, and 47 K *M*, polypeptides with and without treatment with lupin endopeptidase are shown in Fig. 5.

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Abbreviation: BAPA, benzoylarginine-*p*-nitroanilide.

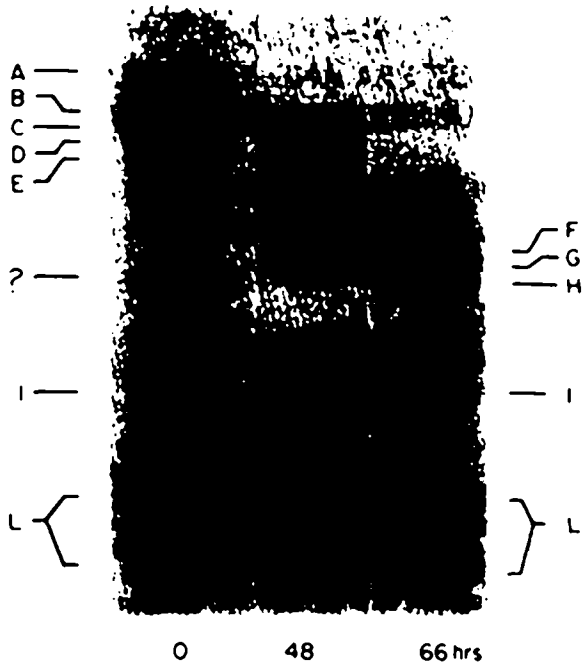

Fig 1 SDS-PAGE analysis in the presence of 2-mercaptoethanol of pea legumin incubated with lupin endopeptidase Legumin (1 mg ml^{-1}) was incubated with lupin enzyme for the time indicated Details of the incubation are given in the Experimental section The following apparent M_rs were determined by comparison with standard proteins (see Experimental): A 'big' α subunits, 42 39 K, B main α subunit, 38 5 K, C, D, E minor α subunits, 37 K, 36 5 K and 36 K, respectively, F, G, H major breakdown products, 31 5 K, 30 9 K and 29 8 K respectively, I 'small' α subunit, 25 3 K, L β subunits, 21 5-19 4 K

Although some of the polypeptides produced by the action of lupin protease on vicilin from immature pea seeds were similar to those present in mature vicilin, the lupin protease did not produce the same overall band pattern. Further, the degradation pattern after *in vitro* incubation with lupin enzyme was similar but not identical to that observed during germination of pea seeds when lower M_r polypeptides become quantitatively more important

DISCUSSION

The lupin endopeptidase used in the present paper is present in developing and mature lupin seeds. It is active on vicilin- and legumin-like proteins of lupin seed [13] but does not affect those animal proteins tested The present results show that the endopeptidase is also active towards other legume storage proteins. The long time required by lupin endopeptidase to complete its action on the protein substrates as well as its activation after several hours of preincubation have been noted elsewhere [13] In pea legumin only the acidic subunits, which contain paired basic amino acid residues [14], are processed, similar results were obtained with lupin legumin [12] The basic subunits of pea legumin do not contain such basic amino acid pairs and they are unaffected by the lupin protease. Moreover, the size of the observed polypeptide fragments deriving from the cleavage of the acidic subunits can be tentatively related to the positions of Arg Arg- pairs in the amino acid sequence Three pairs of arginine residues are located at the C-terminus of the acidic subunit in the most hydrophilic and probably most accessible region of the sequence [14] Their cleavage could give rise to the polypeptide fragments of M_r 35-37 K observed at an early degradation stage of lupin endopeptidase Later, the enzyme cleaves in the proximity

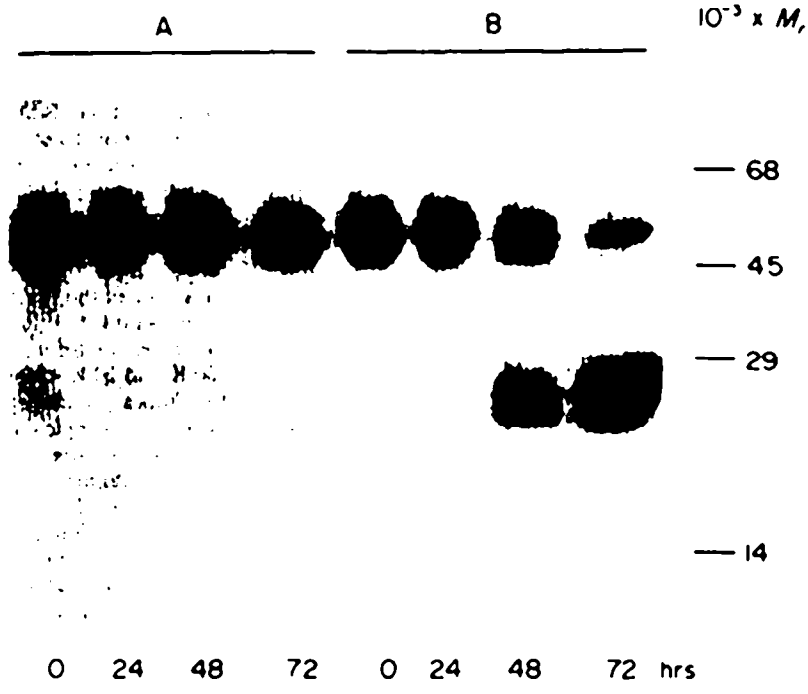

Fig 2 SDS-PAGE analysis of jack bean vicilin incubated without (A) and with (B) lupin endopeptidase. The conditions are the same as in Fig 1 Apparent M_rs shown in the figure refer to standard proteins (see Experimental). No 2-mercaptoethanol was added 30 μg of vicilin were loaded in each lane.

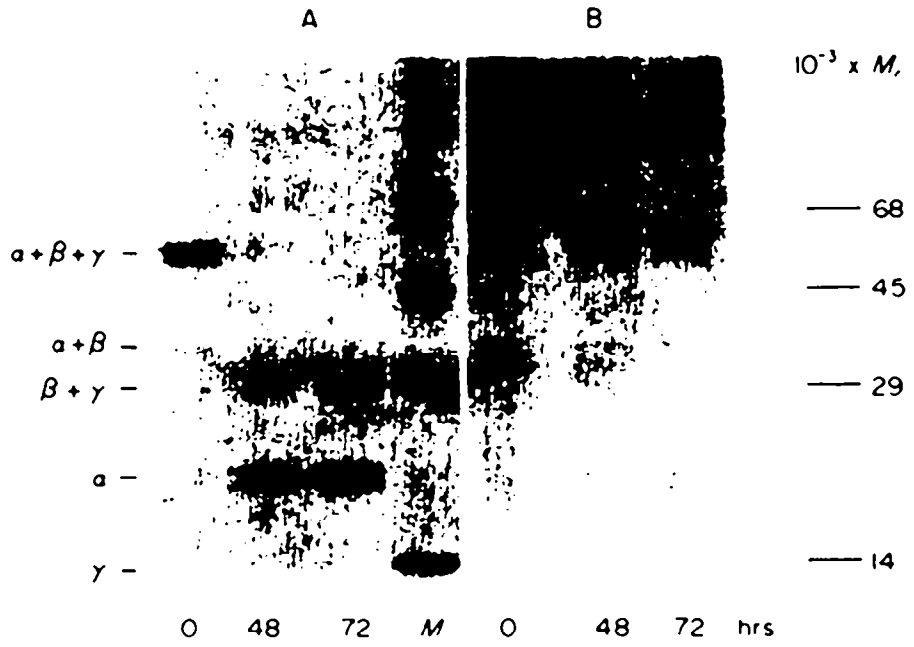

Fig. 3. SDS-PAGE analysis of pea vicilin 50 K M_r polypeptides incubated with (A) and without (B) lupin endopeptidase. The conditions are the same as in Fig. 1. Apparent M_rs shown in the figure refer to standard proteins (see Experimental). No 2-mercaptoethanol was added. 20 μg of protein were loaded in each lane. *M* denotes M_r standard proteins run in this track.

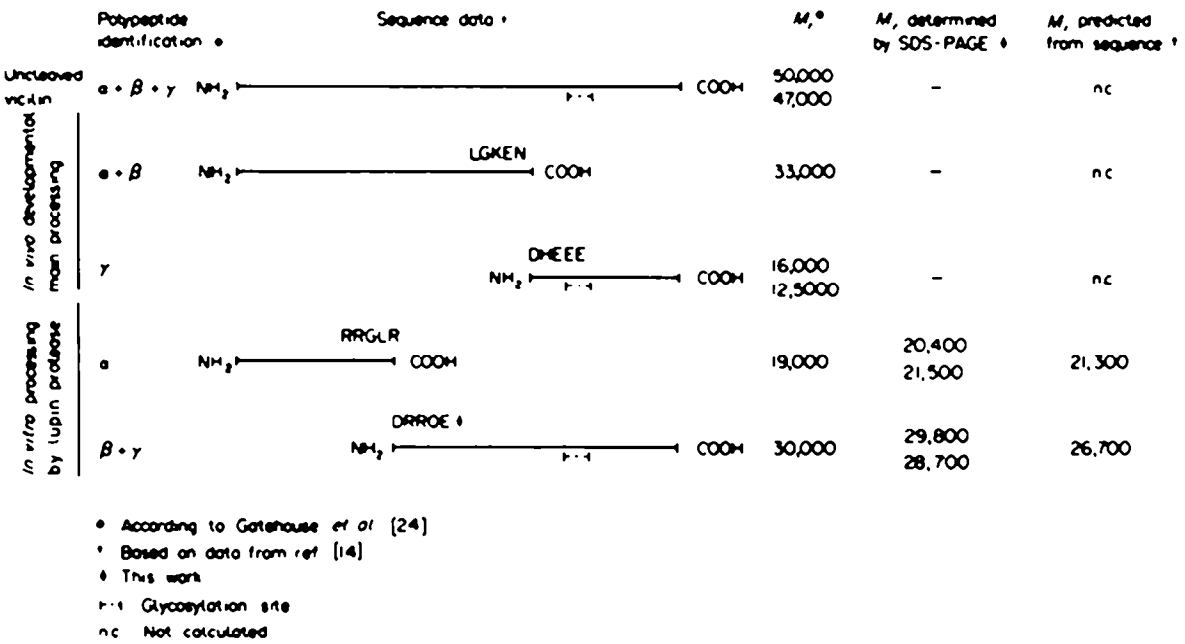

Fig. 4. Sites of *in vivo* and *in vitro* proteolytic cleavage of 50 K M_r pea vicilin polypeptide.

of a pair of arginine residues closer to the *N*-terminus (positions 58 and 59 [14]) with the removal of a larger fragment of M_r around 7 K leading to polypeptides of M_r 29–33 K. A further pair of arginine residues (positions 102 and 103 [14]) do not appear to be affected by the enzyme. The presence of the disulphide bridge at cysteine residue 86 in the acidic subunit linking the basic subunit of pea legumin [14] possibly reduces the accessibility of arginine residues at positions 102 and 103.

The action of lupin protease on pea legumin closely resembles that in the early stages of legumin proteolysis during seed germination [15], suggesting that a similar

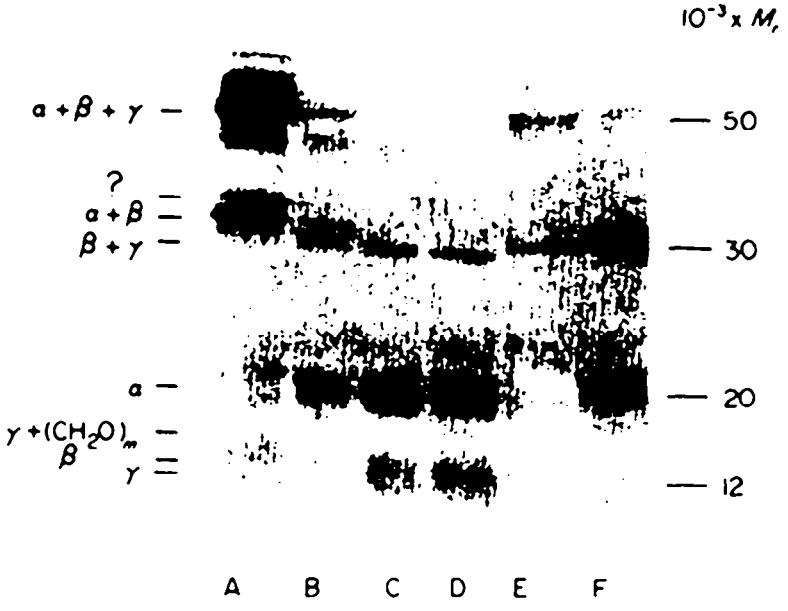

Fig. 5. SDS-PAGE analysis of pea vicilin under different developmental stages of seeds and seedlings and of 50 K M_r vicilin polypeptides processed by lupin endopeptidase. A. Mature pea seed vicilin. B. vicilin from pea seed after 3 days of germination. C. vicilin from pea seed after 5 days of germination. D. vicilin from pea seed after 7 days of germination; E. 11 days after flowering 50 K M_r vicilin subunits. F. 11 days after flowering 50 K M_r vicilin subunits after 72 hr digestion with lupin endopeptidase. Apparent M_rs shown in the figure were obtained by comparison with standard proteins (see Experimental). No 2-mercaptoethanol was added. 15 μg of protein were loaded in each lane, except for lane A where the amount was 40 μg.

enzyme is active during the early stages of germination in peas.

The 50 K M_r and 47 K M_r polypeptides of pea vicilin are degraded by lupin enzyme with a specific site of cleavage at the *N*-terminus of the β + γ fragment where two pairs of arginine residues are present [16]. Covalently linked carbohydrate was found in this fragment indicating that it contained the γ subunit, which is known to be glycosylated [17]. The presence of a carbohydrate moiety on the β + γ subunit can explain the discrepancy between the determined and calculated M_r (Fig. 4). *N*-Terminal analysis of the 30 K M_r subunit obtained with lupin protease gave five amino acid residues coincident with the *N*-terminal sequence of the β + γ subunit. This subunit is a minor component in mature vicilin.

These data suggest that lupin endopeptidase has a specificity for hydrophilic regions of sequence containing paired arginine residues.

The proteolytic breakdown of vicilin by lupin protease is in some respects different to that observed in mature vicilin. This protease clearly does not mimic the effects of the (putative) protease active during pea seed development which cleaves on the *C*-terminal side of specific exposed Asn-X peptide bonds in the legumin [14], vicilin [14] and seed lectin [18] precursors, but its action does resemble that of the protease which cleaves at the α + β subunit junction to give α (M_r 19 K) and β + γ (M_r 30 K) subunits. The final band pattern of mature vicilin in pea may therefore be the result of an enzyme of this specificity cleaving at the α + β subunit junction in some pro-polypeptides and an Asn-specific protease cleaving the β + γ subunit junction in all polypeptides of the 47 K M_r vicilin type.

The action of lupin protease is similar to that of the early stages of vicilin proteolysis on pea seed germination, suggesting that the corresponding pea protease is also present both during seed development and in mature seeds so that it is active in the early stages of germination. In agreement with this conclusion, both developing and mature pea seeds contain BAPA-ase activity [Duranti, M. and Gatehouse, J. A., unpublished results]. As with legumin another protease must be acting as germination proceeds to produce fragments of M_r around 20 K as the major polypeptides after 7 days of germination (see Fig. 5).

Jack bean vicilin also gave proteolytic fragments upon treatment with the lupin enzyme, but a complete amino acid sequence of this protein is not available. However, because of the homology in the amino acid sequence of the tryptic peptides of canavalin with pea vicilin [19], it is likely that hydrophilic sequence regions containing Arg-Arg- residues are located near the middle of the polypeptide chain as in vicilin 50 K M_r polypeptide. During germination of jack bean seeds, canavalin is converted to form A1 of canavalin (M_r 24 K) and to form A2 (M_r 25 K) which in turn is rapidly degraded to A3 and A4 (M_r 12 K and 13 K, respectively) [20]. Furthermore, similar proteolytic fragments were obtained by prolonged treatment with trypsin and chymotrypsin [20] and with lupin protease. Since the action of different proteases produces polypeptide fragments of similar size, the existence of an easily accessible predetermined site of cleavage

in the amino acid sequence of the storage proteins of this family is suggested

EXPERIMENTAL

Purification of the protease from mature seeds of *Lupinus albus*, var. Multolupa was according to Duranti and Cerletti [12] The partially purified enzyme was then incubated with protein substrates under the conditions given below Activity of the enzyme on BAPA was used to calculate the enzyme to protein ratio. for each assay an amount of enzyme soln to give 10 nmol BAPA hydrolysed min^{-1} mg^{-1} of protein substrate was used The freeze dried protein substrates used in this study were mature pea (*Pisum sativum* L.) seed legumin, 50 K *M*, and 47 K *M*, vicilin polypeptides from immature pea seeds (11 days after flowering) and canavalin from jack bean (*Canavalia ensiformis* L.) [19] For comparison of the SDS-PAGE patterns mature pea seed vicilin and vicilin from germinating pea seeds at 3, 5 and 7 days of germination (prepared as described in ref [6]) were also used The amount of protein substrate was determined by the microbiuret method or by absorbance at 280 nm The amount of protein incubated with the protease is indicated in each figure Blanks without added enzyme were run in parallel Incubation experiments were performed under conditions of highest enzyme activity These were 50 mM NaPi buffer, pH 7 4, containing 0 15 M NaCl and 0 01 %NaN_3, the temperature was 37° and the samples were gently stirred during incubation

Aliquots (50 μl) were withdrawn at various times, denatured with 50 μl 0 125 M Tris–HCl buffer, pH 6 8, containing 1 5% SDS, by heating at 100° for 4 min and analysed by SDS-PAGE according to Laemmli [21] with minor modifications. When necessary, 2-mercaptoethanol, final concentration 2 5%, was added Protein bands in the gel were stained with Coomassie blue R 250 The *M*, of the polypeptide fragments produced during incubation was determined by comparing peak location in the laser densitometer scannings at 637 nm as with that of known protein markers, bovine serum albumin (*M*, 68 K), egg albumin (45 K), carbonic anhydrase (29 K) and lysozyme (14 K), all obtained from Sigma Chemical Co., U S A Glycosylated polypeptides were detected in the gels with dansyl hydrazine as described in ref [22] Separation of the polypeptide fragments was by gel permeation on a column of Sephacryl S300 (Pharmacia Fine Chemicals) equilibrated with 70% formic acid Breakdown products of 50 K *M*, and 47 K vicilin polypeptides (5 mg) were denatured with 70% formic acid and loaded onto the column (60 × 1 5 cm) which was eluted at a flow rate of 10 ml hr^{-1} with 70% formic acid Eluted fractions were pooled, dried under vacuum and submitted to *N*-terminal analysis *N*-Terminal amino acid sequence analysis was performed manually using the film method for large peptides as described in ref [23] A reverse phase column on a Jasco apparatus was used Phenylthiohydantoin derivatives of the amino acids were identified

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