Proteolysis of Phaseolin in Relation to Its Structure

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Phaseolin differs from other native legume proteins in that its hydrolysis by trypsin and pepsin stops after a limited number of peptides have been cleaved off. Concomitantly, trypsin splits each phaseolin subunit approximately into halves. The N-terminal sequencing of these final hydrolysis products of high molecular mass showed loss of a tetrapeptide from the N terminus of each subunit, with a second cleavage in the interdomain linker. Other probable sites cleaved by trypsin, which account for the quantity of degraded protein, may be deduced from the tertiary structure of phaseolin and from the specificity of trypsin. Proteolysis by pepsin is limited to cleavage of seven amino acids from the N terminus, and of two to three peptides probably from the disordered C-terminal segment of phaseolin subunits. The cleavage site in the N-terminal sequence has been identified. The peculiarities of the phaseolin structure that may cause its resistance to the proteolytic attack are discussed.

Keywords: *Phaseolin; proteolysis; pepsin; trypsin*

INTRODUCTION

Leguminous seeds are important sources of proteins for food and feed. Their major protein components are 7S and 11S storage proteins. One of the main factors affecting their nutritive value is their susceptibility to hydrolysis by digestive proteinases (Liener and Thomson, 1980). The degree and the rate of hydrolysis of several representative leguminous storage proteins by pepsin are similar to those of the standard light hydrolyzable protein, hemoglobin (Vaintraub et al., 1979). Nielsen et al. (1988) also observed a comparatively fast and complete hydrolysis of soybean and pea storage proteins by pepsin. The hydrolysis of native 7S and 11S proteins by trypsin and chymotrypsin is slower, but the extent of proteolysis is also great (Vaintraub et al., 1976). Nielsen et al. (1988) obtained qualitatively similar results using SDS electrophoresis.

A striking exception is displayed by phaseolin, a 7S protein of dry bean *(Phaseolus vulgaris).* Its hydrolysis by digestive proteinases, including pepsin, stops after cleavage of a small proportion of peptide bonds (Liener and Thompson, 1980; Romero and Ryan, 1978; Vaintraub et al., 1976, 1979). Sequential action of pepsin and trypsin is additive. Hence, pepsin action does not enhance trypsin hydrolysis. The resistance of phaseolin to the action of seven different proteases has been shown qualitatively by SDS electrophoresis. However, SDS electrophoresis showed that all proteinases tested except pepsin caused gradual disappearing of the bands due to subunits. Concomitantly, several bands with M_r approximately half of a subunit appeared, irrespective of the type of proteinase used (Deshpande and Nielsen, 1987; Nielsen et al., 1988). The peptide bonds split by trypsin, chymotrypsin, and papain were identified. They were located in the same region in the middle of each subunit polypeptide chain (Nielsen et al., 1988). No changes in electrophoretic pattern of pepsin hydrolyzates were observed by these authors.

Disruption, upon heating, of the tertiary and quaternary structure of phaseolin leads to a rapid and complete proteolysis (Vaintraub et al., 1979; Deshpande and Damodaran, 1989a). Thus, the very limited hydrolyzability of phaseolin is evidently due to the peculiarities of structure, distinguishing it from other 7S proteins. The conformational stability of pea vicilin is lower as compared with that of phaseolin (Deshpande and Damodaran, 1989b). However, the specific features of phaseolin structure determining its resistance to enzymatic hydrolysis remained unknown.

Phaseolin is a trimer of three types of similar subunits consisting of 397, 411, and 412 amino acid residues (Slightom et al., 1985). The subunits are termed β, α, and α', respectively. Each subunit has two potential sites of glycosylation completely or partially glycosylated (Bollini et al., 1983), which causes the appearance of additional bands during SDS electrophoresis (Lioi and Bollini, 1984).

The high-resolution X-ray structure of phaseolin (Lawrence et al., 1994) was described recently. It has also been determined for another 7S protein, canavalin (Ko et al., 1993), and proves to be very similar to that of phaseolin. On the grounds of these results, together with the primary structure data of 15 subunits of 7S proteins from 9 species, Lawrence et al. (1994) developed a canonical model of the structure of 7S proteins. These achievements open a new way to understanding the peculiar behavior of phaseolin during proteolysis. Here we report the results of the investigation of the hydrolysis of phaseolin by trypsin and pepsin that extend the results previously obtained. The peculiarities of phaseolin hydrolysis are interpreted on the basis of the structural model of Lawrence et al. (1994).

MATERIALS AND METHODS

Reagents. Phaseolin was isolated according to the method of Schlesier et al. (1984) and further purified by DEAE-cellulose chromatography. Trypsin (Serva, Germany) and

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pepsin (Olajne, Latvia) were used in this work. The content of active trypsin was determined by active-site titration (Chase and Shaw, 1970). All other reagents were of analytical grade.

Proteolysis. Solutions (1%) of phaseolin were hydrolyzed by trypsin in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ and pepsin in 0.1 M phosphate−citrate buffer, pH 2.2 and 4.0. Both buffers were adjusted to an ionic strength of 0.5 by adding NaCl. The enzyme/substrate ratio was 1:50, calculated for trypsin on the basis of active enzyme. The incubation temperature was 30 °C. The duration of trypsin hydrolysis was 48 h and that of pepsin 36 h. NaN_3 was added to the incubation mixture at 0.04% concentration. Samples of hydrolyzates were taken periodically. The reaction was stopped by addition of 15% CH_3COOH to give pH 2.5−3.0 for trypsin hydrolyzates and by addition of 4% $Na_2B_4O_7 \cdot 10H_2O$ to give pH 6.5−7.0 for pepsin hydrolyzates. For consecutive hydrolysis by both enzymes 2% phaseolin solution was hydrolyzed by pepsin during 12 h. Then, after the pH of the hydrolyzate had been raised to 7 by addition of 0.5 M NaOH, it was dialyzed against the buffer used for trypsin hydrolysis. Finally, the concentration of protein was determined and trypsin solution was added.

Residual (TCA-insoluble) protein, the content of amino groups in the TCA-soluble fraction, and SDS electrophoretic pattern were determined in the samples. Protein was determined by a dye-binding method (Gofman, 1968; Vaintraub and Yattara, 1995). In the final hydrolyzates the residual protein was separated from the low molecular mass hydrolysis products by gel filtration through a Sephadex G-75 column (1.2 × 57 cm, equilibrated with the hydrolysis buffer). After gel filtration, the protein was determined according to the microbiuret method (Itzhaki and Gill, 1964). The residual protein in the final hydrolyzates was also calculated by subtracting the molecular mass of the peptides presumably split off during proteolysis from that of the intact subunits. The former was determined using the program PHYSCHEM of the PCGENE package.

The molar concentration of peptides formed was estimated in the TCA-soluble fractions of the final hydrolyzates. The amino groups were determined as described earlier using 2,4,6-trinitrobenzenesulfonic acid (Vaintraub et al., 1976). Correction was made for the lysine content of the peptides presumably formed. The molar absorbance of peptides at 340 nm was taken as 11 000 M^{-1} cm^{-1} and that of ϵ-amino groups of lysine as 13 000 M^{-1} cm^{-1} [values determined by Okuyama and Satake (1960) for several dipeptides and ϵ-TNP-lysine, respectively].

Electrophoresis. Nondenaturing gradient pore electrophoresis was carried out in a vertical flat-bed (1 × 115 × 115 mm) gradient (4−30%) polyacrylamide gel. Tris-borate buffer (0.09 M, pH 8.4) was used. The duration of electrophoresis was 4500 Vh. Ferritin, catalase, phaseolin, and bovine serum albumin were applied simultaneously as standards. The percent of the residual protein was calculated from the decrease of its M_r.

For SDS electrophoresis the protein of hydrolyzed samples was precipitated by TCA (final concentration 4%), washed three times with acetone, and then treated according to the method of Laemmli (1970). SDS electrophoresis was carried out in a vertical flat-bed 12% polyacrylamide gel. The ratio of acrylamide to methylenebis(acrylamide) was 200:1. Phosphorylase *b*, bovine serum albumin, ovalbumin, carboanhydrase, Kunitz soybean trypsin inhibitor, and α-lactalbumin were used as standards for M_r determination. The electrophoregrams were stained with Coomassie brilliant blue G-250. The electrophoregrams of initial phaseolin and of pepsin hydrolyzates were quantified by densitometry in the gel scanning and integrating attachment to the spectrophotometer M-40 (Carl Zeiss, Jena, Germany).

N-Terminal Sequencing. The fragments formed during proteolysis were separated by SDS electrophoresis, blotted on PVDF membranes, and sequenced according to a special procedure (Beckman protocols) using the Beckman sequencer LF3400.

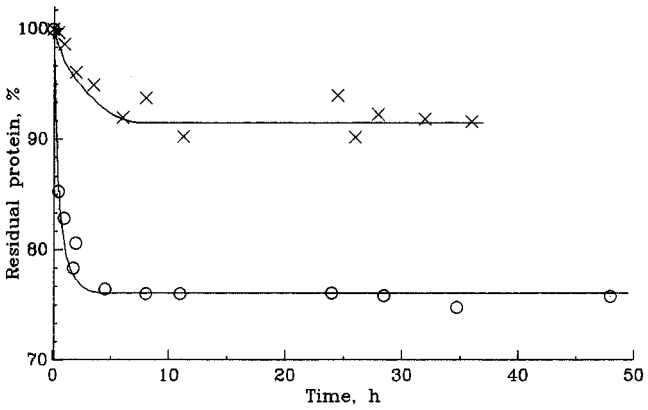

Figure 1. Time dependence of the residual protein concentration during phaseolin hydrolysis by trypsin (○) and pepsin (×). Hydrolysis conditions are detailed under Materials and Methods. The initial concentration is taken for 100%.

Table 1. Residual Protein in the Final Hydrolysates of Phaseolin[a,b]

	method of determination			
hydrolyzing proteinase	precipn by TCA, dye-binding	gel filtrn, microbiuret	nondenatrg gradient pore electrophoresis	calcd from assumed cleavage points
trypsin	76 ± 1	79 ± 6	78 ± 5	78.5
pepsin (pH 4.0)	91 ± 1	94 ± 1	95 ± 3	93.5

[a] Percent of the initial protein. [b] $n = 4-5$.

RESULTS

Phaseolin Degradation. The content of the residual protein decreased under the action of trypsin during the first 6−8 h with a rapidly declining rate. During subsequent hydrolysis it remained unchanged (Figure 1). Similar results were obtained when phaseolin was hydrolyzed by pepsin both at pH 4.0 (Figure 1) and at pH 2.2 (not shown here). However, the degree of proteolysis by pepsin was considerably lower. It was slightly higher at pH 2.2 than at pH 4.0, but the difference was not significant. The final degree of proteolysis attained by both proteinases is given in Table 1, which also shows the results of its determination by other methods used. The final high molecular mass products of trypsin and pepsin hydrolysis will be subsequently referred to as phaseolin-T and phaseolin-P, respectively. The consecutive hydrolysis by pepsin and trypsin was additive. In this case the residual protein amounted to 66.7 ± 1.5% of the initial protein. At the end of proteolysis the calculated number of peptides split off reached 7.7 ± 0.2 per subunit in trypsin hydrolyzates and 5.0 ± 0.1 in those of pepsin.

Electrophoresis of Residual Protein. SDS electrophoresis of the unhydrolyzed phaseolin showed four bands of its subunits (Figure 2a). The intensities of these bands decreased under the action of trypsin, with concomitant appearance of fragments showing approximately half the M_r of the subunits. The fragments appeared already after 1.5 min of hydrolysis. Their M_r was initially in the range of 25−33 kDa and decreased gradually to 21−26 kDa. The original subunit bands disappeared after 1 h of hydrolysis, while the changes in M_r of their fragments continued during 8 h of hydrolysis, i.e., until the decrease of the residual protein occurred. The final electrophoresis pattern is shown in Figure 2a.

On SDS electrophoresis of phaseolin-P only three bands of subunits and no fragments were observed at

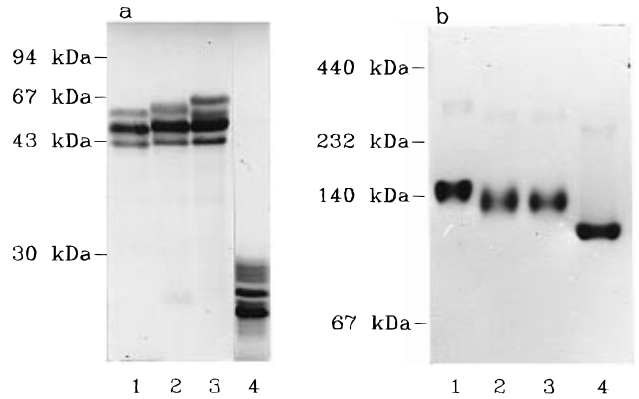

Figure 2. Electrophoregrams of initial phaseolin and its hydrolysis products. (a) SDS electrophoresis: lane 1, phaseolin-P (pH 2.2); lane 2, phaseolin-P (pH 4.0); lane 3, initial phaseolin; lane 4, phaseolin-T. (b) Gradient pore electrophoresis: lane 1, initial phaseolin; lane 2, phaseolin-P (pH 2.2); lane 3, phaseolin-P (pH 4.0); lane 4, phaseolin-T. The conditions of electrophoresis are detailed under Materials and Methods.

Table 2. Comparison of the Relative Content of Electrophoretic Bands of Phaseolin and Phaseolin-P[a,b]

	bands[c]				
protein	1	2	2+3	3	4
phaseolin	22 ± 1	20 ± 1		38 ± 1	20 ± 1
phaseolin-P	24 ± 3		53 ± 3		23 ± 3

[a] Relative percent. [b] $n = 4$. [c] Numbered according to increasing mobility.

both pH 2.2 and 4.0 (Figure 2a). It follows from the comparison of the ratio of electrophoretic bands of unhydrolyzed phaseolin and of phaseolin-P (Table 2) that this disappearance is caused by merging of bands 2 and 3 (the bands are numbered according to the increase of their mobility). There was a slight but definite increase of the mobility of the subunits corresponding to lowering of their M_r by 2−3 kDa. This was more evident at pH 2.2.

Gradient pore gel electrophoresis (Figure 2b) showed that phaseolin retained its quaternary structure after hydrolysis by both trypsin and pepsin. M_r of phaseolin-T decreased from 140 to 109 ± 7 kDa and that of phaseolin-P to 133 ± 4 kDa. The values of residual protein calculated from these data agree with those determined directly (Table 1).

N-terminal sequencing of phaseolin-T showed two types of fragments formed

fragments with N-terminal sequence

E E E E S Q D N P F

fragments with N-terminal sequence

Q D N T I G N E F G

indicating splitting of the bonds R4−E5 in the N terminus and K217−Q218 in the middle of subunits. All modified subunits of phaseolin-P had the same N-terminal sequence

E S Q D N P F Y F,

indicating splitting of the bond E7−E8.

DISCUSSION

Our results confirm the high resistance of most of the phaseolin molecule to proteolytic attack reported by

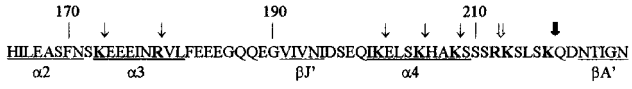

Figure 3. Fragment of the amino acid sequence of phaseolin subunit comprising the interdomain linker and the adjacent parts of domains. The numbers of amino acid residues are given for the mature β-subunit. The structural segments are indicated according to the results of Lawrence et al. (1994). Arrows indicate the cleavage loci: heavy arrow, determined in this work; open arrow, determined by Nielsen et al. (1988); ↓, assumed.

numerous authors (see the Introduction). The quantitative determinations of the residual protein and of the amino groups in the TCA-soluble fraction of the final hydrolyzates are in good accordance with the results obtained earlier (Liener and Thompson, 1980; Romero and Ryan, 1978; Vaintraub et al., 1976, 1979). We also observed the cleavage by trypsin of the phaseolin subunits into two roughly equal fragments reported previously (Liener and Thompson, 1980; Romero and Ryan, 1978; Nielsen et al., 1988). A more prolonged hydrolysis used in this work permitted us to detect a gradual decrease of the M_r of these fragments that was correlated with the decrease of the residual protein. The loss of more than 20% of protein, as well as the decrease of M_r of the fragments, indicates the splitting off by trypsin of an appreciable number of short TCA-soluble peptides. The gradient pore electrophoresis showed that the cleavage of phaseolin subunits and further split off peptides does not break its quaternary structure.

During pepsin hydrolysis of phaseolin no changes of the electrophoretic pattern were observed earlier (Deshpande and Nielsen, 1987; Nielsen et al., 1988). We observed small but definite increases of mobility both of undissociated phaseolin by gradient pore electrophoresis and of its subunits by SDS electrophoresis. So, the limited proteolysis of phaseolin by pepsin is also confirmed by electrophoresis.

By taking account of the primary (Slightom et al., 1985) and tertiary (Lawrence et al., 1994) structure of phaseolin, it is possible to identify the cleaved bonds indicated by sequencing, as well as the most probable loci of other cleavages. The phaseolin β-subunit is composed of two extremely similar structural domains. Each consists of two elements: a compact eight-stranded β-barrel and an extended loop consisting of three short α-helices. The interdomain linker consists of helix 4 and of an unfolded sequence not detected in the X-ray structure (Figure 3). The sequences of 10 N-terminal and 15 C-terminal amino acid residues are also absent from the X-ray pattern. The N-terminal sequence contains one arginine residue. Two lysine residues are located in the C-terminal sequence. Besides these disordered regions, a high-temperature factor is exhibited by the poorly formed N-terminal part of helix 4 and by two short sequences in the loops connecting the β-strands (Lawrence et al., 1994). Helix 3 is exposed to the environment in both domains of the subunits and within the trimer (Lawrence et al., 1990). Subunits α differ from subunits β by two short insertions in the loops linking the structured segments and by an insertion of nine amino acid residues containing one more lysine in the disordered C-terminal sequence. One lysine residue in the interdomain segment is replaced by a glutaminic one.

The K217−Q218 bond cleaved by trypsin is located in the disordered section of the interdomain linker (Figure 3). Nielsen et al. (1988) reported splitting of a different bond, R212−K213, in the same section of the

linker. This discrepancy may be due to the shorter time of the hydrolysis performed by these authors. Probably, the earliest cleavage is that of the R212−K213 bond, causing the formation of fragments detected by SDS electrophoresis. A more prolonged proteolysis leads to the subsequent cleavage at K217−Q218 detected in this work, and a pentapeptide is split off. The second cleavage point determined by sequencing accounts for the removal of the N-terminal tetrapeptide. Naturally, these two peptides cannot account for the 20% loss of protein during trypsin hydrolysis. A number of other peptides must also be split off. It follows from the sequencing results that it may take place only from the C termini of both domains. In all probability two and three peptide bonds are cleaved in the disordered C-terminal sequences of subunits β and α, which contain two and three lysine residues, respectively. Other loci probably accessible to the trypsin attack are the helix 4 and helices 3 in both domains. The former is, evidently, hydrolyzed in canavalin (Ko et al., 1993). If the cleavage in these helices really takes place, the split off peptides would account for the experimentally determined loss of protein during the hydrolysis by trypsin (Table 1). Assuming that all peptide bonds formed by lysine and arginine in the split off sector are cleaved in the final hydrolyzate (Figure 3), the overall number of peptides formed would be 11 for each subunit. These values may be considered close to the number of peptides calculated from the determination of amino groups in the TCA-soluble fraction of hydrolyzate (the conditional character of the values of molar absorbance used in these calculations should be taken into account). Although the agreements of these values support the presumed sites of phaseolin proteolysis, their chance agreement is not fully excluded. Further studies are needed to verify the number and the location of the supposed cleavage sites.

The N-terminal sequence of the phaseolin-P subunits suggests that pepsin splits off seven amino acids from the disordered N-terminal segment. Splitting off the disordered C-terminal segment is also very probable. These splittings would account for the decrease of protein during hydrolysis (Table 1). The shortened subunits remain whole. Evidently, no amino acid residues corresponding to pepsin specificity are found in the interdomain linker that is easily disrupted by a number of other enzymes (Nielsen et al., 1988).

Another cleavage point exists in canavalin, besides those situated in the interdomain linker (Smith et al., 1982). It results in the cleavage of one of the subunit domains and formation of 13 and 12 kDa fragments. The position of the EF loop of low electron density connecting E and F β-strands of the C domain is consistent with the length of these fragments (Ko et al., 1993). This loop produces a prominent protrusion on the surface of the subunit. In all vicilins of known primary structure except phaseolin, the EF loop is even longer than in canavalin (Lawrence et al., 1994). N-terminal sequencing of the fragments formed during post-translational cleavage of pea vicilin (Gatehouse et al., 1983), 7S protein of cacao seeds (Shutov et al., 1996), and soybean β-conglycinin hydrolyzed by trypsin (Shutov et al., 1996) also showed cleavage in the EF loop. No such fragments are detected in hydrolyzates of phaseolin, and the structure of its EF loop is different. It is considerably shorter, and Glu283 situated in the middle of this loop participates in a salt link with Arg53 situated in the AB loop of the N domain (Lawrence et al., 1994). Evidently, these peculiarities of the phaseolin EF loop make it inaccessible to proteinases.

In contrast to other 7S proteins the greatest part of the phaseolin molecule is remarkably resistant to proteolytic attack. It seems reasonable to assume that the splitting of the EF loop is essential for further profound hydrolysis of 7S proteins.

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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