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THE COMPARATIVE ANALYSIS OF *IN VITRO* PHASEOLIN PROTEOLYSIS BY PAPAIN AT DIFFERENT pH VALUES

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Hidroliza fazeolinei native, proteina de rezervă principală din semințele de fasole (*Phaseolus vulgaris* L.), de către papaină la diferite valori ale pH-ului, în diapazonul 4,6-8,0, a fost studiată în condiții *in vitro*. A fost determinat că faseolina este modificată de către proteoliza limitată la toate pH-urile investigate. Această modificare rezultă în formarea fragmentelor ce corespund aproximativ jumătății de subunitate a fazeolinei native, care rămân asociate în structura cuaternară a moleculei, producând fazeolina-Pap. La pH-uri mai înalte de 6,2 o parte din fazeolina-Pap este complet hidrolizată de către papaină, ceea ce indică la faptul că asocierea fazeolinei în structuri supramoleculare la pH acid îi conferă, probabil, rezistență la proteoliză.

Since the feeding of global population represent a major challenge [1] the production and utilization of plant proteins for foodstuffs is of great importance worldwide. The grain crops have seeds that contain a high level of different groups of storage proteins (SP) – albumins, globulins, and prolamins [2]. In legume seeds they are represented by two major globulins: legumins (or 11S) and vicilins (or 7S) [2,3]. SP from legume seeds in general [3] and from common bean seeds in particular [4] have attracted a special attention in recent years because they have important "functional" (physicochemical) properties.

SP from legume seeds have different nutritive value [5 and references therein]. One of the most important factors that affect their nutritive value is their susceptibility to the action of the proteases of the digestive tract [6]. It has been found that the resistance of the major native legume SP to proteolysis have a negative result of nutritive value for the unheated legume seeds [5]. More than that, stable to proteolysis SP of the cupin superfamily are major allergens in vegetal products [7].

In any protein molecule there are many peptide bonds capable of being cleaved by any individual protease provided that these bonds correspond to protease specificity. The rate of a peptide bond cleavage is influenced not only by amino acid residues that form this bond but also by amino acid residues from neighbourhood. The first stage of proteolysis is represented by cleavage of peptide bonds in the most „sensible" place, located at the molecule surface. This represents non-co-operative (or 'zipper') proteolysis [8] that leads to limited modification of the protein. The proteolysis that will follow depends on the influence that has on molecule structure's the cleavage of first peptide bonds and can follow two pathways. In the case when during limited proteolysis the splitting of one/several peptide bond(s) induces destabilisation or unfolding of the protein structure then the rate of the subsequent hydrolysis dramatically increases that leads to unlimited (extensive) proteolysis and the protein is completely degraded. Unlimited proteolysis can be achieved either by co-operative (or 'one-by-one') proteolysis only or by parallel non-co-operative and co-operative (or 'mixed type') proteolysis [8]. But if the limited proteolysis does not destabilise the protein's structure then hydrolysis stops.

The degree and rate of hydrolysis of some representative native legume SP is relatively deep and high [5 and references therein]. The only exception to this rule is phaseolin, the 7S SP from common beans [5, 9 and references therein]. Its hydrolysis by exogenous proteases stops after the cleavage of a small number of peptide bonds that result in limited modification of the molecule [9]. Since phaseolin is mobilised during seed germination it was expected that in seeds during this process are expressed proteinases that can perform a deep hydrolysis of phaseolin. However, the two major cysteine proteinases purified from germinated common bean seeds, CPPh, a papain-like protease [10], and legumain, an Asn-specific proteinase, [11] taken individually perform only a limited hydrolysis of phaseolin. To best of our knowledge, up to now, no profound proteolysis of phaseolin at the action of an individual protease has been achieved. This resistance of phaseolin to the action of individual proteases, both exogenous and endogenous, has been suggested to be due to the peculiarities of its structure that distinguish phaseolin from other legume SP [9, 10, 11]. That is why finding out the exact causes of this unusual property of phaseolin structure represents a theoretical and practical interest.

Native phaseolin, unlike other SP, undergoes a reversible association/dissociation with pH [12]. At acidic pH values from 1.0 to 3.0 it is represented by trimers (referred to as protomer form), while at acidic, but

physiological pH values from 3.8 to 5.4 phaseolin trimers associates into a dodecamer (a tetramer of trimers) [12]. With the increase of pH phaseolin dissociates into trimers at neutral pH values from 6.4 to 10.5, and finally into individual subunits at basic pH values above 11.5. Whether this property of phaseolin molecule to reversibly associate/dissociate has any influence on its susceptibility/resistance to hydrolysis, i.e. protect or render available sites for proteolysis, has not been studied yet.

Phaseolin hydrolysis by different exogenous proteases was performed at their pH optimum either at acidic or at neutral and slightly basic pHs [5, 9], a pH at which phaseolin is in its protomeric form. Phaseolin hydrolysis by endogenous proteases has been performed at their pH optimum at slightly acidic pHs [10, 11], a pH at which phaseolin is predominantly in its dodecameric form. However, no experiments had been performed by using a single protease at different pHs. That is why we decided to investigate the hydrolysis of phaseolin by papain, a cysteine protease that in comparison with other proteases has a distinguishing feature of preserving its activity in a large interval of temperature and pH [13]. It is stable and active in the interval of pH from 4.0 to 10.0 [13], with pH optimum in the region 6.0 – 7.0. The purpose of this work was to compare qualitatively and quantitatively the degradation patterns of native phaseolin at the action of papain in the range of pH from 4.6 to 8.0 at the extremes of which phaseolin is presented in two different forms – dodecamer and protomer. Here we report the results of this investigation that shows that pH influences the susceptibility of phaseolin molecule to papain proteolysis.

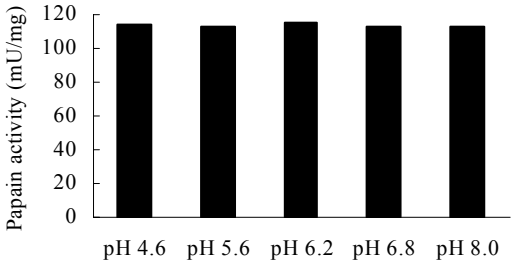
Materials and methods

Reagents. Twice crystallized papain suspended in acetate buffer pH 4.5 (Sigma, USA) was used in this work. Phaseolin was isolated from common bean seeds (*P. vulgaris* L. cv. Moldavian) according to the method of Hall et al. [14]. Bz-Phe-Val-Arg-*p*NA·HCl was from Bachem (Switzerland). All other reagents were of analytical grade.

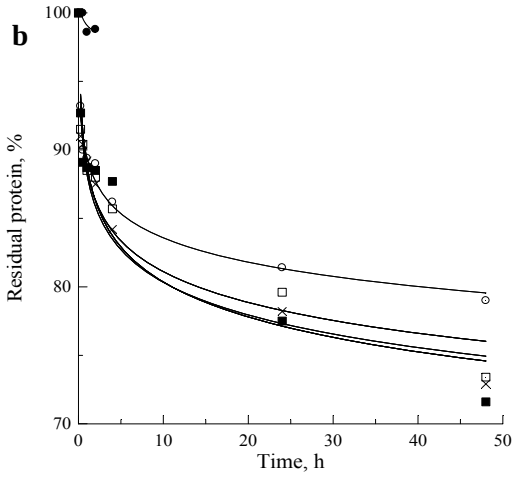
Buffers. The buffer systems selected were 120 mM phosphate-citrate buffers, pHs 4.6, 5.6, 6.2 and 6.8, and 50 mM Tris-HCl buffer, pH 8.0. The buffers contained 500 μM EDTA and 0.04 % NaN_3 and were adjusted with NaCl to ionic strength 0.5.

Papain activity. The papain activity at different pHs was determined with the synthetic substrate Bz-Phe-Val-Arg-*p*NA·HCl as described by Vaintraub and Morari [15] with the incubation of the reaction at 30 °C. The assays showed that papain retained its activity in the pH range 4.6 – 8.0 (Fig. 1a) used for performing phaseolin hydrolysis and that the activity is approximately equal at all pHs.

a



b



c

pH	Method of determination	
	dye-binding[a]	non-denaturating gradient pore PAGE[b]
4.6	-[c]	83
5.6	79	80
6.2	73	81
6.8	73	82
8.0	72	83

Fig.1. The influence of pH on phaseolin hydrolysis by papain. (**a**) Papain activity at pHs used for performing phaseolin hydrolysis. Papain (450 μg/ml) was activated by β-mercaptoethanol (2 μl/ml) for 30 min at 30 °C and then assayed with Bz-Phe-Val-Arg-pNA·HCl (120 μM) as substrate. (**b**) Time dependence of residual protein concentration (percent of the initial protein) during phaseolin hydrolysis by papain at pH 4.6 (●), 5.6 (○), 6.2 (×), 6.8 (□) and 8.0 (■). The initial protein concentration was taken for 100%. (**c**) Residual protein concentration (in %) in the final papain hydrolysates of phaseolin. [a] See Fig. 1b. [b] See Fig. 3f. [c] Can not be determined by the method used.

Proteolysis. Papain was activated by β-mercaptoethanol (5 μl/ml) for 30 min at 30 °C. Phaseolin solution (2%) in appropriate buffer, containing β-mercaptoethanol (5 μl/ml), was mixed with an equal volume of papain solution in the same buffer, and the reaction mixture was incubated at 30 °C. In experiments the enzyme: substrate ratio was 1:50. The duration of hydrolysis was 48 h and samples of hydrolysates were taken periodically.

Proteolysis of phaseolin, in all experiments, was repeated at least twice. Electrophoretic pattern, both SDS and non-denaturing gradient pore, and residual (TCA-insoluble) protein were determined in the samples. In the samples taken for native electrophoresis the reaction was stopped by the addition of 100 μM sodium iodoacetate, an inhibitor of cysteine proteinases.

Residual protein content was determined by a dye-binding method [16]. Replicates (four or five determinations performed on each sample) agreed to within 0.01 absorbance units.

PAGE. SDS/PAGE was carried out in a vertical flat-bed 12.5% PAGE. The protein samples were treated according to the method of Laemmli [17]. Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carboanhydrase (30 kDa), Kunitz soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (GE Healthcare, UK) were used as standards for determination of molecular mass molecular mass (M_r). The electrophoregrams were stained with Coomassie brilliant blue G-250.

SDS/PAGE was also carried out at the ratio of acrylamide to methylenebis(acrylamide) of 200:1 [18].

Non-denaturing gradient pore PAGE was carried out in a vertical flat-bed gradient (4-30%) PAGE using Tris-borate buffer system (90 mM, pH 8.4). The duration of electrophoresis was 4500Vh. Phaseolin (140 kDa) and bovine serum albumin (67 kDa) were used as standards. The percent of the residual protein was calculated from the decrease of its M_r. The gels were stained with Coomassie brilliant blue R-250.

Results and discussion

Our results show for the first time that pH influences phaseolin hydrolysis by papain. Assay of total protein content showed that at pH 4.6 phaseolin hydrolysis is low (Fig.1b) and after 2 h the modified protein starts to fall into precipitate. This differ not only from the action of papain on vetch 7S SP (vicilin) which is completely hydrolysed [not shown] but also from the action of CPPh on phaseolin [10]. That is why we decided to assay the papain action on phaseolin at pH 5.6 (Fig.1b), a pH at which phaseolin is completely hydrolysed by the consecutive action of legumain and CPPh [11]. At this pH modified phaseolin does not fall into precipitate. However, if CPPh hydrolyses only 10 % of phaseolin at both pHs 4.6 and 5.6 [10, 11], then papain hydrolyses 21% of phaseolin at pH 5.6 (Fig.1c). Phaseolin is known to be extremely resistant to proteolysis [5, 9, 10, 11] and until now such a high degree of phaseolin hydrolysis has been observed only at the action of trypsin [9]. Since the hydrolysis of phaseolin by trypsin has been carried out at pH 8.0 [9] we decided to investigate the action of papain on phaseolin also at this pH (Fig.1b). We also assayed the papain action on phaseolin at pHs 6.2 and 6.8 (Fig.1b) a pH diapason were papain has its maximal activity. The obtained results show that papain performs mainly limited proteolysis of phaseolin at all five pHs (Fig.2 and 3). However, the degree of hydrolysis increases with the increase of pH and the content of hydrolysed protein reaches a final degree of 1%, 21%, 27%, 27%, and 28% at pHs 4.6, 5.6, 6.2, 6.8, and 8.0, correspondingly (Fig.1c).

SDS/PAGE showed that the limited proteolysis of native phaseolin by papain results in cleavage of its subunits and concomitant appearance of several bands with M_r approximately half subunit (Fig.2). These fragments appeared already after the mixing of protein with the enzyme and are evident after 0.25 h of hydrolysis. It seems that their number does not change during the time course of hydrolysis. The slight changes of their M_r, i.e. a small increase in their mobility, seem to occur during the entire time course of hydrolysis till 48 h. The pH does not influence this process. The bands, corresponding to native subunits, disappeared after approximately 24 h of hydrolysis. After 48 h of hydrolysis the pattern of fragments formed is similar at all pHs (Fig.2). Two types of fragments are formed: an upper large band 1 and a lower thin band 2. The band 1 has a M_r of approximately 25 kDa. Starting with pH 5.6 this band seems to differentiate by having the lower part more intensely stained. The band 2 has a M_r of approximately 21 kDa.

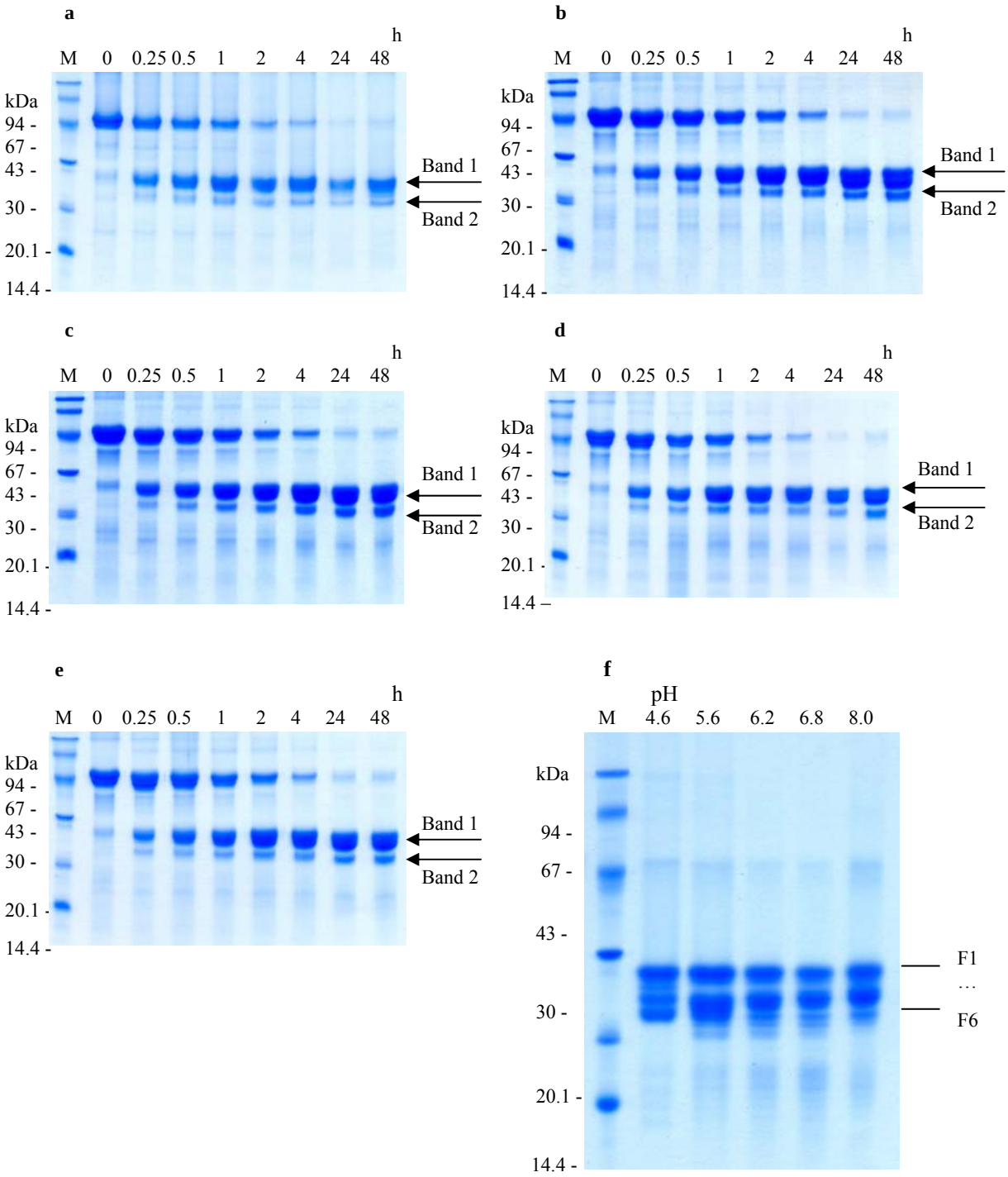


Fig.2. SDS/PAGE electrophoregrams of phaseolin hydrolysed by papain at pHs: 4.6 (**a**), 5.6 (**b**), 6.2 (**c**), 6.8 (**d**), and 8.0 (**e**), and phaseolin hydrolysed 48 h at different pHs (**f**). Proteins samples were taken at the indicated times and were separated on 12.5% SDS/PAGE under reducing conditions (**a-e**). The conditions of SDS/PAGE were changed to the acrylamide:methylenebis(acrylamide) ratio of 200:1 in **f**. On the right are indicated the final fragments designed as band 1 and 2 in **a-e** and as F1-F6 in **f**. M, M_r markers.

Table 1

The Mr (kDa) of fragments in the final (48 h) phaseolin hydrolysates (see Fig. 2f)

Fragments	pH				
	4.6	5.6	6.2	6.8	8.0
F1	27.0	26.9	27.0	26.9	27.4
F2	25.6	-	24.5	24.3	25.0
F3	23.9	24.2	23.1	23.2	23.6
F4	22.4	22.6	22.0	22.3	22.6
F5	-	21.4	21.3	21.2	21.3
F6	-	20.3	20.6	20.6	-

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 [19]. Each subunit has two potential glycosilation sites [20] and both singly and doubly glycosilated species are known to exist *in vivo* [21] which cause the appearance of molecular heterogeneity of phaseolin subunits [22]. As a result when phaseolin is subjected to SDS/PAGE it can be separated into four polypeptides, called size class [21], with M_r in the range from 45 to 52 kDa [20,21]. Not all four polypeptides are present in phaseolin prepared from different strains of *Phaseolus*. Three- and two-banded subunit patterns for phaseolin are also known [14]. It was shown by Stockman et al. [18] that these bands can be more easily separated by changing the acrylamide:methylenebis(acrylamide) ratio to 200:1. We decided to use similar electrophoretic conditions (Fig.2f) in order to separate the fragments formed during phaseolin hydrolysis by papain that could not be separated under standard ratio of 37.5:1 acrylamide: methylenebis(acrylamide) (Fig.2a-2e). Under these conditions six fragments are present in phaseolin hydrolysates. These fragments were labelled F1 through F6 according to decreasing of their M_r (Fig.2f and 2g). It is interesting that the papain action on phaseolin at pH 4.6 result in formation of four fragments (Fig.2f) which resemble the CPPh action on phaseolin at pH 4.6 [10]. The M_r of three of these fragments (F2 – F4) have similar M_r to those generated by CPPh while the first fragment (F1) has a higher M_r. With the increase in pH two changes take place in this pattern of fragments. First, F2 seem to undergo a more profound modification and it almost merges with F3 (especially at pH 5.6), and secondly, two more fragments (F5 – F6) with a lower M_r are formed, thus increasing their number to six (Fig.3f). All six fragments are present only at pHs 6.2 and 6.8 (Fig.2f and 2g). Nielsen et al. [5] have found that after 30 min of hydrolysis at pH 6.2 phaseolin is only slightly hydrolysed by papain. Apart from fragments that correspond to half subunits they also detected two other groups of fragments: one with M_r of approximately 35 kDa and another around 14 kDa [5]. Fragments with M_r lower than half subunits have been also found at the action on phaseolin of subtilisin and pronase E at pH 7.5 [5] and of legumain at pH 5.6 [23]. In our experiments we did not detect such fragments (i.e. fragments with the M_r markedly different from half M_r of native subunit) at any point during the time course of hydrolysis at any pHs. Taking into account the size of fragments as well as the absence of intermediary products it is possible to assume that the cleavage of phaseolin by papain take place in the middle of phaseolin subunits with subsequent splitting off of a few peptides from the N- and/or C-terminuses, which is similar to the results obtained at the CPPh action on phaseolin [10].

Non-denaturing gradient pore PAGE showed that the modification of phaseolin by papain seems not to disrupt the molecule's quaternary structure during the hydrolysis at any pH (Fig.3). The final high M_r products of paseolin hydrolysis by papain will be subsequently referred to as phaseolin-Pap (in order to distinguish it from the final high M_r product formed at the hydrolysis of phaseolin by pepsin and named phaseolin-P [9]). During the time course of proteolysis the M_r of phaseolin-Pap undergoes a gradual decrease at all pHs (Fig.3a-3d). It is interesting to note that this is true even for pH 4.6 (Fig.3b) despite the fact that at this pH phaseolin-Pap start to fall into precipitate after 2 h of hydrolysis. The final (after 48 h of hydrolysis) M_r of paseolin-Pap (Fig.3f) decreases from 140 kDa to 116, 112, 114, 115, and 116 kDa, at pH 4.6, 5.6, 6.2, 6.8, and 8.0, correspondingly which represents a diminishing of M_r by approximately 17, 20, 19, 18, and 17 %,

correspondingly (Fig.1c). These results show that the highest degree of modification of phaseolin molecule is attained at pH 5.6 (Fig.3f and 1c). The diminution of phaseolin-Pap M_r show that the papain cleaves off a considerable number of short peptides from the phaseolin molecule at all pHs. In the case of action of both endogenous cysteine proteases, legumain and CPPh, on phaseolin it also has been established that modified phaseolin, phaseolin-LLP [23] and phaseolin-CPPh [10], correspondingly, retain their quaternary structure. However, while the M_r of phaseolin-LLP decreases only by 2 % (to 137 kDa) [23] and that of phaseolin-CPPh decreases by 9 % (to 128 kDa) [10] the M_r of phaseolin-Pap is much lower, decreasing by as much as 20 % (to 112 kDa) at pH 5.6 (Fig.3f and 1c). Up to now such a deep modification of native phaseolin was observed only at the action of trypsin at pH 8.0 [9].

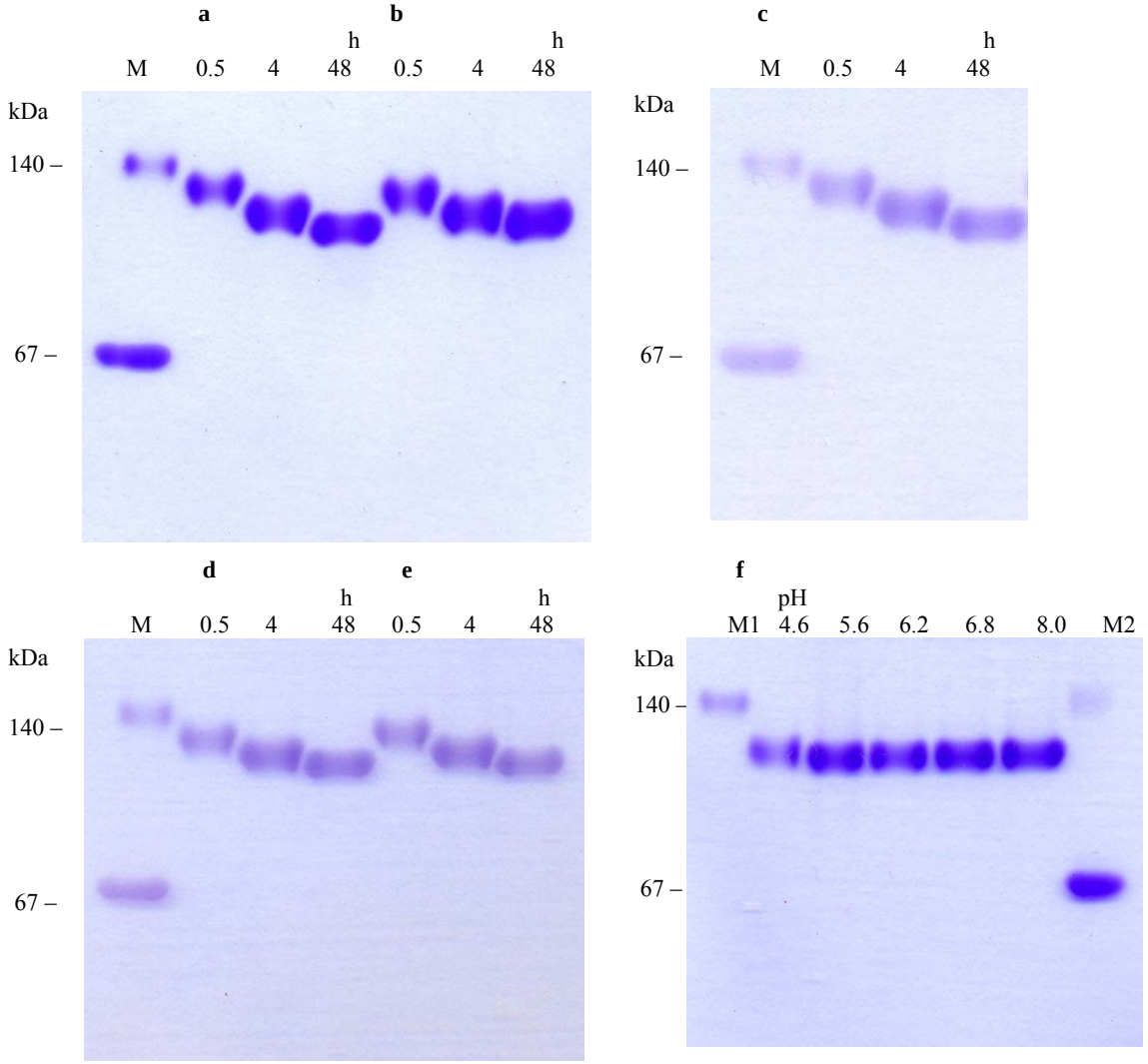


Fig.3. Non-denaturating gradient pore PAGE electrophoregrams of phaseolin hydrolysed by papain at pH: 4.6 (**b**), 5.6 (**a**), 6.2 (**c**), 6.8 (**d**), and 8.0 (**e**), and phaseolin hydrolysed 48 h at different pHs (**f**). The protein samples were taken at the indicated times, and the reaction was stopped by the addition of 100 μM sodium iodoacetate, an inhibitor of cysteine proteinases. M, M_r markers: native phaseolin (M1 in **f**) and BSA (M2 in **f**).

Proteolysis of SP, by both exogenous [8] and endogenous [24] proteinases, takes place in stages that are distinct by mechanism (see the Introduction). As a result SP are completely hydrolysed to short peptides. The results, presented in this work, show that phaseolin hydrolysis by papain seem to take place in two stages: at the beginning, during the first hour of hydrolysis, the phaseolin is hydrolysed about 11 % at pHs 5.6, 6.2, 6.8, and 8.0, while after that the rate of hydrolysis slows down (Fig.1b). However, the native phaseolin subunits

are not modified completely during the first stage of hydrolysis (Fig.2). Phaseolin-Pap formed during this period changes its M_r afterwards (Fig.3). Moreover, in the end, phaseolin hydrolysis stops after the maximum 28 % of protein breakdown (Fig.1c). Thus the mechanism of phaseolin proteolysis by papain differs from the basic mechanism of SP proteolysis [8,24]. And since the same mechanism of phaseolin proteolysis is observed also at the action of both exogenous [9] and endogenous [10,11] proteases it suggests one more time that it is due to the peculiarities of phaseolin structure. Finding the mechanism by which phaseolin molecule is protected from destabilisation still remains a task to be elucidated.

An explanation for phaseolin resistance to the action of proteases was proposed in the basis of differences between its tertiary structure and that of other 7S SP [9,10]. However, as already has been mentioned (see the Introduction), phaseolin protomer reversibly associate and dissociate at different pHs [12]. Naturally arise the question whether this unusual pH-dependent association/dissociation behaviour of phaseolin might influences its susceptibility to proteolysis and explain its unusual resistance to proteolysis. Our results on the action of papain on phaseolin at different pHs confirm the high resistance of most of the phaseolin molecule to the proteolytic attack and show that the cleavage of phaseolin subunits by papain does not result in the destabilisation of phaseolin quaternary structure. It seems that papain, like legumain and CPPh, does not have direct access to the regions of phaseolin molecule essential for keeping its tertiary and quaternary structure. However, our results also show that with the increase in pH the susceptibility of phaseolin to the action of papain increases (Fig.1b and 1c). Although the patterns of phaseolin hydrolysis are similar at all pHs (Fig.2) the loss of total protein is different (Fig.1b, 3f and 1c). More than that the values of residual protein calculated from the data of native gradient pore PAGE differ from those determined directly (Fig.1c). If the content of residual protein at the action of papain reaches a final degree of hydrolysis of 1%, 21%, 27%, 27%, and 28% at pH 4.6, 5.6, 6.2, 6.8, and 8.0, correspondingly (Fig.1b and 1c) then the M_r of Phaseolin-Pap diminishes by approximately 17, 20, 19, 18, and 17 %. (Fig.3f and 1c). These results show that only at pH 5.6 the protein loss determined by these two methods is about the same (Fig.1c). Thus the quantitative determination of the content of residual protein indicates that with the increase in pH at pH values higher than 6.2 a part (approximately 10 %) of phaseolin is susceptible to further unlimited proteolysis and is slowly hydrolysed completely by papain (Fig.1b, 3f and 1c). This differs from the action on phaseolin of both endogenouse, CPPh and legumain [10, 23], and exogenouse, trypsin [9], where the protein loss determined by two methods is in good agreement. Since phaseolin trimer retains its structure after hydrolysis by papain at all investigated pHs (Fig.3f) and since starting with pH 6.2 the difference in protein loss determined by these two methods is about 10 % (Fig. 1c) our results would suggest that the dodecamer dissociation into trimers, which occurs with the increase in pH [12], results in that that more sites in phaseolin molecule become accessible (available) to the papain attack which in its turn leads to that that a part of phaseolin modified at these pHs becomes susceptible to further unlimited proteolysis by papain.

Because the problem of deficiency of protein in alimentation of humans is still actual, the interest expressed for the study of vegetal proteins has never ceased [3]. Alimentation ration of humans across the globe contains 70% vegetal proteins from cereal and legume cultivars. Therefore, food legumes are one of the major food sources and are considered as the major source of dietary proteins among plant species. One of the important issues connected with the resistance of phaseolin to proteolysis is the quality of protein source for food. Digestibility and the content of indispensable amino acids are basic parameters in determining the quality of a protein source for food [25]. The presence of less digestible protein fractions in traditional diets lowers considerably their nutritional value [25]. That is why enhanced digestibility represents one of the targets for legume seed protein improvement [3]. It is well known that in contrast to other 7S SP, native phaseolin is highly resistant to *in vitro* proteolysis by digestive proteinases [5,9]. Its digestion *in vivo* also is reduced [6,26,27]. It has been shown that after disruption of phaseolin structure by heating phaseolin hidrolysis is rapid and complete [5]. However, a recent study has shown that not only unheated phaseolin is resistant to *in vitro* proteolysis but that the susceptibility to pepsin of heat-treated phaseolin also varies across common bean varieties [28]. This indicates that susceptibility to proteolysis affects the nutritional value even of heated phaseolin. Consequently, the resistance of phaseolin to proteolysis by digestive proteases is an important factor contributing to its poor nutritive value and thereby its bioavailability. Therefore finding modalities of enhancing phaseolin susceptibility to proteolysis could be one of the possible criterions of choice in breeding programs aimed at improving the nutritional value of common beans.

Conclusions

In summary, this work show that in the pH range from 4.6 to 8.0 papain action on phaseolin results mainly in limited modification of phaseolin molecule by non-co-operative proteolysis. Our results confirm the high resistance of phaseolin molecule to the proteolytic attack and are consistent with the previous reports [5,9,10,11]. However, they also show, for the first time, that pH influences phaseolin proteolysis by papain. At pH 4.6 only 1% of phaseolin is hydrolyzed while at pH 8.0 - 28%. The comparison of results on residual protein determination by a dye-binding method and native gradient pore PAGE indicates, that at pHs higher than 6.2 a part of modified phaseolin is susceptible to complete proteolysis by papain. Since it is known that phaseolin undergoes reversible oligomerizitaion with pH, it seems that phaseolin dodecamer dissociation at higher pHs makes phaseolin protomer more susceptible to proteolysis by papain. Certainly, more research is needed to be addressed in the future for understanding the relationships between phaseolin property to reversibly associate and dissociate with the changes in pH and the degree of its resistance/susceptibility to proteolysis.

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