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IN VIVO AND *IN VITRO* LIMITED PROTEOLYSIS OF PHASEOLIN: FACTS, SUGGESTIONS AND PROBLEMS

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Degradarea proteolitică a fazeolinei - globulinei 7S de rezervă din semințele *Phaseolus vulgaris* are loc *in vivo* în timpul germinării şi este catalizată de acțiunea succesivă a legumainei Asp-specifice LP şi a enzimei de tip papainic PP. A fost studiată componența fragmentelor cu masă moleculară înaltă care se formează la proteoliza limitată a fazeolinei la acțiunea LP şi la acțiunea succesivă a PL şi a PP *in vivo* şi, de asemenea, în cotiledoanele plantei în creştere.

As a rule, the complete degradation of 11S and 7S seed storage globulins can be achieved under the action of a single endogenous enzyme, namely papain-like proteinase PP [1]. The PP and another endogenous enzyme, an Asn-specific proteinase LP (legumain [2]), are both synthesized during seed germination. Both are responsible for storage globulin degradation, although the LP seems to play only an accessory role in the process. *In vivo* as well as *in vitro* the degradation of storage globulins starts with limited proteolysis that consists in specific processing-like cleavages of several peptide bonds of enhanced susceptibility [3]. After *in vitro* exhaustion of these bonds, further degradation of storage globulins by PP follows a cooperative mechanism that occurs as a pseudo-first order reaction and consists in unlimited one-by-one breakdown of substrate molecules [1,4]. It remains unclear whether the cooperative proteolysis starts from the very beginning of the enzyme action in parallel and irrespective of the initial quick limited proteolysis or the latter brings about structural reformation of storage globulins that triggers the cooperative process.

Among seed globulins, the 7S globulin phaseolin from *Phaseolus vulgaris* seeds gives a unique opportunity to answer this question. Limited proteolysis occurs when phaseolin is subjected to the action of either PP or LP. However, the action of these enzymes individually is restricted to limited proteolysis only. However, phaseolin modified by LP-induced limited proteolysis acquires ability to be completely degraded by PP via cooperative mechanism [1]. Thus, structural reformation of phaseolin resulting from the LP-induced limited proteolysis triggers the involvement of PP in its complete degradation. It might be that the described regulation of storage globulin breakdown induced by limited proteolysis is common for turnover of many other proteins. Therefore, it would be of interest to characterize structural alterations of phaseolin subjected to limited proteolysis by LP (phaseolin-LP) via crystallization and X-ray analysis.

However, it should be taken into consideration that phaseolin-LP subjected to PP attack, similarly to ordinary storage globulins, undergoes quick limited proteolysis [1]. Hence, as before, the question remains as to whether PP-induced limited proteolysis of phaseolin-LP (*e.g*. conversion of phaseolin-LP into phaseolin-LP+PP) is a prerequisite for its degradation via cooperative mechanism.

In this paper, we examine the possibilities to answer this question. The latest experimental and theoretical results on the investigation of the *in vitro* and *in vivo* limited proteolysis of phaseolin are described and discussed.

Materials and Methods

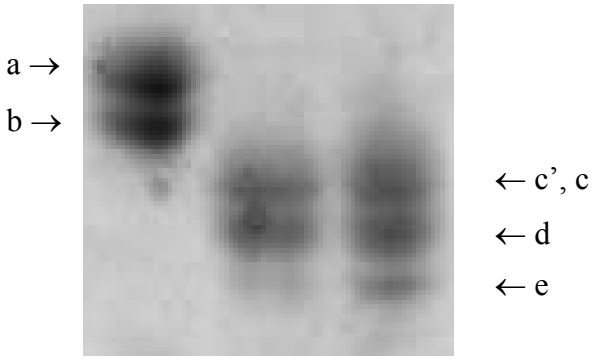
Ph. vulgaris (cv. Moldavian) seeds were soaked in distilled water for 4 h and germinated in the dark at 25ºC. Phaseolin from cotyledons of dry seeds and 6-day old seedlings was isolated by ammonium sulfate fractionation [5]. Phaseolin subjected to limited proteolysis by LP (phaseolin-LP) and to successive action of PP and LP in two variants (phaseolin-PP-LP and phaseolin-LP-PP) were obtained as described earlier [1]. SDS-PAGE was carried out according to Laemmli [6] in 12.5 % gels. Synthetic substrates specific for LP and PP were used as described earlier [1] for determination of the enzyme activities. Polypeptides separated by SDS-PAGE were blotted onto PVDF membranes (Millipore) and subjected to N-terminal sequencing. Chromatographic materials from Pharmacia were used. Electrophoresis patterns were scanned and analyzed using the program Phoretix 1D Gel Analysis v.5.10.

Results and Discussion

Primary structure of subunit halves generated by LP and PP

SDS-PAGE of phaseolin-LP revealed two major fragments generated by cleavage of the bond N220-T221 (Fig.1). Although the protein moiety of the N-terminal and C-terminal fragments ('halves') is differently sized (25.7 kD and 19.3 kD, respectively, in β-phaseolin subunit), they form bands a and b of close apparent molecular masses (26.8 kD and 25.9 kD, respectively) due to glycosylation of the C-terminal fragment.

A



B

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   4       197           211  216 220              389
   |         |             |    |   |                |
tslReeees//idseqikelskhaksssrkslSkqdNtignefgnlter//qqkgrkgafvy β-phaseolin subunit
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tslree (a)	tigne (b)	LP
eeees (c)	kqdn (c')	PP-LP, transient product
eeees (c)	tigne (d)	PP-LP & LP-PP intermediate
eeees (e)	tigne (d)	PP-LP & LP-PP final product

Fig.1. Limited proteolysis of phaseolin by endogenous proteinases LP and PP:
A. SDS-PAGE of phaseolin-LP (lane 1), phaseolin-PP-LP (lane 2) and phaseolin-LP-PP (lane 3);
B. N-terminal sequences (underlined) of polypeptide fragments generated by limited proteolysis of phaseolin by endogenous proteinases LP and PP. In the upper row residues printed in bold correspond to positions P1 of the cleaved bond (capitals, detected cleavages; low case letters, deduced cleavages). The band c' is formed due to direct action of PP on intact phaseolin subunits still present in phaseolin-LP preparation (not shown). Cleavage points R4 and S216 in the position P1 are the same that have been detected earlier [7].

Although the N-termini of the phaseolin-LP+PP fragments were determined (Fig.1B), their C-terminal limits remain unknown. Nevertheless, C-terminal truncation both of N-terminal and C-terminal subunit halves generated by PP action can be qualitatively assumed on the basis of comparison of the SDS-PAGE patterns of phaseolin-LP and intermediate/final products of successive action of the enzymes (Fig.1). Indeed, two differently sized N-terminal halves are formed (bands c and e), and the conversion of phaseolin-LP into phaseolin-LP-PP is coincided with decline in molecular mass of the C-terminal half (bands b and d).

The apparent molecular mass values for the polypeptide fragments from SDS-PAGE data deviate from the expected values (particularly because of glycosylation of the C-terminal half) and therefore cannot be used directly even for estimation of C-terminal limits of subunit halves truncated by PP action in phaseolin-LP+PP. Nevertheless, these limits can be deduced quantitatively using the following approach. *First*, real molecular masses of phaseolin-LP N-terminal (T1-N220) and C-terminal (T221-Y397) polypeptides as well as the loss of molecular mass of the N-terminal polypeptide of phaseolin-LP+PP due to cleavage off of the N-terminal tetrapeptide T1-R4 (Fig.1B) can be calculated from the sequence of β-phaseolin subunit. *Second*, according to SDS-PAGE the size differences between the respective N- and C-terminal polypeptide fragments of phaseolin-LP and phaseolin-LP+PP are minor, and the deviations of their apparent molecular masses from real values should be practically identical. Therefore, using the differences in the apparent molecular masses of phaseolin-LP

and phaseolin-LP+PP N-terminal and C-terminal halves one can characterize quantitatively C-terminal truncation of the halves due to the PP action. Putative C-terminal limits deduced for the phaseolin-LP+PP intermediate and final products correspond to cleavages that are reasonable in light of the known cleavage specificity of PP (S197-E198, S211-R212 and K389-G390, Fig.1B). These cleavage points are the same when the sequence of phaseolin α-subunit is used for calculations.

Minor fragments in phaseolin-LP preparation

According to Zakharov *et al*. [1], the physiologically essential LP-induced proteolysis of phaseolin is restricted to cleavage of a single peptide bond N220-T221 (Fig.1). Other cleavages of Asn-flanked bonds detected earlier [8] were estimated to occur very slowly and have been ignored [1]. Thus, it was assumed that the sum of subunit halves in phaseolin-LP trimer is the very substrate susceptible to cooperative proteolysis by PP [1]. Indeed, PP-contaminated LP was used by Senyuk *et al*. [8], and at least a portion of the minor fragments found in the phaseolin-LP preparation might be formed due to this contamination. However, in all further experiments some of the minor fragments were still detected in phaseolin-LP obtained under selective inhibition of any contaminating PP in the LP preparations by E-64 [1,9]. So, the cleavage of other bonds in addition to the bond N220-T221 can be ignored only in the case of negligible quantities of minor fragments generated by these additional cleavages.

To elucidate whether these quantities are really negligible, we conducted densitometry of the very electrophoretogram of the phaseolin-LP preparation published as Figure 3 a in the paper of Zakharov *et al*. [1] (Fig.2). Two quickly appearing minor bands, F1 and F2 correspond to C-terminally truncated C-terminal subunit halves [8] and account for 15.8 % of the sum of non-truncated N- and C-terminal halves after 8 h of incubation. Using the above described approach for estimation of real molecular masses of the fragments F1 and F2, we found that the cleavage of the bond N370-K371 (Fig.3) might be responsible for their formation both in β- and α-subunits of phaseolin (Table 1). The molar ratio of truncated/non-truncated C-terminal halves during the time course of a 24-h proteolysis is constant and approximated at 1:1.6 (Table 1). So, each increase in the molar quantity of the truncated C-terminal halves along the LP action is proportional to the increase of concentration of non-truncated C-terminal half as an immediate substrate. Thus, the cleavage of the bond N370-K371 appears to be a regular first order reaction. As a conclusion, the suggested cleavage of the bond N370-K371 is regular and formation of the fragments F1 and F2 cannot be ignored.

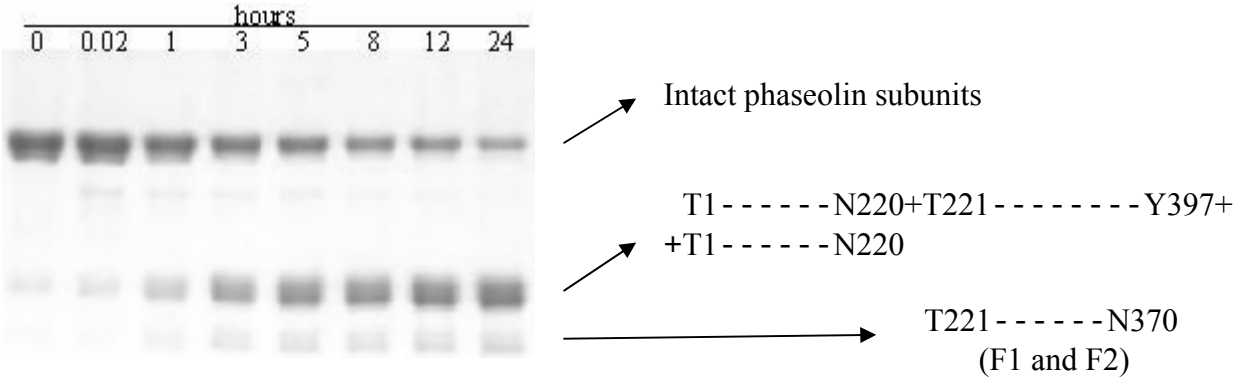

Fig.2. Time-course of phaseolin-LP formation.
The SDS-PAGE pattern is reproduced from Fig.3a of the paper by Zakharov *et a*l. [1] (minor fragments F3-F5 not shown). Presumed composition of two groups of fragments (non-truncated subunit halves and C-terminally truncated C-terminal halves F1 and F2) is shown at the right. The SDS-PAGE pattern was used here for description of limited proteolysis kinetics (see Fig.7).

A recent phaseolin-LP preparation obtained in the presence of E-64 reveals five minor fragments (Fig.4). They correspond to the fragments F1 and F2 discussed above and probably to the fragments F3 (20.8 kD), F5 (17.1 kD) and F6 (15.5 kD) of identical N-termini S236 that have been described by Senyuk *et al*. [8] (here we use the same fragment nomenclature). Formation of the fragment F3 coincides with a 5.1-kD loss of apparent molecular mass of the initial C-terminal half. Fragment F3 probably corresponds to the sequence fragment S236-N370 (4.8-kD loss of calculated real molecular mass).

Table 1

Minor fragments F1 and F2 formed due to cleavage of the bond N370-K371 in phaseolin-LP C-terminal halves cannot be ignored

Minor fragments	*F1*	*F2*
Loss of apparent M of C-halves due to formation of minor fragments, kD	3.3	4.1
Loss of real M of C-halves due to cleavage of the bond N370-K371, kD*	3.2	4.2
*Truncated C-terminal halves, molar fraction along 24-h reaction***	0.39 ± 3.6%	
*Molecular mass of phaseolin-LP, % from intact phaseolin ****	98	

M, molecular mass (apparent and real values calculated from SDS-PAGE data and from subunit sequences, respectively). * It was suggested that the fragments F1 and F2 are derived from differently sized β- and α-subunits, respectively. ** It was taken into account that the band of N-terminal and C-terminal subunit halves not separated in the paper [1] also contain non-truncated N-terminal half complementary to truncated C-terminal half (Fig.2). *** According to densitometry, averaged phaseolin-LP trimer after 24-h proteolysis is considered to contain 0.7 moles of intact subunits (Fig.2), 1.4 moles of subunits cleaved into halves of the same summary molecular mass (~50 kD), and 0.9 moles of subunit halves with decreased by 3.7 kD molecular mass (average for β- and α-subunits) due to detachment of the C-terminal fragment K371-Y397.

```
               220        230             240       250       260       270
                 |        * |               |         |         |         |
ph lskqd--------Nti-gnefgnltertd--Ns----lnvlissiemeegalfvphyyskaivilvvnege
cg issedepfnlrsrnpiysnnfgkffeitpeknpqlrdldiflssvdinegalllphfnskaivilvinegd
          Z       A'     A         h0       B         C          D

          280              290       300       310       320       330
            |                |         |         |       *   |         |
ph ahvelvgpkg----nk---etleyesyraelskddvfvipaaypvaikatsnvnftgfginannnnrnlla
cg anielvgikeqqqkqkqeeeplevqryraelseddvfvipaaypfvvnatsnlnflafginaennqrnfla
    E                        F         G         H         I          J

      340       350       360       370       380       390
        |         |         |         |         |         |
ph gktdnvissigraldgkdvlgltfsgsgdevmkliNkqsgsyfvdahhhqqeqq-kgrkgaf------vy
cg gekdnvvrqie-----rqvqelafpgsaqdverllkkqresyfvdaqpqqkeegskgrkgpfpsilgaly
          h1        h2        h3        J'
```

Fig.3. Sequences of C-terminal domains of phaseolin β-subunit (ph) and *Glycine max* β-conglycinin β-subunit (cg). Sequence numbering corresponds to that of phaseolin β-subunit. Secondary structures [10,11] are underlined (capitals, β-strands, h, α-helices). Asn residues of phaseolin glycosylation sites are indicated by asterisks. Asn residues shown in bold are discussed in the text.

The origin of the fragments F5 and F6 is unclear. The size difference between the initial C-terminal half and the fragments F5 and F6 is too high for estimation of their C-terminal limits. To do justice, it should be noted that Asn residues are present in the region upstream from N370 (Fig.3). However, it is difficult to imagine susceptibility of this region, as it is too close to the β-strand I involved in inter-domain hydrophobic interactions [10]. It seems unbelievable that the formation of the fragments F5 and F6 is due to limited specificity of the LP (this has never been shown; with the single exception of the cleavage of Asp-flanked bonds that occurs very slowly [9,12]). It should be noted that the formation of the fragments F5 and F6 occurs long before completion of cleavage of the highly susceptible bond N220-T221. Both of the fragments are formed *in vivo* in seedling cotyledons [8] in presence of non-LP activities. So, the presence of foreign activity in the LP preparation cannot be excluded even in presence of E-64. For instance, incomplete inhibition (only 94% of initial activity) of proteinase A (a papain-like proteinase from cotyledons of *Vicia sativa* seedlings) was found in the presence of E-64 [13].

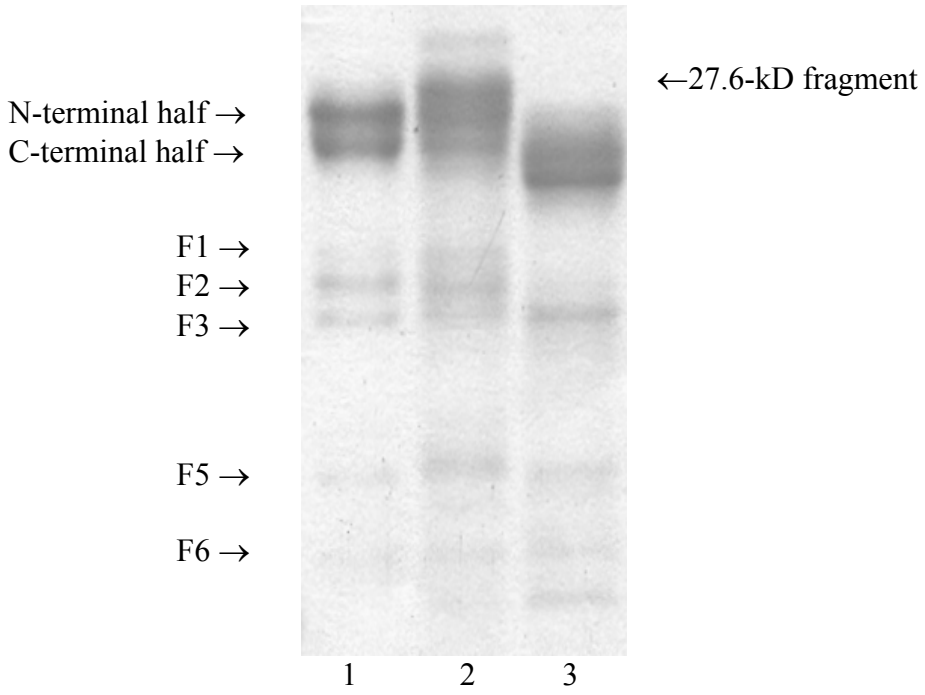

Fig.4. SDS-PAGE of phaseolin subjected to *in vitro* and *in vivo* limited proteolysis.
Lane 1, phaseolin-LP. Lane 2, phaseolin isolated from cotyledons of 6-day old seedlings. Total protein was extracted with 0.1 M Tris-HCl buffer pH 9, 0.5 M NaCl, 0.1 M sodium iodoacetate, and further subjected to ammonium sulfate fractionation for phaseolin isolation [5]. The upper minor band corresponds to contamination removable by Q Sepharose chromatography. Lane 3, phaseolin isolated from the same seedling cotyledons. Prior to the isolation, the cotyledons were homogenized with 0.1 M sodium acetate, pH 5.6, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 0.02 % sodium azide, and incubated for 24 h at 30ºC. After centrifugation, the extract was subjected to ammonium sulfate fractionation for phaseolin isolation [5].

Only traces of minor fragments that might be derived from the phaseolin-LP N-terminal half (cleavage of the bond N103-P104 [8]) were detected by SDS-PAGE (not shown in Fig.4).

Mass spectrometry of the high- and low-molecular mass products generated by LP action on phaseolin should be helpful to define whether the presumed specific cleavage of the bond N370-K371 really occurs, whether polypeptides T221-N235 and K371-Y397 are detached (see Fig.5 that summarizes all known and suggested cleavages of phaseolin subunits), and whether other cleavages that generate minor fragments F5 and F6 are Asn-specific.

The major difficulty is to define the minimal LP-induced alterations of phaseolin primary structure that are sufficient for its physiologically essential tertiary structure alterations. Most quick cleavages of phaseolin subunits by LP (N220-T221) and PP (S216-K217) are too close to each other to believe that the structure of phaseolin-PP inaccessible for cooperative proteolysis is stabilized only by presence of the four residues KQDN in between these cleavage points (Fig.1B). Thus, additional Asn-dependent cleavages specifically determined by phaseolin primary structure must be necessary for its tertiary structure reformation. Cleavage of the bonds N235-S236 and N370-K371 coincident with detachment of the polypeptides T221-N235 and/or K371-Y397, respectively (Fig.5) might be sufficient even in the case where these cleavages occur only in a single subunit of the phaseolin trimer because these detachments would bring about loss the β-strands A'A and J' (Fig.3).

Interestingly, when phaseolin is subjected to the action of proteinase B, an Asn-specific proteinase (legumain) from cotyledons of *Vicia sativa* seedlings [12], only the fragments F1 and F2 are formed in addition to N-/C-terminal subunit halves in phaseolin-LP(B) preparation (data not shown). It would be tempting to determine whether phaseolin-LP(B), like phaseolin LP can be hydrolyzed by PP via cooperative mechanism. If so, formation of other fragments in addition to F1/F2 should be either only accessorial or completely nonessential.

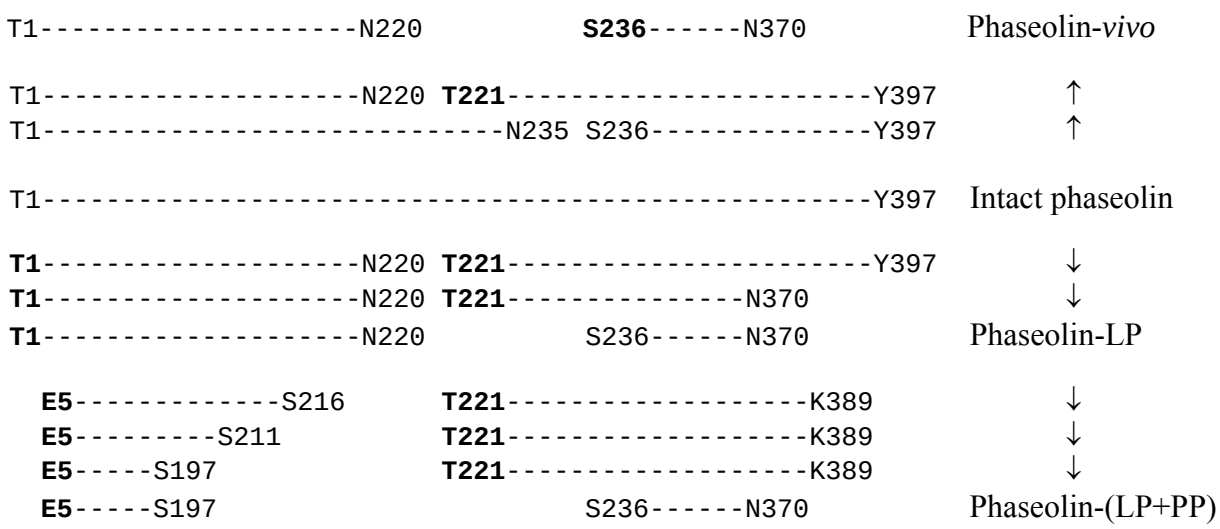

Fig.5. Schematic presentation of proved (N-termini in bold) and suggested cleavages of phaseolin β-subunit that forms successive products of *in vivo* and *in vitro* limited proteolyses.

Specificity of phaseolin structure in relation to limited proteolysis

Both the structure [10] and degradation regularity [1] of phaseolin are peculiar among the 7S seed storage globulins. For instance, *Glycine max* β-conglycinin β-subunit homo-trimer is absolutely inaccessible to LP attack [1]. Therefore, it would be interesting to compare the primary and higher order structures of these globulins in the three regions presumed to be specifically susceptible to LP attack in the phaseolin trimers (Fig.3).

First region, N220 setting. Phaseolin lacks the β-strand Z that stabilizes inter-domain interactions in all 7S globulins of known 3-D structures and probably in all other 7S globulins [10]. It cannot be excluded that the absence of the strand is responsible for accessibility of the region. According to Protein Database accession pdb|2PHL, the residue N220 form the very beginning of the A' β-strand in subunits A and B of β-phaseolin homo-trimer, whereas in the subunit C both N220 and T221 are free. It might be that even in the homo-trimer the susceptibility of the bond N220-T221 to LP attack is different in A/B and C subunits. (This speculation will be further discussed in the section describing kinetics of phaseolin limited proteolysis.) Remarkably, in most of the known sequences of 7S globulins the residue corresponding to the N220 residue of phaseolin is substituted by some other type amino acid residue. In few cases when this Asn residue is not substituted (including β-conglycinin, Fig.3), it forms an Asn-Pro bond.

Second region, N370 setting. The N370 residue corresponds to the very end of the α-helix 3 only in the protomer C. In most of other 7S globulins (including β-conglycinin, Fig. 3) the N370 is substituted with a non-Asn residue.

Third region, N235 setting. According to Lawrence *et al*. [10], the N235 belongs to the AB loop, which is peculiarly short in the phaseolin structure. In β-conglycinin (and many other 7S globulins) an Asn residue matching with N235 form the peptide bond Asn-Pro.

In summary, the lack of susceptibility of the β-conglycinin to LP attack might be understandable enough only in the case when the presence of Pro in position P1' of an Asn-flanked bond (Fig.3) prevents (or at least hinders) its cleavage. As it was discussed earlier [3], this might be the case. In all other instances where Asn-Pro bond is present in an otherwise highly susceptible site of a 7S and 11S globulin its cleavage never occurs *in vivo* under the action of VPE (Asn-specific proteinases from developing seeds). A possible exception, the cleavage of the N103-P104 bond in phaseolin subunit has been found [8]. However, this cleavage occurs very slowly.

Separation of natural forms of phaseolin different in subunit composition

To study of the polypeptide composition of phaseolin subjected to limited proteolysis is complicated by the non-uniformity of subunits that form hetero-trimers as major components of the phaseolin preparation isolated by ammonium sulfate fractionation [5]. Thus, it would be desirable to isolate natural phaseolin homo-

trimers as has been done for β-conglycinin [14]. SDS-PAGE of the unfractionated phaseolin preparation reveals upper and lower bands of subunits (Fig.6). These bands probably correspond to the α- and β-groups of subunits that can be exemplified by sequences gb|AAC23610 and pdb|2PHL, respectively. These sequences are identical except for two hydrophilic insertions in the α-group sequence relative to the β-group (*i.e.* GQEQE in the N-terminal domain and KGSHQQEQQ in the C-terminal domain).

The summary phaseolin preparation can be further separated into two fractions by hydrophobic interacttion chromatography on Phenyl Sepharose (Fig.6). The more hydrophobic fraction A consists of the upper band and lacks the lower band, while the more hydrophobic fraction B is enriched by the lower band. While the fraction A is not homogenous, it does appear to be composed of only α-type subunits identical in size and closely similar in sequences. This fraction is possibly suitable for crystallization and structural analysis of X-ray crystallography (a structure for the α-type subunits is not presently available). It is likely, that the fraction B corresponds to a mixture of β/α-hetero-trimers and β-homo-trimers.

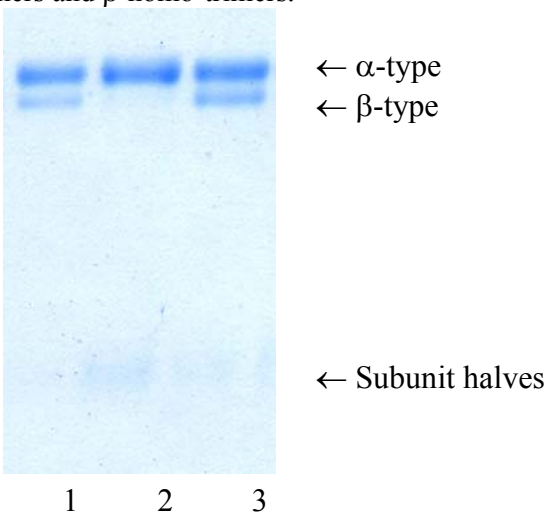

Fig.6. Phaseolin isolated by ammonium sulfate fractionation can be further fractionated using Phenyl Sepharose CL-4B chromatography.
Lane 1, initial phaseolin. Lane 2, fraction A (non-adsorbed fraction eluted in starting conditions, 0.1 M Tris-HCL buffer, pH 8, 4 M NaCl, slow loading of diluted phaseolin solution). Lane 3, fraction B (eluted by 1 M NaCl in the same buffer). Minor lower bands correspond to subunit halves formed in developing seeds due to the action of vacuolar processing enzyme.

Both the fractions (as well as the initial phaseolin preparation) contain polypeptides detectable by Western blotting [8] and identical in electrophoretic mobility to subunit halves generated by LP *in vitro*. These polypeptides are detectable even when phaseolin is isolated with sodium iodoacetate added to inhibit all cysteine proteinases including LP (data not shown). Thus, these hydrolysis products probably resulted from the *in vivo* action of the Asn-specific vacuolar processing enzyme VPE that is responsible for the maturation of 11S globulin present in *Ph. vulgaris* cotyledons in small quantities [15].

Limited and cooperative proteolyses of phaseolin in seedling cotyledons

High levels of both LP- and PP-type activities were found using specific synthetic substrates [1] in phaseolin preparations isolated from cotyledons of 6-day old seedling. Thus, to characterize the true state of phaseolin altered *in vivo* by limited proteolysis (phaseolin-*vivo*), an additional precaution, the inclusion of 0.1 M iodoacetate, was taken to prevent *in vitro* alterations of the protein during the isolation procedure. Phaseolin-*vivo* isolated under these conditions formed bands similar to those of subunit halves produced *in vitro* by LP, and an additional upper band (27.6 kD) that previously was not detected by SDS-PAGE (Fig.4, lane 2). None of known or predicted cleavages generated by PP would appear to be responsible for the formation of such a polypeptide. Most probably, this polypeptide corresponds to the fragment T1-N235 (calculated molecular masses 27.3 kD and 27.9 kD for β- and α-type subunits, respectively) formed by the direct action of LP on intact phaseolin subunits but without the cleavage of the bond N220-T221 (which is most susceptible *in vitro*).

This observation reflects the first distinction that can be made between the *in vivo* and *in vitro* proteolytic processes, *i.e*. a difference in relative susceptibilities of the N220-T221 and N235-S236 bonds to limited proteolysis.

All of the five minor fragments found in phaseolin-LP preparation are also formed *in vivo* (thus, in the presence both of LP and PP).

When phaseolin-*vivo* is subjected to additional *in vitro* attack by LP and PP activities simultaneously present in a cotyledon homogenate, the SDS-PAGE pattern of the phaseolin-(*vivo*+*vitro*) produced (Fig.4, lane 3) resembles that of the phaseolin-(LP+PP) produced *in vitro* (shown above, Fig.1). This *in vitro* phaseolin-(LP+PP) is composed of C-terminally truncated N-terminal and C-terminal halves, as well as N-terminal halves with further C-terminally truncations (presumably E5-S216, T221-K389 and E5-S197, respectively, see Fig.5). Thus, the polypeptide products present in the phaseolin-(LP+PP) obtained *in vitro* are not detectable in phaseolin-*vivo* (compare lanes 2 and 3, Fig.4). This phenomenon reflects the second distinction of *in vivo* and *in vitro* processes.

There are two possible explanations for this second distinction. *First*, the PP-induced limited proteolysis of either intact phaseolin or of phaseolin-LP does not occur *in vivo* at all. In this case phaseolin-LP is the real substrate for the cooperative proteolysis carried out by PP. *Second*, the phaseolin-*vivo*, presumably generated by LP action alone, represents a stable intermediate that then undergoes limited proteolysis by PP resulting in the formation of an unstable phaseolin-(LP+PP) as a transient product quickly removed (and is thus not detected) by extensive hydrolysis by PP via a cooperative mechanism. This second speculation implies that the cooperative proteolysis *in vivo* occurs faster than that observed *in vitro*.

Although the second speculation looks most plausible, it still remains unclear whether phaseolin-LP or phaseolin-(LP+PP) is the actual substrate of the extensive *in vivo* cooperative proteolysis. To get preliminary answer, one can compare kinetics of proteolysis of both of these substrates by pepsin at pH 4. Native phaseolin is practically inaccessible to pepsin, whose action is restricted to cleaving off of a short N-terminal peptide [16]. It is expected that either phaseolin-LP or only phaseolin-(LP+PP) will acquire the ability to be hydrolyzed by pepsin via cooperative mechanism. Genuine phaseolin-*vivo* (Fig.4, lane 2) would be an additional very interesting substrate to test with pepsin.

Kinetics of limited proteolysis of phaseolin by LP

During the action of LP on phaseolin, the losses of protein due to truncation of the C-terminal subunit halves were negligible (Table 1). Therefore, the gradually decreased values of relative band intensity of the intact phaseolin subunits (Fig.2) can be used in a first approximation to describe the kinetics of cleavage of the N220-T221 bond. Such a single cleavage should be a pseudo-first order reaction. However, the plot lg P *vs* time is curvilinear (Fig.7). Two extremes can be used for explanation of this phenomenon.

First extreme, the plot lg P *vs* time is curvilinear only because of inactivation of the enzyme during incubation at least after one hour (Fig.7A). However, this inactivation looks too quick. Moreover, the concavity of the activity curve implies an unbelievable gradual stabilization of the residual activity. Therefore, it is very probable that the initial curvilinear part of the plot lg P *vs* time cannot be explained only by inactivation of the enzyme.

Second extreme, no losses of the enzyme activity. The linear part of the plot lg P *vs* time (8-24 h, Fig.7B) reflects a slow first order reaction, and the initial curvilinear part indicates parallel occurrence of slow and quick first order reactions. This assumption implies the presence of two kinds of phaseolin trimers (or two kinds of subunits in the trimers) that differ in their susceptibility to cleavage at the N220-T221 bond. It might be that presumed these two kinds of phaseolin trimers correspond to the fractions A and B of intact phaseolin described above that differ in their subunit composition and hydrophobicity (Fig.6). In this context it should be noted that special efforts should be made to obtain phaseolin-LP preparation free from intact subunits [1].

Intermediate between two extremes. Let us assume that the second extreme is believable but should be corrected because of partial inactivation of the enzyme during prolonged incubation. If so, in the case of a slight inactivation of the enzyme the extrapolation of putative linear part of the plot to zero time (line III) should yield a lg P value approximated to 1.82, *i.e.* a value of P approximated to 66.7% (Fig.7B). This would imply that the cleavage of the N220-T221 bond occurs quickly in only one third of phaseolin subunits. It cannot be excluded that even in phaseolin homo-trimers the subunits are non-uniformly susceptible to limited proteolysis because in only one subunit of the trimer (subunit C, see above) the residues that form N220-T221 cleavage site are not involved in formation of the A' β-strand and therefore are not protected.

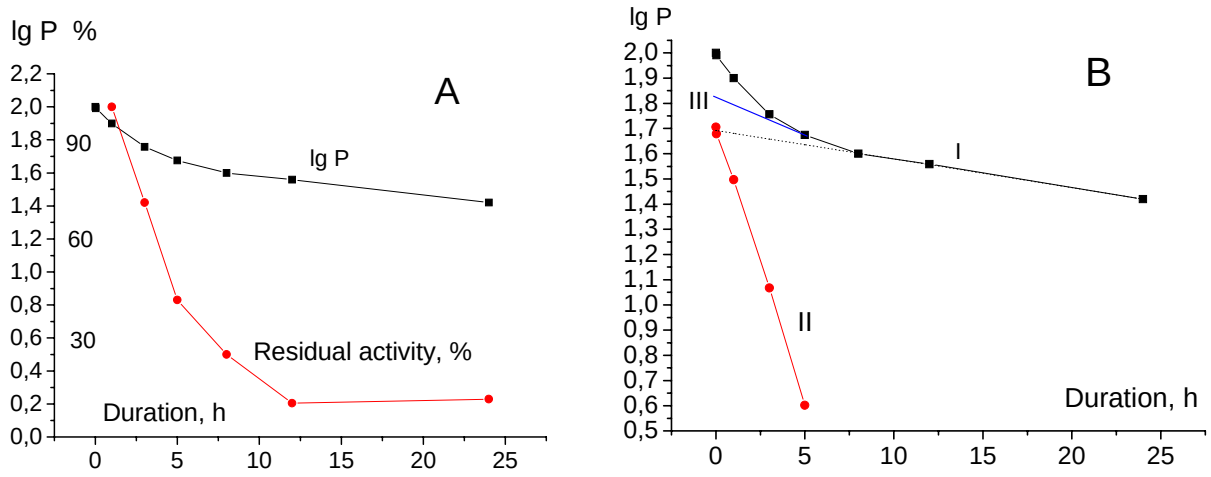

Fig.7. Kinetics of conversion of phaseolin subunits into subunit halves due to cleavage of the N220-T221 bond. P, concentration of intact phaseolin subunits (%). A, *first extreme*. The values of the residual LP activity correspond to rate constants of the reaction during each of time intervals calculated as per cent from the initial rate constant during first hour of incubation. B, *second extreme*. I, logarithm of the experimental P values that reflect a slow first order reaction after 8 h of incubation. II, lg P values of a quick first order reaction calculated as logarithms of differences between experimental P values and those obtained from linearly extrapolated lg P values. The line III illustrates a rough but interesting speculation that represents an intermediate between *first* and *second* extremes (see the text).

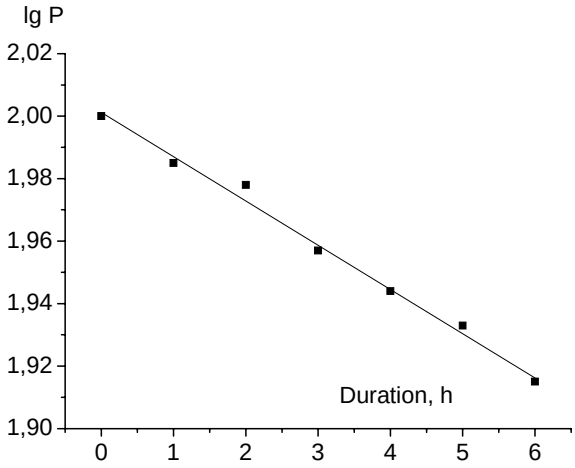

Fig. 8. Time-course of limited proteolysis of phaseolin fraction B by *Vicia sativa* proteinase B.

To be sure that cleavage of the N220-T221 bond can be described experimentally as a pseudo-first order reaction, we analyzed the time-course of formation of subunit halves generated by the action of *Vicia sativa* proteinase B on phaseolin fraction B composed of both β- and α-type subunits (lower and upper bands. Respectively, see Fig.6). Linearity of the plot lgP *vs* time was observed starting from the very beginning of the reaction (Fig.8). A contravention of the linearity was observed during further incubation. Interestingly, the ratio α/β-type subunits in the residual non-cleaved phaseolin was increased from 2.0 at the very beginning of the reaction up to 2.8 after 24-h incubation. It implies that the relative quantity of the α-type homo-trimers present in non-homogeneous fraction B increases during proteolysis. Thus, the α-type subunits might be less susceptible to cleavage of the bond N220-T221 than the β-type subunits.

In line with this suggestion, the rate of proteinase B-induced limited proteolysis of phaseolin fraction A composed of only α-type subunits (see Fig.6) is essentially lower than that of the fraction B (data not shown). In this context it should be noted that during the action both of *Ph. vulgaris* LP and *V. sativa* proteinase B on

the unfractionated phaseolin preparations (as well as during the *in vivo* limited proteolysis) a gradual increase of relative quantities of the α-type subunits is observed in the residual protein. It is likely that the enhanced susceptibility of the N220-T221 bond really depends on the presence of β-type subunits in phaseolin trimers. This suggestion might explain the parallel occurrence of slow and quick first order reactions in the case of the unfractionated phaseolin preparation as a substrate (Fig.7B).

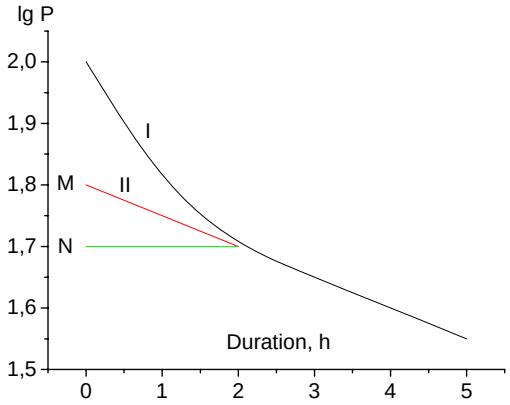

Fig.9. Principle possibility to ascertain whether cooperative proteolysis depends on limited proteolysis.
P, protein concentration (% of initial). I, experimental plot of lg P *vs* time; II, extrapolation of the linear part of the plot I to zero time. According to this extrapolation, the cooperative proteolysis starts from the very beginning of the reaction with a constant rate independent of the limited proteolysis that occurs in parallel. Thus, the extrapolated M value is equal to logarithm of the Mr of the residual protein as % from the initial Mr after completion of limited proteolysis [4]. In the case where cooperative proteolysis starts only after completion of limited proteolysis, the decrease of P is only due to detachment of peptides from protein molecule. Hence, the value N is equal to logarithm of the Mr of the residual protein (as % of the initial Mr). To chose between these two extremes, one can compare real molecular mass of the residual protein calculated from its sequence and those obtained from M and N values. In combination with SDS-PAGE data, any deviation of both the M/N extremes from the real Mr of the residual protein after completion of limited proteolysis might define alterations of substrate structure that allow cooperative proteolysis to start.

Investigation of the kinetics of cooperative proteolysis might be helpful in answering the general question of whether an initial limited proteolysis of an ordinary storage globulin (see the Introduction) is a prerequisite for its complete degradation via a cooperative mechanism. In the case of such a simple enzyme/substrate system like trypsin (with a well-defined primary specificity) and glycinin A3B4 homo-hexamer, limited proteolysis coincided with the detachment of short peptides can be described unequivocally. Hence, the precise molecular mass of the final product of limited proteolysis (glycinin-T) can be calculated from its primary structure. In parallel, the molecular mass of glycinin-T can be determined experimentally assuming parallel occurrence both of quick limited and slow cooperative proteolyses at the beginning of the reaction further altered by exclusive cooperative process [4]. As illustrated by Fig.9, a deviation of the experimentally determined molecular mass value of glycinin-T from the calculated real value could be regarded as an indication that limited proteolysis is a prerequisite for cooperative process.

Concluding remarks

Only a part of the overall paradigm on limited proteolysis of phaseolin described in this paper is based on experimentally proved facts. These are:

- LP-induced cleavage of the N220-T221 bond that *in vitro* is most susceptible;
- PP-induced truncation of both subunit halves T1-N220 and T221-Y397, that generates the phaseolin-(LP+PP) structure;
- Different susceptibility *in vitro* of the N220-T221 bond in α-type and β-type subunits of phaseolin.
- The difference between limited proteolyses that occur *in vivo* in simultaneous presence of LP and PP, and *in vitro* under successive action of the same enzymes.

Other parts of the paradigm are suggestions and speculations (cleavage points additional to those proved by N-terminal sequencing, see Fig. 5; explanations of peculiarity of limited and cooperative proteolyses of phaseolin compared to other 7S globulins, explanations for distinction between patterns of *in vitro* and *in vivo* limited proteolyses etc.). Moreover, the major question remains non-answered as to whether phaseolin-LP or phaseolin-(LP+PP) is the actual substrate for cooperative proteolysis carried out by PP. Nevertheless, we deem that the suggestions and even speculations combined in this paper together with univocal experimental results would be useful to encourage further investigations of phaseolin degradation.

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