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RESEARCH PAPER

A comparative study of the role of the major proteinases of germinated common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* (L.) Merrill) seeds in the degradation of their storage proteins

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Abstract

Two types of cysteine proteases, low-specificity enzymes from the papain family and Asn-specific from the legumain family are generally considered to be the major endopeptidases responsible for the degradation of seed storage proteins during early seedling growth. The action of the corresponding enzymes (CPPh1 and LLP, respectively) from common bean (*Phaseolus vulgaris* L.) on phaseolin (the common bean storage protein), and on the homologous soybean (*Glycine* max (L.) Merrill) storage protein, β-conglycinin, was studied. Under the action of LLP, proteolysis of phaseolin was limited to cleavage of its interdomain linker. No cleavage of the interdomain linker occurred in β-conglycinin with LLP. LLP action was restricted to splitting off the disordered N-terminal extensions of α and α′ subunits. No extensive hydrolysis (degradation to short TCA-soluble peptides) of either protein occurred under the action of LLP. CPPh1 cleaved the phaseolin subunits into roughly half-sized fragments at the onset of proteolysis. The cleavage was accompanied by a small (8–10%) decrease of protein. No decrease of protein occurred with further incubation. Thus the two most active proteinases detected in common bean seedlings individually were incapable of the extensive degradation of phaseolin. Extensive hydrolysis of phaseolin was only achieved by the consecutive action of LLP and CPPh1. Similar cleavages occurred during the action of CPPh1 on β-conglycinin. However, by contrast with phaseolin, CPPh1 by itself accomplished the extensive hydrolysis of β-conglycinin. The differences in the course of proteolysis of the proteins studied were determined by their structural peculiarities.

Key words: β-conglycinin, legumain-like proteinase, papain-like proteinase, phaseolin, proteolysis, seed storage protein degradation.

Introduction

During the development of the legume seed on the parent plant, storage proteins are accumulated in protein storage vacuoles, especially in the cotyledons. Following germination the storage proteins are hydrolysed to free amino acids, which serve as precursors for the synthesis of new proteins and other nitrogen-containing compounds in the seedling. Endopeptidases play key roles in storage protein degradation, producing oligopeptides. The latter are, in turn, hydrolysed by exopeptidases to free amino acids. Some of these peptidases are present in the protein storage vacuoles and the lytic vacuoles derived from them, especially serine carboxypeptidases (Wilson, 1986; Shutov and Vaintraub, 1987). Other peptidases, especially aminopeptidases, are localized in the cytosol and act on oligopeptides

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Abbreviations: CP, cysteine proteinase; CPPh1, *Phaseolus vulgaris* cysteine proteinase; dai, day after the start of imbibition; DTT, dithiothreitol; E-64, *trans*-epoxysuccinnyl-L-leucylamido-(4-guanidino) butane; LLP, *Phaseolus vulgaris* legumain; TCA, trichloroacetic acid.

transported out of the lytic vacuoles into the cytosol (Wilson, 1986; Shutov and Vaintraub, 1987).

Cysteine proteinases (CPs) (EC 3.4.22) are the major endopeptidases present in the cotyledons during early seedling growth, and are presumed to be largely responsible for the mobilization of the storage proteins (Shutov and Vaintraub, 1987; Müntz, 1996). They fall into two families, the papain-like CPs and the legumain-like CPs. The former exhibit low specificity for the peptide bonds cleaved (Abe *et al*., 1978; Shutov *et al*., 1984*a*; Asano *et al*., 1999), while the latter are specific for the cleavage of Asn-X peptide bonds.

A multiplicity of papain-like proteinases has been ascertained in plants (up to 30 are predicted in *Arabidopsis thaliana* L. Heynh. based upon the genome sequence by the MIPS database). However, not all the CPs predicted by genomic analysis are necessarily expressed in seeds. Müntz and coworkers have identified four papain-like CPs in germinating vetch (*Vicia sativa* L.) seeds (Fischer *et al*., 2000). The most active of them (proteinase A) was purified earlier (Shutov *et al*., 1984*b*). Similar papain-like CPs have been isolated from the seeds of a number of species (reviewed by Wilson, 1986; Shutov and Vaintraub, 1987; Müntz, 1996). In general, these major papain-like CPs are synthesised *de novo* in growing seedlings, have acidic pH optima corresponding with the acidic milieu of the lytic vacuole, and the periods of their maximal activity and of massive degradation of storage proteins coincide. It was shown *in vitro* that they are capable of the extensive hydrolysis of native storage proteins. A limited number of specific cleavages are inflicted upon the substrate molecule at the onset of proteolysis, producing high molecular weight, trichloroacetic acid (TCA)-insoluble fragments. Further proteolysis proceeds until the protein is converted into short TCA-soluble peptides. The mean number of residues in the peptides obtained by the exhaustive proteolysis of storage proteins by endogenous CPs was four (mainly dipeptides) for vetch (Shutov *et al*., 1984*b*), three for mung bean (*Vigna radiata* (L.) Wilczek) (Baumgartner and Chrispeels, 1977) and wheat (*Triticum aestivum* L.) (Shutov *et al*., 1984*a*), and two for sunflower (*Helianthus annuus* L.) (Vaintraub and Ropot, 1988). In all cases no free amino acids were found, excluding the presence of contaminating exopeptidases. These results substantiate the important role of the major papain-like proteinases in storage protein degradation.

An exception is the major papain-like proteinase of germinated common bean (*Phaseolus vulgaris* L.) seeds. It has been isolated and described under different names by Boylan and Sussex (1987) and Rotari *et al*. (1997). For the sake of nomenclature unity it will be referred to as CPPh1 (emb|CAB 17074) to indicate the plant source and its established homology with the vetch papain-like proteinase CPR1 (Fischer *et al*., 2000). CPPh1 action on phaseolin, the main 7S storage protein of common bean, is limited to the cleavage of phaseolin subunits into two fragments (Boylan and Sussex, 1987) corresponding with the two domains that form the subunits of seed 7S proteins (Lawrence *et al*., 1994). The formation of TCA-soluble peptides or the corresponding decrease of protein was not detected by Boylan and Sussex (1987). However, Rotari *et al*. (1997) showed that the splitting of the subunits of phaseolin is accompanied by the formation of a number of TCA-soluble peptides, resulting in the loss of 8.5% protein. No further hydrolysis was detected thereafter. Therefore, CPPh1 by itself is incapable of carrying out the extensive hydrolysis of phaseolin.

The function of legumains in developing seeds, where they carry out the processing of the precursor forms of the legumin-like storage proteins, is well established (Müntz and Shutov, 2002). However, the role of legumains in germinated seeds is less clear. They were purified in germinated seeds of vetch (*V. sativa* L.; Shutov *et al*., 1982), moth bean (*Phaseolus aconitifolia* (Jacq.) Marechal; Kembhavi *et al*., 1993), black gram (*Vigna mungo* (L.) Hepper; Okamoto and Minamikawa, 1995), and common bean (*P. vulgaris*; Senyuk *et al*., 1998). The legumain of the mung bean removes the propeptide of the precursor of SH-EP, the major papain-like proteinase of the black gram seedlings (Okamoto and Minamikawa, 1995). However, the action of the *Vigna mungo* legumain on the endogenous seed storage proteins was not examined. The legumain of germinated vetch seeds (proteinase B) acted on vetch legumin, but only after the legumin has been subjected to limited proteolysis by a papain-like proteinase (Shutov *et al*., 1981). In a recent work the ability of proteinase B to carry out the extensive hydrolysis of native storage proteins was affirmed (Fischer *et al*., 2000). These results, however, were not confirmed later (AD Shutov, personal communication). According to Senyuk *et al*. (1998) common bean legumain (LLP) (emb|CAB 17078) hydrolyses phaseolin to TCA-soluble peptides. However, it is not clear if this hydrolysis was due in part to a possible contaminating papain-like proteinase (such as CPPh1) in the legumain preparation. The hydrolysis was carried out without added inhibitor E-64 that does not affect legumain, but inhibits traces of CPPh1. Moreover, N-terminal analysis of fragments formed during phaseolin hydrolysis showed the splitting of the bond Arg^4-Glu^5. This cleavage is incompatible with the strict specificity of legumain, but is characteristic of CPPh1 action (Rotari *et al*., 1997).

In this paper, the reinvestigation of the action on phaseolin of the two major endopeptidases from germinated common beans is reported. The previously reported inability of CPPh1 to perform the extensive hydrolysis of phaseolin (Rotari *et al*., 1997) was confirmed, while no such action by LLP was found in the presence of E-64. So, neither of the two most active proteinases found in germinated common bean seeds taken separately can accomplish the extensive degradation of phaseolin. In this

respect, the possibility of the extensive degradation of phaseolin by the concerted action of both proteinases was investigated. The limited proteolysis of phaseolin by legumain leads to the further degradation of phaseolin by CPPh1. A study of the degradation of soybean β-conglycinin by these proteinases performed in parallel allowed the distinctive features of phaseolin proteolysis to be related to the peculiarities of its structure. In the course of this work a new method was developed that permitted the simultaneous isolation of LLP and CPPh1 and led to the higher recovery and purer preparations of both proteinases.

Materials and Methods

Materials

Phaseolin was isolated from dry common bean (*P. vulgaris* cv. Moldavian) seeds according to Schlesier *et al*. (1979). The β_3 and $\alpha\alpha'$ forms of soybean (*G. max* cv. Amsoy 71) β-conglycinin were purified by the method of Morita *et al*. (1996). The $\alpha\alpha'$ preparation was primarily the $\alpha_2\alpha'_1$ based upon staining intensity on SDS-PAGE. Dithiothreitol (DTT) and bacitracin were from Serva (Heidelberg, Germany), E-64 from Sigma (St Louis, MO), Bz-Asn-*p*-nitroanilide and Bz-Phe-Val-Arg-*p*-nitroanilide from Bachem (Heidelberg, Germany). Phenyl-Sepharose CL-4B, purchased from Pharmacia (Uppsala, Sweden), Bacitracin-Sepharose, made by coupling bacitracin to cyanogen bromide-activated Sepharose 4B (Pharmacia), and Fractogel-650-TMAE(M) from Merck (Darmstadt, Germany) were used for chromatography. All other reagents used were analytical grade.

Determination of proteases activity

The synthetic substrates Bz-Asn-*p*-nitroanilide and Bz-Phe-Val-Arg-*p*-nitroanilide were used for the determination of the proteolytic activity of LLP and CPPh1, respectively. Samples of 25 µl (CPPh1) or 75 µl (LLP) enzyme solution were adjusted to 225 µl by the addition of 0.12 M phosphate-citrate+0.18 M NaCl+2 mM DTT+0.5 mM EDTA, pH 5.6 ('Incubation Buffer'). The reaction was started by the addition of 25 µl of either 10 mM Bz-Asn-*p*-nitroanilide or 5 mM Bz-Phe-Val-Arg-*p*-nitroanilide·HCl. The assay mixture was then incubated for 30 min at 30 °C, and then acidified by the addition of 500 µl of 6% (v/v) acetic acid. The absorbance of the liberated *p*-nitroaniline was measured at 405 nm. The enzyme activity was expressed in terms of mU (the amount of enzyme releasing 1 nmol of *p*-nitroaniline min^{-1}).

Enzyme purification

Common bean seeds (*P. vulgaris* L. cv. Moldavian) were soaked in distilled water for 2 h and germinated on filter paper in the dark at 25 °C. Fresh cotyledons, 100 g, were collected on the sixth day after the start of imbibition (dai), ground into an homogeneous paste, and extracted with cold water (1:3 w/v). This and all subsequent purification procedures were carried out at 4 °C and all solutions contained 2 mM DTT and 0.5 mM EDTA. After centrifugation (6000 *g*, 60 min) the proteins of the supernatant were salted out by $(NH_4)_2SO_4$ at 90% saturation. The precipitate was redissolved in a minimal quantity of 0.01 M Na acetate+0.2 M NaCl, pH 5.8 ('Standard Buffer') and applied to a Phenyl-Sepharose column (3.4×7.5 cm) equilibrated with the same buffer. The column was washed with the Standard Buffer and then eluted with water. Both enzymes were found to elute in the water fractions. The enzyme-containing fractions were pooled, adjusted to 0.2 ionic strength by the addition of 4 M NaCl, and loaded onto a Bacitracin-Sepharose column (2.6×24 cm) equilibrated with Standard Buffer. The column was washed with Standard Buffer, and both enzymes eluted together with water.

The separation and final purification of the two enzymes was achieved by anion exchange chromatography. The fractions containing both enzymes eluted from the Bacitracin-Sepharose column by water were pooled, 2 M Na acetate, pH 5.8, added to bring the pool to 0.1 M in Na acetate, and loaded onto a Fractogel-TMAE column (1.4×10 cm) equilibrated with 0.1 M of Na acetate, pH 5.8. The column was washed with the same buffer and eluted stepwise by 0.2 M, 0.3 M, and 0.4 M Na acetate of the same pH. LLP was eluted at 0.2 M buffer while CPPh1 eluted at.4 M. Active fractions with each enzyme were combined, concentrated by ultrafiltration using an Amicon Ultra-15 Centrifugal Filter Device (Millipore, Bedford, USA) and stored at –20 °C.

Phaseolin and β-conglycinin digestion

To determine the course of phaseolin or β-conglycinin digestion by LLP, equal volumes of 2% (w/v) protein solution in Incubation Buffer containing 0.02% NaN_3 and enzyme solution (100–250 mU ml^{-1} in 0.2 M Na acetate+2 mM DTT+0.5 mM EDTA, pH 5.8) were mixed. The reaction mixture was then incubated for 24 h at 30 °C. Digestions with CPPh1 were performed in the same manner with 0.4 M buffer. E-64, an inhibitor of papain-like cysteine proteinases was added at 10 µM concentration when the action of LLP alone was studied, in order to ensure the inhibition of traces of CPPh1 that might not be detected by the methods used for checking the purity of LLP preparation. All digestions were repeated at least twice. Samples (8 µl) were taken from the incubation mixture at each specified time interval for determination of residual (TCA-insoluble) protein and for SDS-PAGE.

Determination of residual (TCA-insoluble) protein

The determination was performed by a dye-binding method (Vaintraub and Yattara, 1995). Four replicates carried out on each time interval agreed within 0.01 absorbance units. The dependence of the logarithm of residual protein concentration on proteolysis time was plotted. The rate constants were equal to the slope of the linear part of the plot that correspond to the pseudo-first order reaction characteristic of the co-operative stage of proteolysis (Vaintraub, 1998). The substrate was considered resistant to extensive hydrolysis if the difference between the rate constant value and zero was statistically insignificant (Snedecor, 1957).

Gel electrophoresis

SDS-PAGE was carried out in 1 mm thick 15% (w/v) gels by the method of Laemmli (1970).

Determination of N-terminal sequence of proteolytic fragments of β-conglycinin subunits

The SDS-PAGE gels were electroblotted to a PVDF membrane (Millipore, Billerica, MA USA). The blots were stained with Amido black (Merck, Darmstadt, Germany), and the excised bands subjected to N-terminal sequence analysis at the Cornell University Bio-Resource Center using an applied Biosystems (Foster City CA) Procise Model 492 Sequencing System.

Accessibility

The accessibility of an amino acid residue (X) is defined as the percentage of its surface accessible to the solvent. The accessibility of X in the extended pentapeptide GGXGG is taken for 100%. This method differentiates between the exposed and buried amino acid residues. The accessibility of Asn and adjacent residues located in

and near the interdomain link was determined using the program Deep View/Swiss-Pdb Viewer (Guex and Peitsch, 1996).

Results

Assessment of the purity of the isolated enzymes

The final preparation of LLP exhibited a single major band on SDS-PAGE (Fig. 1) and two minor bands (not seen on the figure). The major band was judged to be at least 95% based upon its staining intensity. The minor bands are of greater mobility, and the most intensive of them had the same N-terminal sequence as LLP (data not shown). All bands reacted with vetch legumain antiserum (data not shown). In all probability they represent LLP autolysis products. The CPPh1 exhibited a single band on SDS-PAGE. No cross-contamination of LLP by CPPh1 or vice versa was detectable by SDS-PAGE and by immunoblotting with corresponding antisera (data not shown). This was confirmed by assays with the synthetic substrates specific for legumain and for papain-like cysteine proteinases described above.

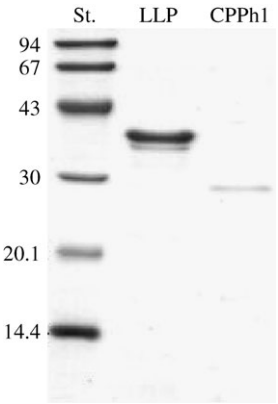

Fig. 1. SDS-electrophoresis of the purified *P. vulgaris* cysteine proteinases LLP and CPPh1. St, molecular mass standards (kDa).

Hydrolysis of phaseolin by LLP and CPPh1

No statistically significant decrease of TCA-insoluble protein occurred during the action of LLP on phaseolin (Fig. 2A). However, examination of the reaction by SDS-PAGE (Fig. 3A) demonstrated that LLP cleaved the phaseolin subunits in half to produce roughly equal fragments. The cleavage was incomplete even after 24 h, but was brought to completion by the addition of another aliquot of LLP and further incubation (not shown here). Several other very faint bands of lower molecular mass, similar to those detected by Senyuk *et al*. (1998), were gradually formed in the course of prolonged LLP proteolysis (Fig. 3A). They were clearly seen when the 24 h hydrolysis was extended to 48 h, with a second aliquot of enzyme added (Fig. 3C, lane M).

The present study of the action of CPP on phaseolin confirmed the previously obtained results (Rotari *et al*., 1997). At the onset of proteolysis CPPh1 cleaved the phaseolin subunit into roughly half-sized fragments of similar molecular mass; the cleavage rate being greater than that observed by Rotari *et al*. (1997) due to a higher concentration of enzyme (data not shown). The cleavage was accompanied by a small (8–10%) decrease of protein (Fig. 2B). Neither degradation to smaller size fragments nor the decrease of protein occurred with further incubation, even if a second aliquot of CPPh1 was added at 24 h and the incubation was prolonged.

Consecutive hydrolysis of phaseolin by CPPh1 and LLP

To determine if the previous modification of phaseolin by LLP was necessary for the subsequent action by CPPh1, or vice versa, the two proteolytically modified substrates were first prepared. Samples of phaseolin were treated with CPPh1 or LLP, according to the protocol described in the Materials and methods to produce CPPh1- and LLP-modified phaseolin, respectively. To ensure the completion

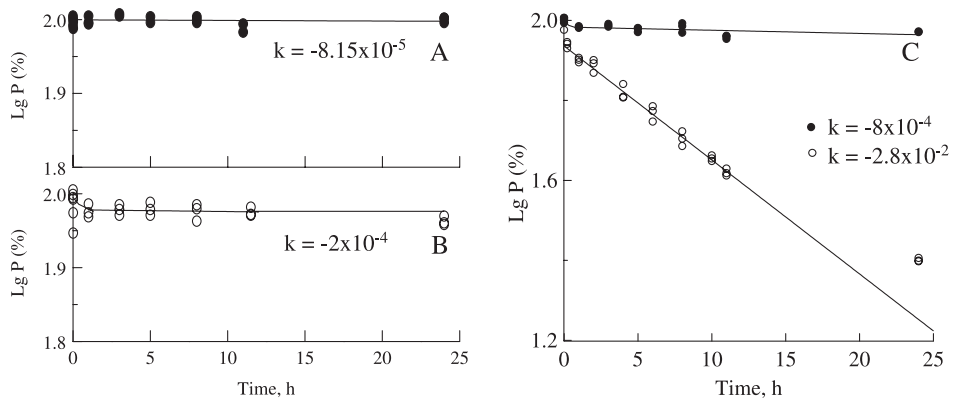

Fig. 2. Kinetic curves of phaseolin proteolysis: (A) by LLP, (B) by CPPh1 and (C) of CPPh1-modified phaseolin proteolysis by LLP (closed circles) and of LLP-modified phaseolin proteolysis by CPPh1 (open circles). The mass concentration of the residual protein (P) is expressed as logarithm of the percentage of the initial protein concentration; k, pseudo-first order rate constants of the co-operative proteolysis.

of each of the two modifications the incubations were extended to 48 h, with a second aliquot of corresponding enzyme added after the first 24 h. E-64 and LLP were eliminated from the LLP-modified phaseolin reaction mixture by gel filtration on Sephadex G-75 fine. CPPh1 contained in CPPh1-modified phaseolin was inhibited by adding E-64 at 10 μM concentration. The preparations of CPPh1- and LLP-modified phaseolin were then subjected to the action LLP and CPPh1, respectively.

No significant losses of protein were observed when LLP acted on phaseolin previously modified by CPPh1 (Fig. 2C). Examination of the reaction mixture by SDS-PAGE (Fig. 3B) revealed a slow formation of minor fragments similar to those produced by LLP itself. It seems, however, that prior treatment by CPPh1 enhanced the rate of their formation (compare Fig. 3A and B).

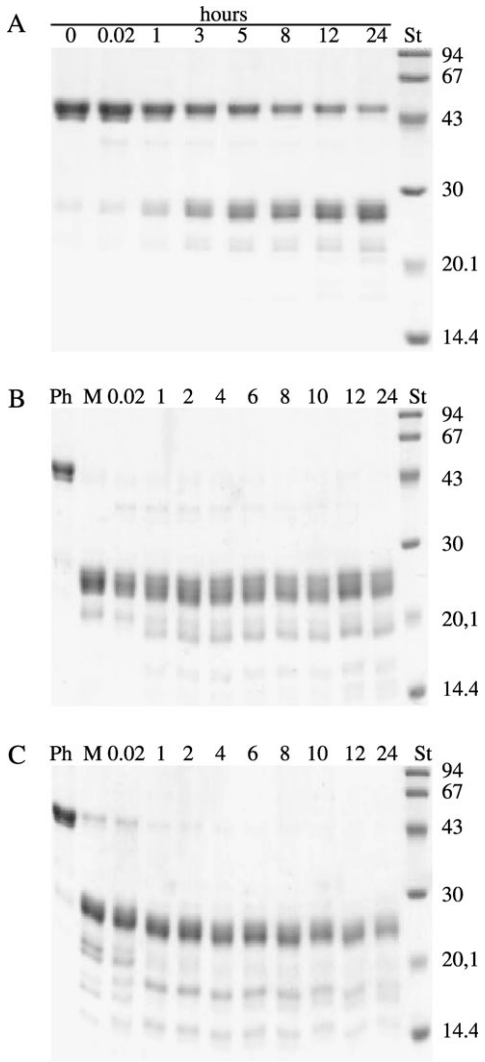

Fig. 3. Time-course of phaseolin degradation as revealed by SDS-PAGE. (A) LLP action; (B) LLP action on CPPh1-modified phaseolin; (C) CPPh1 action on LLP-modified phaseolin. MPh, modified phaseolin; St, molecular mass standards (kDa). Experimental conditions are as detailed in the Materials and methods.

By contrast, CPPh1 catalysed the extensive hydrolysis of phaseolin modified by LLP (Fig. 2C). In the experiment shown in Fig. 2C, 58% of the phaseolin was hydrolysed to TCA-soluble peptides in 10 h and 75% in 24 h. SDS-PAGE (Fig. 3C) also detected the decrease of protein concentration while the fragment composition remained essentially unchanged after the first hour of the reaction, indicating a predominantly co-operative-type of proteolysis (Vaintraub, 1998).

Action of LLP and CPPh1 on the αα′ and β_3 forms of β-conglycinin

No decrease in TCA-insoluble protein was observed during the incubation of both αα′ and β forms of β-conglycinin with LLP (Fig. 4). However, CPPh1, in contrast to phaseolin, degraded them. At the beginning this process exhibited a rapid rate of a non-co-operative type of proteolysis. It was later superseded by the co-operative proteolysis that resulted in the extensive hydrolysis of the β-conglycinin.. The kinetics of the extensive proteolysis of both β-conglycinin forms is similar and only the time-course of hydrolysis of the αα′ subunits is shown on Fig. 4. Only 9.5% and 4% of αα′ and β_3 forms, respectively, remained unhydrolysed after a 24 h hydrolysis.

Intact α and α′ subunits disappeared gradually during LLP proteolysis (Fig. 5A). Two bands of M_{app} 48 kDa and 19 kDa appeared, indicating the splitting off of the long disordered N-terminal extension of these subunits at the Asn residue adjacent to the β subunit-like fragment (Lawrence *et al*., 1994). The split proceeded slowly and intact subunits were detected even after 24 h hydrolysis. The addition of a new portion of enzyme and prolonged incubation results in the completion of the splitting.

No changes in electrophoretic pattern were detected during the action of LLP on β_3 β-conglycinin (data not shown).

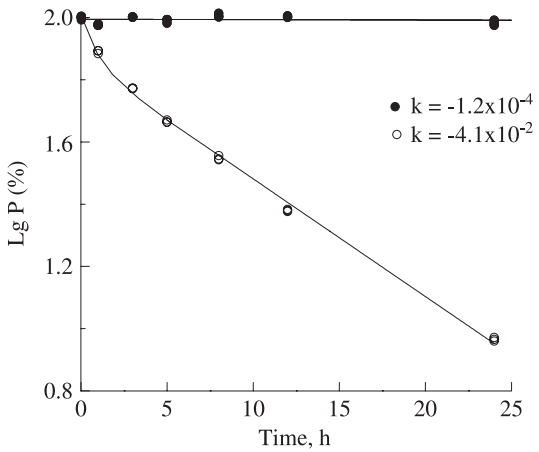

Fig. 4. Kinetic curves of αα′ β-conglycinin proteolysis by LLP (closed circles) and by CPPh1 (open circles). For other details see Fig. 2.

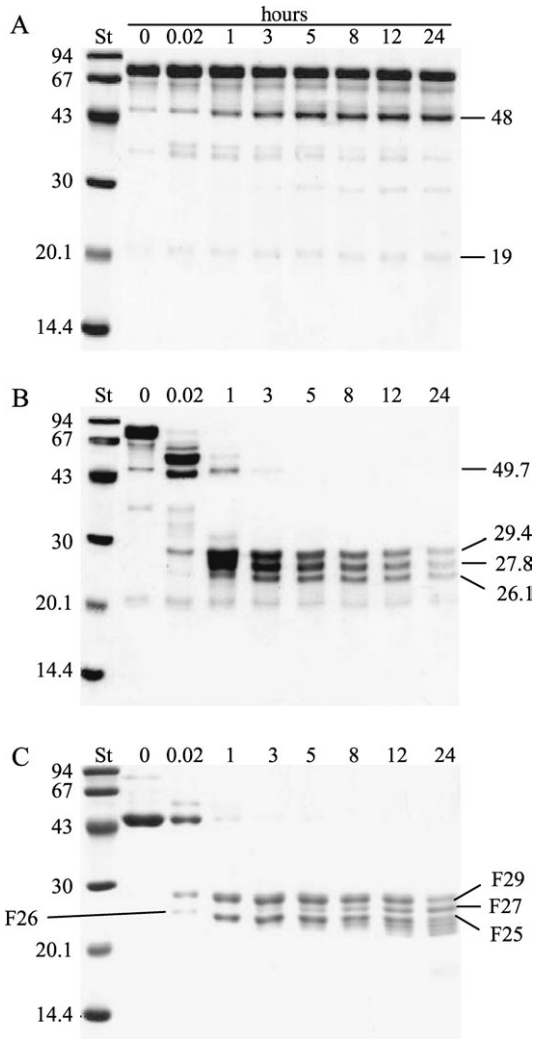

Fig. 5. Time-course of β-conglycinin degradation as revealed by SDS-PAGE. (A) Action of LLP on αα′ β-conglycinin; (B) action of CPPh1 on αα′ β-conglycinin; (C) action of CPPh1 on β_3 β-conglycinin. St, molecular mass standards (kDa). Experimental conditions are as detailed in Materials and methods.

The onset of the proteolysis of α and α′ subunits by CPPh1 brought about an almost instantaneous hydrolysis of their N-terminal extension leading to the formation of β subunit-like 49.7 kDa fragments (Fig. 5B). The latter were split further and during 1 h proteolysis almost entirely transformed into three final fragments of M_{app} 29.4, 27.8, and 26.1 kDa. No additional fragments identifiable by SDS-PAGE were observed during prolonged reaction. Slowing down the process by using a 10-fold diluted CPPh1 preparation allowed the detection of three intermediary products formed during the splitting off of the N-terminal extension (data not shown). One of these (59.4 kDa) was relatively stable and transiently accumulated before its conversion into the β subunit-like fragments.

SDS-PAGE (Fig. 5C) showed that during the non-co-operative stage of hydrolysis CPPh1 initially splits the β subunits into two fragments with M_{app} 29.4 kDa and 26.4 kDa (F29 and F26). Fragments with M_{app} 27.6 kDa and 25.8 kDa (F27 and F25) and several poorly resolved very faint bands in the region of M_{app} 25–22 kDa appeared later. Concurrently, the decrease in the intensity of F29 and F26 occurred. The N-terminal sequences of F26 and F25 were identical. They corresponded with that of the N-terminus of the intact β subunit with the N-terminal tetrapeptide LKVR split off. Evidently these fragments were derived from the N-terminal domain, fragment F26 being further shortened at its C-terminus. N-terminal sequences of fragments F29 and F27 (TISSED... and SRNPI..., respectively) showed that they originate from the C-terminal domain of the β subunit and the fragment F27 was formed by splitting off 12 amino acids from the N-terminus of fragment 29.

Discussion

The concerted action of LLP and CPPh1 is essential for the extensive degradation of phaseolin

According to the previous study LLP triggers the degradation of phaseolin and is capable by itself of its further extensive hydrolysis (Senyuk *et al*., 1998). However, these results were in need of verification. The electrophoretic purity of the LLP preparation used was characterized as 'close to homogeneity' only. Moreover, N-terminal analysis of fragments formed during phaseolin hydrolysis indicated the presence of an admixture of CPPh1. This consideration prompted the reinvestigation of the action of both proteinases on phaseolin using thoroughly purified proteinase preparations. Applying an improved method, the isolation of pure enzyme preparations was successful. The absence of contamination of the LLP and CPPh1 preparations by each other was proved using sensitive specific substrates and immunoblotting.

Using the highly purified enzymes confirmed the inability of CPPh1 to perform the extensive hydrolysis of phaseolin determined earlier (Boylan and Sussex, 1987; Rotari *et al*., 1997). However, by contrast with the previous results (Senyuk *et al*., 1998) it was found that LLP performs only a limited proteolysis of phaseolin. No decrease of protein was detected during prolonged action of LLP even after the addition of a new portion of enzyme. Therefore, each of the most active proteinases detected in germinated common bean seeds individually is incapable of extensive degradation of phaseolin. However, extensive hydrolysis of phaseolin was achieved by the consecutive action of LLP and CPPh1.

These results indicate that LLP is really the enzyme triggering the degradation of phaseolin, but it is incapable of the further hydrolysis of phaseolin to oligopeptides. The latter function is assumed by CPPh1. Thus the concerted action of LLP and CPPh1 seems to be essential for the extensive proteolysis of phaseolin. It is also indicated by the

results of Senyuk *et al*. (1998), where the use of LLP contaminated by CPPh1 brought about the extensive hydrolysis of phaseolin. It is possible that LLP takes part in the hydrolysis of some intermediary products of the CPPh1 action. Indeed, the vetch legumain (proteinase B) acts on vetch storage proteins that have been partially hydrolysed by proteinase A (the most active papain-like proteinase from germinated vetch seeds) (Shutov *et al*., 1981).

Comparison of the action of different proteinases on 7S storage proteins of common bean, soybean, and vetch

By contrast with LLP, the action of CPPh1 on other 7S seed proteins is quite different. CPPh1 carries out extensive hydrolysis of soybean β-conglycinin (present work) and also of vetch vicilin (Rotari *et al*., 1997). The action of several other exogenous proteinases is similar. The action of trypsin and chymotrypsin (Vaintraub *et al*., 1976) and pepsin (Vaintraub *et al*., 1979) on phaseolin is limited to the splitting of a small number of peptide bonds causing the loss of only 10–20% of protein, while these enzymes hydrolyse to short TCA-soluble peptides the storage proteins of a number of other leguminous seeds (Vaintraub *et al*., 1976). The structure of phaseolin proved to be more rigid than that of pea vicilin by several physical methods (Desphande and Damodaran, 1990). Phaseolin also differs from other 7S proteins in its stability at pH 2.0 (Hall *et al*., 1977). Evidently the resistance of phaseolin to the action of proteinases of different specificity is mainly due to the peculiarities of its structure.

The action of CPPh1 on β-conglycinin closely followed that of soybean papain-like proteinase C2 (Seo *et al*., 2000). Phaseolin is split into the similar fragments both by proteinase C2 (Seo *et al*., 2000) and by CPPh1 (Rotari *et al*., 1997). Similar hydrolysis products are formed during the action of CPPh1 and vetch proteinase A on vetch vicilin (Rotari *et al*., 1997; Fischer *et al*., 2000). Thus, the homologous proteinases from different plants appear to attack the same site of the storage proteins.

Connection between the structure of phaseolin and β-conglycinin and the differences in their proteolysis

Each proteinase is capable of splitting one to several accessible, flexible segments of a particular substrate protein, provided that these segments are situated on the surface of the protein molecules and contain peptide bonds corresponding to the proteinase specificity. If such cleavage(s) destabilize(s) the protein structure then unfolding of the substrate protein occurs leading to subsequent extensive hydrolysis. These considerations allow plausible explanations to be proposed for the differences observed in the course of proteolysis of different 7S proteins.

According to the canonical model of the seed 7S proteins structure developed by Lawrence *et al*. (1994), each of their subunits consists of two similar domains linked by an unstructured segment. In its turn, each domain is formed of a β-barrel and an α-helical region. Besides the inter-domain linker, certain loops joining the strands of β-barrels and the disordered terminal segments may be subjected to initial proteinase attack.

Although the long extensions of both α and α′ subunits of β-conglycinin split off by LLP are disordered, they are not hydrolysed further since they contain only one Asn residue located at the C-terminal end of each extension (Lawrence *et al*., 1994).

According to Senyuk *et al*. (1998), LLP splits the phaseolin subunits into their two modules at the bond Asn^{220}–Thr^{221}. It might be assumed that the admixture of CPPh1 contained in the LLP preparation used influences this result. However, the splitting also occurs when a pure LLP preparation is applied, and it can take place only at the site determined by Senyuk *et al*. (1998), as the Asn^{220} residue is the sole Asn in the disordered amino acid sequence that links both modules. It is located at the border of the C-terminus of the linker and the strand A′ of the C-terminal module (Fig. 6). Therefore, the splitting of phaseolin subunits by LLP really occurs at the bond Asn^{220}–Thr^{221} solely corresponding to the strict specificity of LLP. The high accessibility of Asn^{220} (64%) and moderate accessibility of the following Thr^{221} (32%) are in accordance with this assertion. Neighbouring asparagines N^{224} and N^{228} belonging to strands A′ and A were practically inaccessible (accessibility 15% and 3%, respectively).

The lower molecular mass fragments similar to those detected by Senyuk *et al*. (1998) also appear under the action of LLP devoid of CPPh1. They are barely visible on Fig. 3A, but are clearly seen when 24 h hydrolysis was extended to 48 h, with a second aliquot of enzyme added (Fig. 3C, lane M). It seems, however, that the prior action of CPPh1 enhances the rate of their formation (compare Fig. 3A and B).

No splitting of β-conglycinin subunits occurs. In α and α′ subunits of β-conglycinin, as well as in all other 7S proteins of known primary structure, Asn is replaced by other amino acids and is detected only in β subunits of β-conglycinin (Lawrence *et al*., 1994). However, in the latter, Asn^{237} is followed by Pro (Fig. 6). The cleavage of the Asn^{103}–Pro^{104} link was detected in phaseolin (Senyuk *et al*., 1998). However, the rate of its cleavage is extremely

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β-Phaseolin     210 SSRKSLSKQD--------NTI-GNEFGNLTERTD
α-Conglycinin   333 SSRKTISSEDKPFNLRSRDPIYSNKLGKFFEITP
β-Conglycinin   218 SSRKTISSEDEPFNLRSRNPIYSNNFGKFFEITP
                                  Z       A′    A
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Fig. 6. Representation of the potential sites for LLP cleavage (based upon the primary specificity of LLP) in and near the interdomain linker (denoted in bold) in phaseolin and β-conglycinin subunits. The site that is actually cleaved is denoted by regular font, the inaccessible ones are in italic. The structural segments are indicated according to Maruyama *et al*. (2001).

low in spite of the high accessibility of Pro^{104} (54%). The very low accessibility of Pro^{238} in β subunits of β-conglycinin (15%) evidently accounts for the non-cleavage of β-conglycinin subunits at this bond. Another putatively scissile link near the interdomain linker of β-conglycinin is the Asn^{232}–Leu^{233} link located in the strand Z lacking the C-terminal module of phaseolin (Maruyama *et al*., 2001). However, both amino acid residues are practically inaccessible (accessibility 7% and 2%, respectively). No cleavage of vetch vicilin was observed under the action of vetch legumain on vetch vicilin (AS Shutov, personal communication).

CPPh1 does not split the EF loop of the C-terminal module of phaseolin. However, CPPh1 does split the corresponding EF loop of vetch vicilin, which is then subjected to further extensive proteolysis by CPPh1 (Rotari *et al*., 1997). On this basis it was suggested that the cleavage of the EF loop is the prerequisite of further extensive hydrolysis of seed 7S proteins (Rotari *et al*., 1997). However, extensive hydrolysis of β-conglycinin by CPPh1 occurs, although none of the fragments that should be generated by splitting the EF loop were detected during the CPPh1 hydrolysis of the α, α′, and β subunits. Evidently, the resistance of phaseolin to CPPh1 attack is caused by different particularities of its structure.

The main event occurring during the proteolysis of phaseolin by LLP is the splitting of the covalent bond between the two domains of the phaseolin subunits resulting from the cleavage of the Asn^{220}–Thr^{221} bond. During the subsequent action of CPPh1 the sites Ser^{216}–Lys^{217} and in all probability Ser^{204}–Lys^{205} are cleaved (Rotari *et al*., 1997) resulting in the splitting off of one or two peptides. It may be supposed that these events produce changes in phaseolin structure inducing its susceptibility to further profound hydrolysis by CPPh1. *In vivo*, both enzymes are located together in the same subcellular compartment (Tiedemann *et al*., 2001) and the order of their action initiating the profound proteolysis by CPPh1 could be inverted also.

Similarity of degradation process of phaseolin in vitro *and* in vivo

The degradation of storage proteins in the storage tissues is detected in many plants only after 2–3 dai, while the majority of storage protein breakdown occurs 4–8 dai (Müntz, 1996). In common bean cotyledons the decrease in protein content was also observed after 2 dai (Boylan and Sussex, 1987). Using the immunohistochemical method, Tiedemann *et al*. (2000) showed massive phaseolin degradation at 5 dai. By comparison, the activity of both CPPh1 (Rotari, 1996) and LLP (Senyuk *et al*., 1998) was also detected at 2 dai, and then increased dramatically to a maximal level at 5–7 dai. Therefore, the temporal patterns of storage protein degradation and the appearance of the two enzymes studied here is consistent with these enzymes catalysing this degradation *in vivo*.

The first step of phaseolin proteolysis *in vitro* is the rapid split of its subunits into two halves that is carried out by both CPPh1 and LLP. A much slower rate of further extensive degradation leads to the accumulation of half-subunits. SDS-PAGE shows a concomitant very slow degradation of the latter into fragments of lower molecular mass performed by LLP. The fragments formed are similar, irrespective of the prior action of CPPh1.

The SDS-PAGE of phaseolin isolated from the seedling cotyledons at 6 dai shows a very similar pattern (Senyuk *et al*., 1998). Apart from a quantity of unmodified protein, the overwhelming majority consists of half-subunits, and only a lesser content of smaller fragments is detected. The molecular mass and the N-terminal sequence of smaller fragments observed *in vivo* correspond to those produced *in vitro* by the action of LLP on the phaseolin isolated from dry seeds (Senyuk *et al*., 1998). As shown in the present work the admixture of CPPh1 may influence only the rate of formation of these fragments.

Further extensive CPPh1 hydrolysis of modified phaseolin is of the co-operative type that does not produce relatively stable lower molecular mass fragments detectable by SDS-PAGE (Vaintraub, 1998). Indeed, CPPh1 did not form such fragments *in vitro*, and phaseolin isolated from cotyledons harvested at 6 dai did not contain fragments identifiable as having been generated by CPPh1.

Thus the combined data support the suggestion that the degradation of phaseolin *in vivo* proceeds in a manner similar to the *in vitro* reactions described here.

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