CHARACTERISATION OF PHASEOLIN MODIFIED BY LEGUMAIN

Vitalie I. ROTARI, Diana MORARI, Tatiana STEPURINA

Laboratory of Plant Biochemistry

Fazeolina se deosebeşte de alte proteine de rezervă din seminţele plantelor leguminoase prin faptul că hidroliza ei, atât la acţiunea enzimelor proteolitice endogene, cât şi a celor exogene, se opreşte după modificarea limitată a moleculei, care rezultă în formarea fragmentelor cu masă moleculară ce corespunde jumătăţii subunităţilor fazeolinei. Legumaina este singura enzimă a cărei acţiune asupra fazeolinei diferă de alte proteinaze. Mai mult decât atât, modificarea fazeolinei de către legumaină iniţiază degradarea ei profundă. Fazeolina modificată de legumaină este compusă din fragmente de două tipuri care rămân legate necovalent în structura terţiară şi cuaternară. Situsurile clivării sunt localizate numai pe una din suprafeţele trimerului fazeolinei ce indică că particularităţile structurii fazeolinei pot influenţa accesibilitatea legăturilor peptidice la atacului proteolitic.

During germination the seed storage proteins (SP) are hydrolysed to free amino acids, which serve as precursors for the synthesis of new proteins in the seedling. Endopeptidases play key roles in SP degradation, producing oligopeptides. The latter are, in turn, hydrolysed by exopeptidases to free amino acids.

Cysteine proteinases (EC 3.4.22) are the major endopeptidases present in the cotyledons during early seedling growth, and are generally considered to be largely responsible for the mobilization of the SP [1]. They fall into two families, the low-specificity enzymes from the papain family and Asn-specific enzymes from the legumain family. The former exhibit low specificity for the peptide bonds cleaved while the latter are specific for the cleavage of Asn-X peptide bonds. Legumains have almost strict specificity for Asn in the P1 positions with only Asp being tolerated there but at a 100 times lower rate [2]. Due to their narrow specificity and wide distribution [3], these proteases may play a role as regulatory enzymes.

It has been recognized that SP mobilization during germination is a regulated process and a hypothetical scheme of SP degradation in germinating legume seeds was proposed [1]. In this scheme the central role in SP mobilization was attributed to cysteine proteinases termed A and B, which are synthesized *de novo* during seed germination. Proteolytic modification of storage proteins is the first stage in their degradation in germinated seeds [1]. In a number of species type A proteinases, which are members of the papain family [4], were assumed to start SP degradation modifying them by limited proteolysis making SP available to the attack by other proteinases. They are also capable of further hydrolysis of the modified storage proteins to short peptides. However once being modified SP are broken down co-operatively by both proteinases A and B and by carboxypeptidases. So, the complete hydrolysis of protease A modified SP is achieved by the orchestrated action of proteases synthesized *de novo* and by those pre-existing in seeds [1]. It was claimed that proteinase B, which belongs to the legumain family, does not act on unmodified SP, such as vicilin and legumin [5]. According to this hypothesis, proteinase A should play a central role in controlling the initiation of SP breakdown.

Three cysteine proteinases designated CPPh [6], legumain [7] and CPA [8] were found in germinated kidney bean (*Phaseolus vulgaris* L.) seedlings. CPPh and CPA were shown to be papain-like cysteine endopeptidases [8] belonging to the clan CA [4] whereas legumain [7] belong to the clan CD of cysteine endopeptidase [9]. The sequence data showed that these endopeptidases are related to the corresponding proteinases from cotyledons of germinating vetch (*Vicia sativa* L.) seeds [8]. Their action on kidney bean vicilin-like 7S storage globulin phaseolin, that is the major SP in this legume, is limited to the cleavage of protein subunits into high-molecular mass fragments and to the splitting off a small number of short peptides [6,7,8]. Thus the three 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 legumain and CPPh [10].

The investigation of the role of proteases from the kidney bean in the degradation of phaseolin showed that the above mentioned hypothetical scheme needs some modifications because only limited proteolysis was achieved by incubating these proteinases individually with phaseolin as a substrate. New results [11] also questioned the triggering role of proteinase A in mobilisation of SP in vetch. Several other papain-like cysteine endopeptidases were detected in germinating vetch [12] at a much earlier stages of germination than protei-

nase A and might be the initiators of SP degradation. It may be expected that such a controlling proteinase should have a narrow cleavage specificity like the lugumains. This seems to be the case in kidney bean where phaseolin modified by legumain becomes susceptible to complete hydrolysis at the action of CPPh [10]. Thus the caracterisation of phaseolin modified by legumain is of great interest for the finding of the peculiarities that makes it susceptible to the action of other endopeptidases. Here we report the results of the investigation of the action of legumain on phaseolin that extend the previously obtained results. The phaseolin structural model of Lawrence et al. [13] is used for visualizing the location of the detected cleavage sites.

Materials and Methods

Reagents. Phaseolin was isolated according to the method of Schlesier et al. [14]. Legumain was isolated as previously described [10]. The content of active legumain was determined with the synthetic substrate Bz-Asn-*p*-nitroanilide.

Proteolysis. Phaseolin solution (2%) in 120 mM phosphate-citrate buffer, containing 180 mM NaCl, 2 mM DTT, 500 μM EDTA, and 0.02% NaN_3, pH 5.6 was mixed with an equal volume of legumain solution (100–250 mU ml^{-1}) in 200 mM sodium acetate buffer, containing 2 mM DTT, 500 μM EDTA, and 0.02% NaN_3, pH 5.8 and the reaction mixture was incubated for 24 h at 30 ^oC. The incubation mixtures for the digestion of phaseolin contained 10 μM E64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), an inhibitor of papain-like cysteine proteinases.

Polyacrylamide gel electrophoresis (PAGE). For sodium dodecyl-sulfate (SDS) electrophoresis (SDS-PAGE) the protein samples were treated according to Laemmli [15]. The SDS-PAGE was carried out in a vertical flat-bed 12.5% gel with the ratio of acrylamide to methylenebis(acrylamide) of 200:1 [16]. Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), Kunitz soybean trypsin inhibitor (20.1 kDa), and α-lactoglobulin (14.4 kDa) were used as standards for molecular mass (M_r) determination. The gels were stained with Coomassie brilliant blue G-250 according to the standard procedure.

Nondenaturing gradient pore electrophoresis was carried out in a vertical flat-bed gradient (4-30%) PAGE using 90 mM Tris-borate buffer system, pH 8.4. The duration of electrophoresis was 4500Vh. Ferritin (440 kDa), catalase (232 kDa), and bovine serum albumin (67 kDa) were used as standards. The percent of the residual protein was calculated from the decrease of phaseolin molecule's M_r. The gels were stained with Coomassie brilliant blue R-250 according to the standard procedure.

2-D electroforesis was carried out in a vertical flat-bed nondenaturing gradient pore (4-30%) PAGE in the first direction and the protein bands were excised from the gel and run in a flat-bed 12.5% PAGE with the ratio of acrylamide to methylenebis(acrylamide) of 37.5:1 in the second direction.

Protein modeling. Tertiary and quaternary structures of the phaseolin molecule was drawn from PDB record 2PHL [17] by use of the Swiss-PdbViewer program [18].

Results and Discussion

The modification of phaseolin by legumain seems to trigger its complete hydrolysis [2,7,10]. This modification results in the formation of fragments with molecular masses ranged from 12 to 27 kDa (Fig.1A). These fragments broadly fall into two groups:

1. The first group is represented by the fragments with M_r that roughly corresponds to that of a half phaseolin subunit. Fragments of this group resemble those observed during the hydrolysis of phaseolin by both endogenous papain-like [6,19] and exogenous [20,21] proteases.

2. The second group comprises the fragments that have molecular masses less than half phaseolin subunit. Phaseolin fragments of these sizes were observed only under the action of endogenous legumain [2,7,10]. While during the *in vitro* proteolysis of phaseolin by legumain intermediate fragments were formed some of which disappeared or decreased significantly during extended proteolysis [7] the action of legumain in presence of E64, an inhibitor of papain-like cysteine proteases, results in a stable pattern of formed fragments [2].

The fragments of the first group were labeled F1h through F4h while those of the second group F1 through F6 (according to decreasing apparent M_r). The comparison of the fragments of the second group (Fig. 1A) with the previously reported results [7] showed that they are very similar despite of slight difference in their molecular masses (Table 1). This comparison permits to confirm the same three cleavage sites identified

previously [7]: N103-P, N220-T, and N235-S (here and forward the amino acids are numbered according to phaseolin β numbering) which belong to the FG loop in the N-terminal β-barrel domain and to the A' strand and AB loop in the C-terminal β-barrel domain of the phaseolin subunit respectively, according to the structure model of Lawrence et al [13]. The fragment F3 does not correspond to any of previously identified fragments but its apparent M_r is similar to a fragment of phaseolin that is formed during *in vivo* digestion and results from the cleavage site N235-S. Consequently, in all cases, the cleavage sites were flanked by Asn in P1 position which corresponds to the almost strict Asn-specificity of legumain-like cysteine endopeptidases [2].

Phaseolin is a trimer of three types of similar subunits termed β, α, and α' consisting of 397, 411, and 412 amino acid residues, respectively [13]. Each subunit has two potential glycosilation sites [22] and both singly and doubly glycosylated species are known to exist *in vivo* [23] which cause the appearance of molecular heterogeneity of phaseolin subunits [24]. As a result when phaseolin is subjected to SDS-PAGE it can be separated into four polypeptides, called size class [25], with range in M_r from 45 to 52 kDa. It was shown that these bands can be more easily separated by changing the acrylamide:methylenebis(acrylamide) ratio to 200:1 [26].

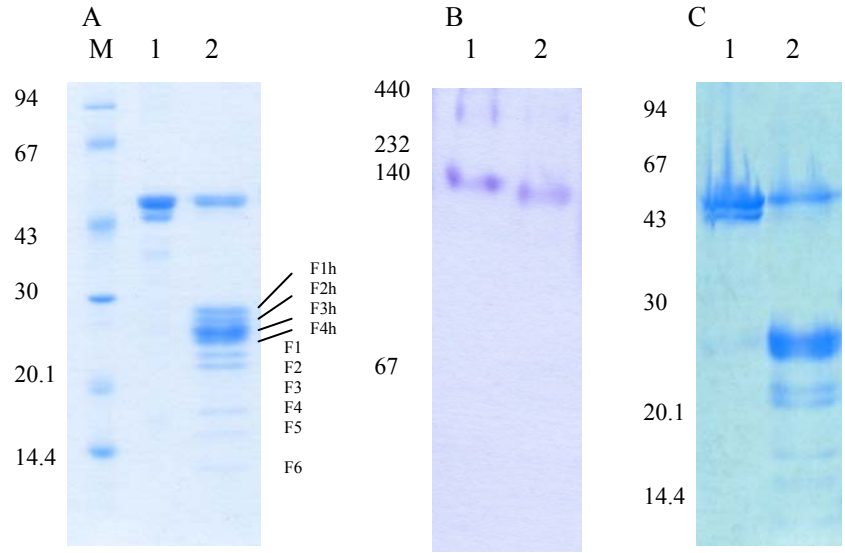


Fig.1. Electrophoretic pattern of the residual protein during phaseolin proteolysis by legumain.

A – SDS electrophoresis: On the right are indicated the fragments detected by SDS electrophoresis in the phaseolin modified by legumain;
B – Gradient pore electrophoresis;
C – 2-D electrophoresis;
Experimental conditions are detailed in Materials and methods.
Lane M – molecular weight standards; lane 1 – native phaseolin; lane 2 – phaseolin modified by legumain.
On the left are indicated the molecular masses of the standards (in kDa).

We decided to use similar conditions for the separation of the fragments of the first group that could not be separated under standard ratio of 37.5:1 acrylamide/methylenebis(acrylamide) [2,7,10]. Under these conditions four fragments belonging to the first group with have the apparent molecular masses in the range from 24 to 28 kDa were identified (Table 1).

It is peculiar that in the case of CPPh action on phaseolin also four fragments corresponding to half phaseolin subunit were detected. They, by two, belong to N- and C-terminal domains and were suggested to be due to heterogeneity in the glycosilation and by CPPh cleavage of C-terminus of the formed fragments. The molecular masses of fragments generated by legumain are higher that those generated by CPPh and while four fragments generated by CPPh are of approximately equal quantity in phaseolin modified by legumain the fragment F3h evidently predominates. In order to draw any conclusion from this it is necessary to determine their N-terminal sequence or/and their exact M_r by mass spectrometry.

Table 1

The fragments of phaseolin modified *in vitro* by legumain in presence of E64

Fragments	M_r, kDa	Similar to the identified fragment by Senuyk et al. [7]		
		Fragments	M_r, kDa	Cleavage site
F1h	28.1			
F2h	27.1			
F3h	25.6			
F4h	24.3			
F1	22.9	F1	22.4	N220-T
F2	21.6	F2	21.5	N220-T
F3	18.6	?		
F4	17.5	F5	17.1	N235-S
F5	15.7	F6	15.0	N235-S
F6	13.4	F8	12.8	N103-P

On the basis of determined high-resolution X-ray structure of phaseolin together with the primary structure of 15 subunits of 7S proteins, Lawrence et al. [13] developed a canonical model of the structure of 7S proteins. According to this model each phaseolin subunit consists of two domains of similar size and structure. In its turn, each domain consists of two elements: a compact eight-stranded β-barrel and an extended loop consisting of three short α-helix. The interdomain linker consists of helix 4 and of an unfolded sequence, comprising residues 211-219, that was not detected in the X-ray structure. The sequences of 10 N-terminal and 15 C-terminal amino acids residues are also absent from the X-ray structure [13].

Proteins from plant seeds are important for food and feed. Among the factors that affect their nutritive value one of the most important is their susceptibility to hydrolysis by proteases [29]. In contrast to other 7S SP, native phaseolin is highly resistant to proteolysis *in vitro* [6,20, 21,27,28,29]. The greatest part of the phaseolin molecule is remarkably resistant to proteolytic attack. Its degradation generally stopped after a short-term limited proteolysis producing a high molecular-mass oligomeric product [6, 21]. At the action of both endogenous [6,19] and exogenous [20,21] proteases its limited proteolysis stops after a small number of peptides have been cleaved off. It is only completely hydrolyzed under the concerted action of two endogenous proteases – legumain and CPPh [10] which is in contrast to the consecutive action of exogenous proteases that is additive and does not proceed to the complete hydrolysis [21].

An explanation of phaseolin resistance to the action of proteases was proposed on the basis of the differences of its tertiary structural features from that of other homologues 7S proteins [6,7,21]. In the case of legumain, from the finding of cleavage sites and assumptions, a four-step scheme was proposed by Senyuk et al. [7] for the cleavage of phaseolin subunits by this endopeptidase:

1. Phaseolin degradation presumably starts via cleavage in an inter-module linker of the phaseolin monomer since cleavage products having half the M_r of a monomer were generated first. Thus the first cleavage products are derived from both the N-terminal and the C-termional domains of phaseolin subunits.

2. The A'-A β segment is detached from the C-terminal β-barrel domain.

3. Later, legumain attacks α helices and the disordered segment of the C-terminus that results in the formation of fragments with lower molecular masses with the same N-terminal sequences.

4. Afterwards or in parallel, the FG loop of the N-terminal β-barrel domain is cleaved off.

By cleavage at the sites located in the C-domain: N220-T in the A' β strand, and N235-S in the AB-loop, the A'-A β segment, having a calculated molecular mass of 1.6 kDa, must have been dissected from the β-barrel domain of the C-domain. This segment includes the N-glycosilated site N228 [13] which can be glycosilated by two different glycans: $Man_7(GlcNAc)_2$ and $Xyl\text{-}Man_3(GlcNAc)_2$ [23]. These glycans would add to the 1.6 kDa polypeptide chain approximately 1.7 and 1.1 kDa, correspondingly. The loss of these fragments would result in changes to the M_r of the native phaseolin molecule as well as it could destabilize the quaternary structure of the phaseolin.

According to native gradient-pore PAGE (Fig. 1B) M_r of phaseolin decreased during hydrolysis by legumain from 140 to 137 kDa, indicating the loss of 2% protein. Therefore it showed that phaseolin retained its quaternary structure after hydrolysis by legumain. More than that these results indicate that the loss of

protein would constitute about 1 kDa per subunit which means that the A'-A β segment is not detached from the phaseolin molecule. All the fragments generated by legumain action remain bound noncovalently in the phaseolin molecule. Our results confirm the high resistance of most of the phaseolin molecule to proteolytic attack of legumain. However the data concerning the content of residual protein during legumain action on phaseolin indicates that a few peptides are cleaved off (Fig. 1B). Whether they are due to the cleavage at the C-terminus of these fragments as suggested previously by Senyuk et al. [7] would be clear only if the exact molecular masses of these fragments is determined by mass spectrometry.

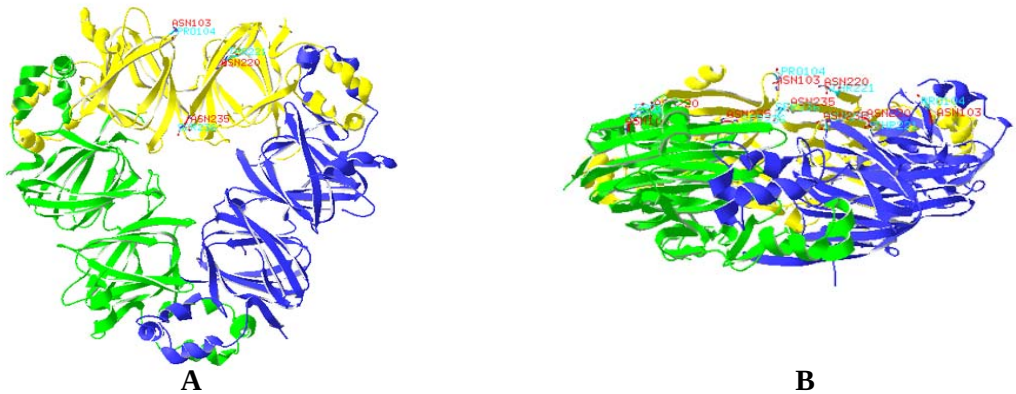
The final high molecular mass product of phaseolin hydrolysis by legumain will be subsequently referred to as phaseolin-L. It's M_r is higher by 10 kDa than the M_r of the phaseolin modified by CPPh (subsequently referred to as phaseolin-CPPh) [6]. CPPh action on phaseolin result in the splitting of the subunits of phaseolin that is accompanied by the formation of a number of TCA-soluble peptides, resulting in the loss of 8.5% protein. In both cases during proteolysis, the residual phaseolin protein retains its oligomeric structure as indicated by non-denaturing gradient-pore PAGE. But in comparison to phaseolin-CPPh where only fragments corresponding to half phaseolin subunits were detected by SDS-PAGE phaseolin-L is composed of fragments of two types which indicated the cleavage in the subunit's domains. This raised another question and namely whether in the phaseolin-L are present all the fragments detected by SDS-PAGE.

The 2D PAGE (Fig.1C) showed that indeed all the fragments detected by SDS-PAGE are part of the phaseolin-L molecule. This result showed that the cleavage of phaseolin subunits and their further split off does not break quaternary structure. So, the nature of the limited proteolysis of phaseolin by legumain was confirmed by PAGE (SDS, native gradient-pore and 2D).

The methods used in this study do not permit to get an exhaustive information about the phaseolin-L. For example it is difficult to account for 2% protein loss detected by native gradient-pore PAGE. It was suggested previously that legumain could cleave off some peptides from the C-terminus of the formed fragments [7], but for clarifying this, the N-terminal sequencing of all fragments detected by SDS-PAGE and determination of their exact molecular masses by mass spectrometry is highly advisable.

The modification by limited proteolysis of phaseolin by legumain makes phaseolin molecule susceptible to the attack of CPPh [10]. The mechanic by which this happens is still not known. We used the structural model of phaseolin [13] to show the localization of the sites of legumain cleavage of phaseolin (Fig.2).

As we have already mentioned these cleavage sites are located in FG loop in the N-terminal β-barrel domain and in the A' strand and AB loop in the C-terminal β-barrel domain of the phaseolin subunit. These regions are an essential part of phaseolin structure. In comparison to phaseolin-L the cleavage sites of phaseolin-CPPh can not be mapped on phaseolin structure because they are located in flexible regions with are not present (no electron density was discerned for these regions) in the refined structure of phaseolin [13].



A **B**

Fig.2. Localisation of legumain cleavage sites in the quaternary structure of the phaseolin molecule.

The structure was drawn from PDB record 2PHL by use of the Swiss-Pdb Viewer program [18].
A – Phaseolin trimer viewed down the molecular threefold axis
B – The picture in A is rotated 90° in the plane of the view
The subunits are colored in different colors. The determined legumain cleavage sites are indicated by labeling the Asn residues in P1 position and the residues in the P1' position. Cleavage sites are indicated in only one subunit in A and in all subunits in B.

As it can easily be seen all the cleavage sites are located in the middle of the subunit's tertiary structure (Fig. 2A). And what is more interesting they all are located on the same side of the phaseolin molecule's quaternary structure (Fig. 2B).

The phaseolin trimer is a disc of diameter 90 Å and thickness 35 Å [30,31]. Unlike other 7S storage globulins phaseolin converts to an 18 S tetramer at acid pH [32], and images recorded under these conditions suggest that four of the 7 S trimer discs associate to form the faces of a regular tetrahedron [30]. This tetramer of trimers turned out to be the form of the molecule in the crystals of phaseolin [31]. These results indicate that constraints imposed on seed proteins by their role in sustaining the germinating plant may have imposed restrictions on the structure of phaseolin that are to accumulate in the protein-storage bodies of seeds. Whether the observed arrangement of legumain cleavage sites on phaseolin structure is determined by the accessibility of these sites that results from the packing of phaseolin trimers into tetramers could be answered only by the X-ray structure of phaseolin-L.

Our results confirm the high resistance of most of the phaseolin molecule to proteolytic attack by legumain. This has also been reported for the action of other endogenous [6,19] and exogenous [20,21] proteases. SDS-PAGE showed that all proteases tested except pepsin [21] caused gradual disappearing of the polypeptide bands due to subunits. Concomitantly, irrespective of the type of protease tested, several bands with molecular masses approximately half of a subunit appeared [6,19,20,21]. The split peptide bonds identified were all located in the middle of the subunit [6,20,21]. While the hydrolysis of native storage protein is slow the hydrolysis of heat denaturated storage proteins is rapid and complete which is caused by the disruption of the tertiary and quaternary structures [20,33]. Consequently the limited proteolysis of phaseolin, that distinguish it from other storage proteins, is evidently due to the peculiarities of its structure. The specific features of phaseolin structure probably determine its resistance to enzymatic hydrolysis.

At the best of our knowledge no proteolytic enzyme, carrying out the profound hydrolysis of native phaseolin, were detected. However it was found that preliminary modification of phaseolin by legumain is necessary for the subsequent action by CPPh [10]. CPPh catalysed the extensive hydrolysis of phaseolin-L [10]. SDS-PAGE however did not detected changes in the fragment composition indicating that further degradation of phaseolin down to trichloroacetic acid-soluble peptides occurs without changes in the number and relative amounts of the fragments generated by legumain. They remained essentially unchanged during the reaction [10], indicating a predominantly co-operative-type of proteolysis [34].

Incubation of phaseolin with legumain generated a fragment pattern different from those observed with other proteases but it is similar to the pattern of fragments observed for phaseolin partialy modified *in vivo* that was isolated from cotyledons of six day germinated kidney bean [7]. In both cases the splitting sites in phaseolin subunit were on C-side of the same asparagine residues. No such fragment similarity was found after incubating native phaseolin with CPPh. This pattern was also confirmed by immunoblotting of phaseolin mobilization during and after kidney bean germination [7]. The results suggest that is very likely that legumain is responsible for the initiation of phaseolin proteolysis *in vivo.*

The cysteine endopeptidases found in cotyledons of germinating kidney beans are similar to those detected in cotyledons of germinating vetch [8]. Whereas the papain-like cysteine endopeptidase CPPh degraded *in vitro* vicilin from vetch profoundly, it's action on phaseolin is limited. This indicates differences in the structure-function relations between homologous proteinases and homologous storage globulins as substrates from vetch and kidney bean, respectively. Phaseolin that was isolated from dry kidney bean cotyledons is also modified *in vitro* by the Asn-specific cysteine endopeptidase legumain. This is in contrast to previously reported conclusion that vetch proteinase B, homologous to kidney bean legumain, could not digest neither vicilin nor legumin from dormant vetch seeds *in vitro* [1,5].

Similarities of *in vivo* and *in vitro* cleavage products of phaseolin strongly suggest that legumain could initiate the degradation of phaseolin in the cotyledons of germinating kidney bean. This is in contrast to the hypothesis of Shutov and Vaintraub [1] mentioned above, according to which proteinase B cannot attack native globulins from mature legume cotyledons but only globulins that already underwent limited proteolysis by a papin-like cysteine endopeptidase. Outwardly, it seems that, the action of legumain on phaseolin differs essentially from that of the proteinase B on SP [1,5]. Thus, legumain may control the initiation of phaseolin breakdown in kidney bean, whereas the other cysteine endopeptidases together with carboxypeptidases might contribute to further degradation of this 7S storage globulin.

These results indicate that legumain is the enzyme that triggers the degradation of phaseolin. The modifications caused by legumain makes the phaseolin molecule susceptible to the attack of CPPh. The elucidation of the mechanism by which this proteolysis is achieved is of major interest.

Any 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. So, the cause of the difference detected should be looked for in the peculiarities of phaseolin structure. Perhaps the determination of X-ray structure of phaseolin-L and/or phaseolin-CPPh could prove decisive in the solution of this conundrum.

References:

1. Shutov A.D. and Vaintraub I.A. Degradation of storage proteins in germinated seeds // Phytochemistry. - 1987. - Vol.26. - P.1557-1566.
2. Rotari V., Dando P. M. and Barrett A. J. Legumain forms from plants and animals differ in their specificity // Biol. Chem. - 2001. - Vol.382. - P.953-959.
3. Barrett A.J. Mammalian legumain. - In: Handbook of Proteolytic Enzymes, Barrett A.J., Rawlings N.D., Woessner J.F., eds. Academic Pr Inc, 1999.
4. Rawlings N.D., Morton F.R., Barrett A.J. MEROPS: the peptidase database // Nucleic Acids Res. - 2006. - Vol.34 (Database issue). - P.D270-272. - (http://merops.sanger.ac.uk)
5. Becker C., Shutov A.D., Nong V.H., Senyuk V.I., Jung R., Horstmann C., Fischer J., Nielsen N.C. and Müntz K. Purification, cDNA cloning and characterization of proteinase B, an asparagines-specific endopeptidase from germinating vetch (*Vicia sativa* L.) seeds // Eur. J. Biochem. - 1995. - Vol.228. - P.456-462.
6. Rotari V., Senyuk V., Jivotovskaja A., Horstmann C. and Vaintraub I. Proteinase A-like enzyme from germinated kidney bean seeds. Its action on phaseolin and vicilin // Physiologia Plantarum. - 1997. - Vol.100. - P.171-177.
7. Senyuk V., Rotari V., Becker C., Zakharov A., Horstmann C., Müntz K. and Vaintraub I. Does an asparaginyl-specific cysteine endopeptidase trigger phaseolin degradation in cotyledons of kidney bean seedlings? // Eur. J. Biochem. - 1998. - Vol.258. - P.546-558.
8. Senyuk V., Rotari V., Becker C., Zakharov A., Horstmann C., Müntz K., Vaintraub I. Cysteine proteinases from kidney bean seedlings // Analele Ştiinţifice ale Universităţii de Stat din Moldova. - 1998. - P.143-147.
9. Chen J.-M., Rawlings N.D., Stevens R.A.E., Barrett A.J. Identifcation of the active site of legumain links it to caspases, clostripain and gingipains in a new clan of cysteine endopeptidases // FEBS Letters. - 1998. – Vol.441. – P.361-365.
10. Zakharov A., Carchilan M., Stepurina T., Rotari V., Wilson K., and Vaintraub I. 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 // J.Exp. Bot. - 2004. - Vol.55. - P.2241-2249.
11. Becker C., Senyuk V.I., Shutov A.D., Nong V.H., Fischer J., Horstmann C. and Müntz K. Proteinase A, a storage globulin degrading endopeptidase of vetch (*Vicia sativa* L.) seeds, is not involved in early steps of storage protein mobilization // Eur. J. Biochem. - 1997. - Vol.248. - P.304-312.
12. Becker C., Fischer J., Nong V.H. and Müntz K. PCR cloning and expression analysis of cDNAs encoding cysteine proteinases from germinating seeds of *Vicia sativa* L. // Plant Mol. Biol. - 1994. - Vol.26. - P.1207-1212.
13. Lawrence M.C., Izard T., Beuchat M., Blagrove R.J. and Colman P.M. Structure of phaseolin at 2.2 Å resolution. Implications for a common vicilin/legumin structure and the genetic engineering of seed storage proteins // J. Mol. Biol. - 1994. - Vol.238. - P.748-776.
14. Schlesier B., Manteuffel R., Armin R., Jüttner G. A simple method for preparation of phaseolin // Biochem. Physiol. Pflanz. - 1984. - Vol.179. - P.665-678.
15. Laemmli U.K. Cleavage of structural proteins during the assembly of the hed of bacteriophage T4 // Nature. - 1970. - Vol.227. - P.680-685.
16. Knowland J. Protein synthesis directed by the RNA from a plant virus in a normal animal cell // Genetics. - 1974. - Vol.78. - P.383-394.
17. Berman H.M., Westbrook J., Feng Z., Gilliland G., Bhat T.N., Weissig H., Shindyalov I.N., Bourne P.E. The Protein Data Bank // Nucleic Acids Research. - 2000. - Vol.28. - P.235-242. - (http://www.pdb.org/)
18. Guex N. and Peitsch M.C. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling // Electrophoresis. - 1997. - Vol.18. - P.2714-2723. - (http://www.expasy.org/spdbv/)
19. Boyland M.T. and Sussex I.M. Purification of an endopeptidase involved with storage protein degradation in *Phaseolus vulgaris* L. cotyledons // Planta. - 1987. - Vol.170. - P.343-352.

20. Nielsen S.S., Deshpande S.S., Hermodson M.A., Scott M.P. Comparative digestibility of legume storage proteins // J. Agr. Food Chem. - 1988. - Vol.36. - P.896-902.
21. Jivotovskaya A., Senyuk V., Rotari V., Horstmann C. and Vaintraub I. Proteolysis of phaseolin in relation to its structure // J. Agr. Food Chem. - 1996. - Vol.44. - P.3768-3772.
22. Doyle J.J, Schuler M.A., Godette W.D., Zenger V., Beachy R.N. and Slightom J.L. The glycosilated seed storage proteins of *Glycine max* and *Phaseolus vulgaris*. Structural homologies of genes and proteins // J. Biol. Chem. - 1986. - Vol.261. - P.9228-9238.
23. Sturm A., van Kuik J.A., Vliegenthart J.F.G., and Chrispeels M.J. Structure, position, and biosynthesis of the high mannose and the complex oligosaccharide side chains of the bean storage protein phaseolin // J. Biol. Chem. - 1987. - Vol.262. - P.13392-13403.
24. Lioi L., Bollini R. Contribution of processing events to the molecular heterogeneity of four banding types of phaseolin, the major storage protein of *Phaseolus vulgaris* L. // Plant Mol. Biol. - 1984. - Vol.3. - P.345-353.
25. Bollini R, Vitale A. and Chrispeels M.J. In vivo and in vitro processing of seed reserve protein in the endoplasmic reticulum: Evidence for two glycosylation steps // J. Cell Biol. -1983. - Vol.96. - P.999-1007.
26. Stockman D.R., Hall T.C. and Ryan D.S. Affinity chromatography of the major seed protein of the bean (*Phaseolus vulgaris* L.) // Plant Physiol. - 1976. - Vol.58. - P.272-275.
27. Vaintraub I.A., Bassüner R. and Shutov A.D. The action of trypsin and chymotrypsin on the reserve proteins of some leguminous seeds // Nahrung. - 1976. - Vol.20. - P.15-21.
28. Romero J. and Ryan D.S. Susceptibility of the major storage protein of the bean, *Phaseolus vulgaris* L., to in vitro enzymatic hydrolysis // J. Agric. Food. Chem. - 1978. - Vol.26. - P783-788.
29. Liener I.E., Thamson R.M. *In vivo* and *in vitro* studies of the digestibility of the major storage protein of the navy bean (*Phaseolus vulgaris*) // Qual. Plant. Foods Hum. Nutr. - 1980. - Vol.30. - P.13-25.
30. Tulloch P.A. and Blagrove R.J. Electron microscopy of seed-storage globulins // Arch. Biochem. Biophys. - 1985. - Vol.241. - P.521-532.
31. Lawrence M.C., Suzuki E., Varghese J.N., Davis P.C., Van Donkelaar A., Tulloch P.A. and Colman P.M. The three-dimensional structure of the seed storage proteins phaseolin at 3 Å resolution // EMBO J. - 1990. - Vol.9. - P.9-15.
32. Sun S.M., McLeester R.C., Bliss F.A. and Hall T.C. Reversible and irreversible dissociation of globulins from *Phaseolus vulgaris* seed // J. Biol. Chem. - 1974. - Vol.249. - P.2118-2121.
33. Deshpande S.S., Damodaran S. Heat-induced conformational changes in phaseolin and its relation to proteolysis // Biochim. Biophys. Acta. - 1989. - Vol998. - P.179-188.
34. Vaintraub I.A. Kinetics of co-operative proteolysis // Nahrung. - 1998. - Vol.42. - P.59-60.

Acknowledgement: *This research was supported by a CGP award MOB1-2822-CS-06 from CRDF.*

Prezentat la 20.06.2007