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Calcium Ions Make Phytohemagglutinin Resistant to Trypsin Proteolysis

DIANA MORARI, TATIANA STEPURINA, AND VITALIE I. ROTARI*

Laboratory of Plant Biochemistry, State University of Moldova, Mateevici str. 60, MD-2009 Kishinev, Republic of Moldova

To investigate the mechanism of phytohemagglutinin (PHA) susceptibility or resistance to the action of proteolytic enzymes, its in vitro proteolysis by trypsin was studied. It was found that Ca^{2+} gives resistance to the native PHA molecule to trypsin proteolysis. In the absence of Ca^{2+} trypsin performs a thorough hydrolysis of PHA. At the first stage of trypsin hydrolysis of PHA the formation of a relatively stable high molecular mass product occurs (PHA-T) as a result of non-co-operative proteolysis. At the second stage, the degradation of PHA-T occurs, and this degradation is performed by parallel co-operative proteolysis. This type of proteolysis differs from the action of trypsin on phaseolin, the main storage protein from common bean (*Phaseolus vulgaris* L.). The implications of Ca^{2+}influence of PHA hydrolysis by trypsin are discussed.

KEYWORDS: Phytohemagglutinin; *Phaseolus vulgaris*; proteolysis; trypsin; Ca^{2+}

INTRODUCTION

Proteins from vegetal sources represent an important component of foodstuffs (*1*). Production of animal proteins is relatively inefficient when compared with the production of plant proteins, and animal proteins are an expensive source of dietary protein. More seed protein than animal protein can be produced on a unit of land. A significant part of the human world population relies on cereals and legumes as a staple food. Legumes have some of the higher protein-containing seeds, and legume seed storage proteins have attracted in particular much attention in past years because they have important "functional" (physiochemical) properties that are significant to their use in foods (*1*). Lectins, besides the 11S and 7S reserve globulins, represent another group of seed proteins, which are deposited in large quantities in the seeds of some species of plants (*2*). They are particularly abundant in legumes, and in some species they account for 1–8% of the total protein in vacuoles of the cotyledons and at a lower level they also accumulate in the embryonic axes (*3*). For example, PHA in common bean seeds amounts to 1200 mg per 100 g of seeds (*2*), which constitutes about 10% of total protein (*4*). Lectins are widely distributed in other organs of plants as well (*2*).

Lectins are sugar-binding proteins (*5*) that agglutinate cells and/or precipitate glycoconjugates. Over more than 100 years of lectin research the views about them have evolved from being regarded as hemagglutinins to biological recognition molecules (*6*) and sugar receptors that decipher sugar code (*2*). Legume lectins are one of the largest lectin families, most of which are found mainly in the seeds, and represent the most studied group of lectins (*7*). The exact functions of these lectins in vivo are not known yet (*8*), but defense against predators and interactions with bacteria for establishing symbiosis have been proposed (*3, 6, 8*). Legume lectins resemble each other in their physicochemical properties, although they differ in their carbohydrate specificities. They consist of two or four subunits, which is characteristic of most lectins, with relative molecular mass (M_r) of about 30 kDa, and each subunit has one carbohydrate-binding site (*7, 8*). The structural similarities of these lectins have been reported by primary structure analysis and by X-ray crystallographic studies (*7*). Despite this strong similarity at the level of primary and tertiary structures, their quaternary structures vary widely. The quaternary structure of these lectins forms the basis of a higher level of specificity and is of importance for their biological activities (*7*).

Many lectins are toxic and may therefore protect the plant from being eaten away by predators (*3, 8*). Many tests have shown that the feeding of experimental animals with lectins results in retardation of growth, diarrhea, and interference with the absorption of nutrients in the intestine (*9, 10*); they also might alter the host resistance to infections, and under certain circumstances they can be highly allergenic (*9, 11*). Gibbons and Dankers noted that many food plants contain active lectins (*12*). A survey of the edible portions of fresh and processed diet foods in the United States has found that lectin activity was present in 30% of foodstuffs tested (*11*), showing by this that dietary exposure to plant lectins is widespread. Although it is considered that cooking might abrogate lectin activity, it was found that some lectins are resistant to thermic treatment (*9, 11, 13*). For example, PHA from kidney beans can resist mild cooking and retain lectin activity (*13*). Besides that the heat processing itself can also result in the formation of antinutritional factors in foods (*14*). Several investigators noted also year-to-year and batch-to-batch variations in the lectin

* Corresponding author [telephone +(373-22)-577-529; fax +(373-22)-24-42-48; e-mail rotarivi@yahoo.com].

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content of foods (*11*) which indicates that occasionally lectins are likely to occur even with foods normally considered to be safe.

Despite containing high amounts of lectins, legume seeds, as have been mentioned already, are important sources of protein for food and feed. Beans of *Phaseolus* are important food crop and are cultivated worldwide. With ever rising costs of meat, it is expected that beans will be consumed more in human nutrition. Dry beans contain up to 25% protein on a dry weight basis, which are composed up to 30% from water-soluble albumins and the remaining are represented by globulins. The nutrient composition of dry beans makes them ideally suited for healthy diets (*15*). However, some toxicological aspects, especially linked with the presence of PHA, limit the usage of beans from *Phaseolus* as food for humans.

The common bean seeds have been known to be toxic to animals (*13*), and many investigations have shown that there exists a definitive relationship between the poor nutritive value of a raw bean diet and the content of PHA. Feeding trials on rats and poultry have demonstrated the toxicity of purified PHA (*16, 17*), and consequently PHA has been regarded as a major antinutritional factor of raw beans (*18*). This toxicity is due to the poorly digestible PHA that was shown to interact with intestinal cells in vivo and to cause disruption of many cells (*10, 18*).

PHA occurs in 90% of all bean cultivars and in all wild accessions (*3*). Lectins such as PHA occur in many legume species and probably are present in all species of the genus *Phaseolus* (*19*). PHA consists of five proteins, each of which is a tetramer of 126 kDa composed of subunits of two types: L with M_r 32 kDa, which has a leucoagglutinating property, and E with M_r 34 kDa, which has an erythroagglutinating property (*3*). The subunits are termed PHA-L and PHA-E, correspondingly. The PHA tetramers are built by the subunits in all possible combinations: L_4, L_3E, L_2E_2, LE_3, and E_4 (*20*).

The selection criteria used in most research on varietal selection are high yields and resistance to disease. In recent years attention has been focused also on the nutritive quality and removal of antinutritional factors is one of the targets for legume seed protein improvement (*21*). Lectins are among the factors considered as antinutritional, and the selection of dry bean varieties with a high nutritive value (i.e., a low content of antinutritional factors) is of great importance. Because in *Phaseolus vulgaris* PHA represents the main toxic component, it even was attempted to develop a breeding program aimed at the removal of PHA (*22*).

One of the main factors affecting the nutritive value of proteins is their susceptibility to hydrolysis by digestive proteinases. It has been shown for several representative leguminous storage proteins that they are hydrolyzed profoundly by digestive proteinases (*23*). A striking exception is displayed by phaseolin, the 7S protein of dry common bean. Its hydrolysis, by both exogenous digestive proteinases (*23, 24*) and endogenous proteinases (*4, 25*), stops after cleavage of a small number of peptide bonds. The resistance of phaseolin to the action of different proteinases has been explained to be due to the peculiarities of its structure (*24, 25*).

It is known that legume seed proteins have different nutritive values. Whether pure digestibility of these proteins accounts for these differences in nutritive values has not been established, but it has been found that the degree of digestibility of the major storage proteins agrees with the results of nutritive value for the unheated legume seeds (*23*). Some lectins, for example, the wheat germ agglutinin and the lectin from tomato, are known to be resistant to the action of proteolytic enzymes (*9, 11*). That is why the study of the mechanism of PHA proteolysis is of interest for the clarification of its sensitivity or resistance to the action of proteases in general and those of the digestive tract in particular. Here we report the results of the investigation of PHA hydrolysis by trypsin and compare it to the mechanism of hydrolysis of legume seed storage proteins (*26*). The PHA hydrolysis is interpreted as being influenced by calcium ions, and the possible implications of this finding are discussed.

Table 1. Tris-HCl Buffer [0.05 M, pH 8.0, Containing 0.04% NaN_3 ("Standard Buffer")] Used as Initial Buffer for Preparation of Buffers for Trypsin Hydrolysis with the Addition of the Following Supplements: 0.02 M $CaCl_2$, 0.5 mM EDTA, 0.5 M NaCl

	supplements		
buffer	0.02 M $CaCl_2$	0.5 mM EDTA	0.5 M NaCl
buffer−$CaCl_2$-1	+	−	+
buffer−$CaCl_2$-2	+	−	−
buffer−EDTA-1	−	+	+
buffer−EDTA-2	−	+	−

MATERIALS AND METHODS

Reagents. Trypsin from bovine pancreas (Serva) was used in this work. The content of active trypsin was determined by active-site titration (*27*). All other reagents were of analytical grade.

Preparation of Proteins. PHA and phaseolin were isolated from common bean seeds (*P. vulgaris* L. cv. Moldavian). PHA was isolated according to the method of Karmanski (*28*). PHA-P (Biochem) was used as standard. Phaseolin was isolated according to the method of Schlesier et al. (*29*).

Proteolysis. Tris-HCl buffer [0.05 M, pH 8.0, containing 0.04% NaN_3 ("standard buffer")] was used as initial buffer for the preparation of the buffers for trypsin hydrolysis. These buffers contained 0.02 M $CaCl_2$, 0.5 mM EDTA, and 0.5 M NaCl in different combinations (**Table 1**). Buffer-1 differed from buffer-2 by containing NaCl.

Protein solution (2%) in appropriate buffer was mixed with an equal volume of trypsin solution in the same buffer, and the reaction mixture was incubated at 30 °C. In experiments the enzyme/substrate ratio was 1:50 calculated for trypsin on the basis of active enzyme. The duration of hydrolysis was 48 h, and samples of hydrolysates were taken periodically.

Proteolysis of both proteins, PHA and phaseolin, in all experiments, was repeated at least twice. SDS electrophoretic pattern and residual (TCA-insoluble) protein were determined in the samples. Protein was determined by a dye-binding method (*30*). Replicates (four to five determinations performed on each sample) agreed to within 0.01 absorbance units.

Electrophoresis. For SDS electrophoresis the protein samples were treated according to the method of Laemmli (*31*). SDS electrophoresis was carried out in a vertical flat-bed 12.5% polyacrylamide gel. 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) were used as standards for M_r determination. The electrophoregrams were stained with Coomassie brilliant blue G-250 according to the standard procedure.

Non-denaturing gradient pore electrophoresis was carried out in a vertical flat-bed gradient (4–30%) polyacrylamide gel using a Tris-borate buffer system (0.09 M, pH 8.4). The duration of electrophoresis was 4500 Vh. 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 according to the standard procedure.

RESULTS

PHA Hydrolysis. The PHA sample isolated by the purification scheme used (*28*) represents one main band, probably

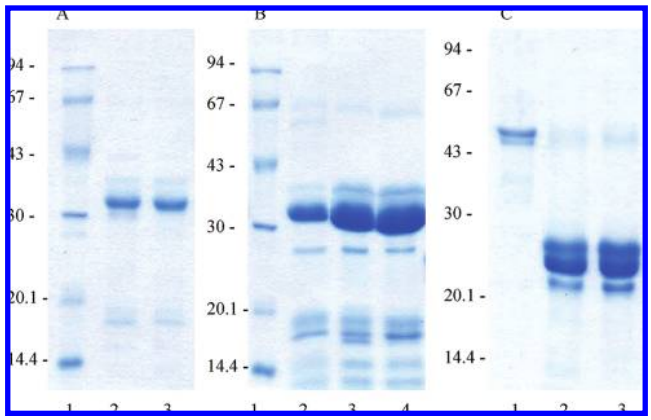

Figure 1. Electrophoregrams of initial (native) PHA and phaseolin and their hydrolysis products. (**A**) SDS electrophoresis of native PHA: lane 1, protein standards; lane 2, PHA-P (Biochem); lane 3, purified PHA. (**B**) SDS electrophoresis of PHA hydrolyzed by trypsin: lane 1, protein standards; lane 2, PHA in standard buffer; lane 3, PHA in buffer−$CaCl_2$-2; lane 4, PHA in buffer−$CaCl_2$-1. (**C**) SDS electrophoresis of phaseolin hydrolyzed by trypsin: lane 1, native phaseolin; lane 2, phaseolin in buffer−$CaCl_2$-1; lane 2, phaseolin in buffer−EDTA-1. The M_r values of standard proteins (in kDa) are shown on the left for all electrophoregrams. The conditions of electrophoresis are detailed under Materials and Methods.

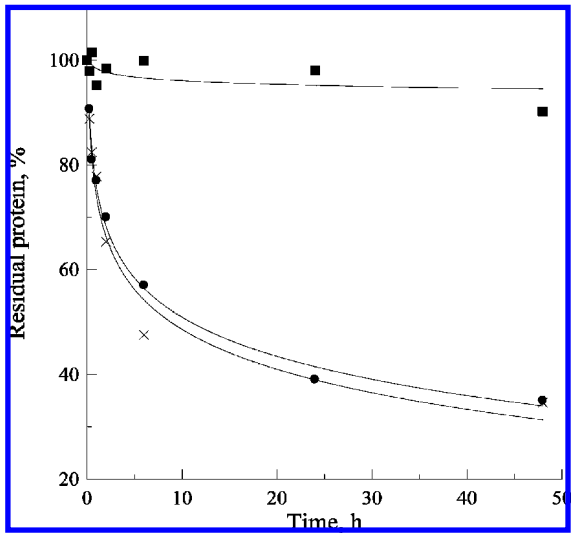

Figure 2. Influence of $CaCl_2$ and EDTA on PHA hydrolysis by trypsin: time dependence of residual protein concentration during PHA hydrolysis by trypsin in buffer−$CaCl_2$-1 (■), standard buffer (×), and buffer−EDTA-2 (●). Hydrolysis conditions are detailed under Materials and Methods. The initial concentration is taken for 100%.

consisting of two polypeptides, PHA-L and PHA-E, that cannot be separated because of the predominance of one band, with the M_r of 32.4 kDa (**Figure 1A**). It is known that the relative amount of the PHA polypeptides may vary in different genotypes, and there even are known bean accessions that contain only one PHA subunit (*22*). The purified PHA sample is similar to PHA-P standard (**Figure 1A**). Apart from the PHA band(s), there are also present a few minor bands in the isolated protein sample, but these bands are also present in the PHA-P standard and probably are specific for it.

The content of initial PHA decreases under the action of trypsin, although at a slower rate than that of phaseolin. At the enzyme to protein ratio of 1:50 there is still present a large part

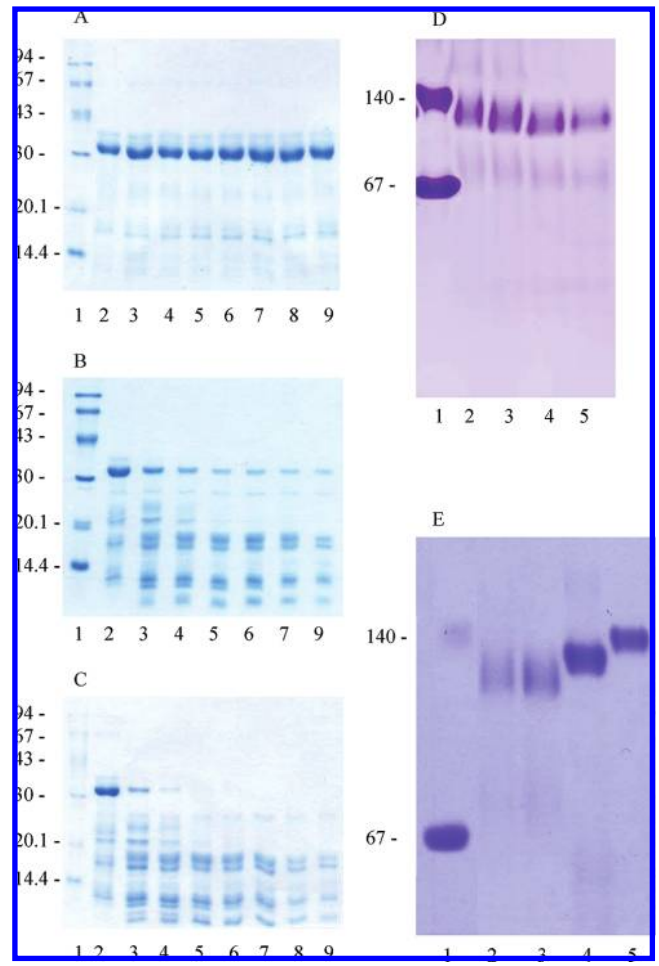

Figure 3. Electrophoregrams of PHA time course hydrolysis by trypsin: (**A**) SDS electrophoresis of PHA hydrolysis in buffer−$CaCl_2$-1; (**B**) SDS electrophoresis of PHA hydrolysis in standard buffer; (**C**) SDS electrophoresis of PHA hydrolysis in buffer−EDTA-2. Lane 1, protein standards; lanes 2–9, PHA hydrolyzed by trypsin 0, 0.25, 0.50, 1, 2, 6, 24, and 48 h, respectively. The M_r values of standard proteins (in kDa) are shown on the left for all SDS electrophoregrams. (**D**) Non-denaturing gradient pore electrophoresis of PHA hydropysis by trypsin in Buffer-EDTA-2: lane 1, protein standards: phaseolin and BSA, M_r (in kDa) values are shown on the left; lanes 2–5, PHA hydrolyzed by trypsin for 0, 0.25, 1, and 6 h. (**E**) Non-denaturing gradient pore electrophoresis of PHA-T: lane 1, BSA; lane, 2 PHA hydrolyzed by trypsin in standard buffer for 6 h; lane 3, PHA hydrolyzed by trypsin in buffer−EDTA-2 for 6 h; lane 4, PHA; lane 5, phaseolin. The M_r values of standard proteins, phaseolin and BSA (in kDa), are shown on the left. The conditions of electrophoresis are detailed under Materials and Methods.

of the initial PHA even after 48 h of hydrolysis, whereas phaseolin subunits are completely modified (**Figure 1B**,**C**). PHA hydrolysis is prevented by the Ca^{2+} (**Figures 1B** and **3A**), whereas EDTA enhances it (**Figure 3C**). It seems that Ca^{2+} influences only PHA proteolysis by trypsin because it has no effect on phaseolin modification by trypsin. After 48 h of hydrolysis, the native subunits of phaseolin are modified completely both in buffer−$CaCl_2$-1 and in buffer−EDTA-1 and the pattern of fragments formed is the same in both samples.

In a separate experiment we found that the PHA is completely hydrolyzed by trypsin also at an enzyme to protein ratio of 1:100, whereas at the ratio of 1:300 the hydrolysis does not take place (not shown). Because in the presence of $CaCl_2$ the PHA becomes resistant to hydrolysis (**Figures 1B**, **2**, and **3A**), we chose to work with the enzyme to protein ratio of 1:50 for all buffers.

The prevention of PHA hydrolysis by Ca^{2+} and its enhancement by EDTA are also confirmed by the decrease of the content of total protein under the action of trypsin. The content of the residual protein after 48 h of hydrolysis decreased to about 35% in both standard buffer and buffer−EDTA-2. A detailed analysis showed that in both standard buffer and buffer−EDTA-2 the content of the residual protein decreased rapidly during the first 2 h of hydrolysis, and after that, it slowed (**Figure 2**). Thus, two stages of PHA hydrolysis by trypsin, in both buffers, are evident from **Figure 2**. In the course of the first one the hydrolysis rate is relatively high. Its duration is about 2 h under the hydrolysis conditions used. During the second stage the hydrolysis rate is slower, but it is not linear. Evidently, the non-co-operative proteolysis occurs during the first stage, whereas during the second stage, probably, both co-operative and non-co-operative proteolyses occur in parallel. The final degrees of proteolysis attained in standard buffer and buffer−EDTA-2 are similar, whereas in buffer−$CaCl_2$-1 the final degree decreased only to 90% after 48 h of hydrolysis (**Figure 2**).

Electrophoresis of Residual Protein. SDS electrophoresis of the unhydrolyzed PHA showed one band (probably consisting of overlapping intensities of two closely migrating PHA subunits, one of which predominates) (**Figure 1A**). The intensity of this band decreased under the action of trypsin both in the absence (standard buffer) and in the presence of EDTA (buffer−EDTA-2), with concomitant appearance of fragments with M_r in the range from 25.7 to 10.2 kDa (**Figure 3B**,**C**). In buffer−EDTA-2 the fragments appeared already after the mixing of protein with the enzyme and are very evident after 0.25 h of hydrolysis. The original subunit band(s) disappeared after 1 h of hydrolysis, and after 6 h of hydrolysis, it seems that the number of formed fragments stops changing. The final electrophoresis pattern is shown in **Figure 3**.

The hydrolysis of PHA by trypsin both in standard buffer and in buffer−EDTA-2 resulted in the generation of four major stable fragments. The major breakdown products can broadly be divided in two groups: The first group is formed by the fragments with M_r in the range of 16–17 kDa, which approximately is half the M_r of original subunits. The M_r values of the fragments of the second group are in the range of 12–10 kDa.

If the intensities of all four fragments are similar in buffer−EDTA-2, then in standard buffer the intensities of the fragments of the first group predominates. Apart from that, in standard buffer another fragment (fifth) with the M_r of about 25 kDa can be also observed.

SDS electrophoresis showed that the breaking up of the polypeptide chains of PHA into fragments occurs during the first stage of trypsin proteolysis in buffer−EDTA-2 (**Figure 3C**). In the absence of EDTA this process is slowed, and the band corresponding to the native PHA subunit is present even after 48 h of hydrolysis (**Figures 1B** and **3B**). $CaCl_2$ almost completely prevents the degradation of native PHA subunits (**Figures 1B** and **3A**). In the presence of $CaCl_2$ the initial band stays almost intact during the hydrolysis, although two minor bands with M_r of about 17 kDa are formed. During the second stage of hydrolysis only some minor qualitative changes of modified PHA take place. These changes could explain the absence of linearity for the second stage of hydrolysis observed from the kinetic data (**Figure 2**).

Gradient pore gel electrophoresis showed that PHA retained its quaternary structure after hydrolysis by trypsin in buffer −EDTA-2 (**Figure 3D**). The final high molecular mass product of trypsin hydrolysis will be subsequently referred to as PHA-T. The M_r of PHA-T decreased from 123 to 114 kDa, which corresponds to 93% of protein. PHA-T after hydrolysis in standard buffer also represents a 114 kDa protein (**Figure 3E**), whereas in the presence of $CaCl_2$ the PHA molecule remains intact (not shown).

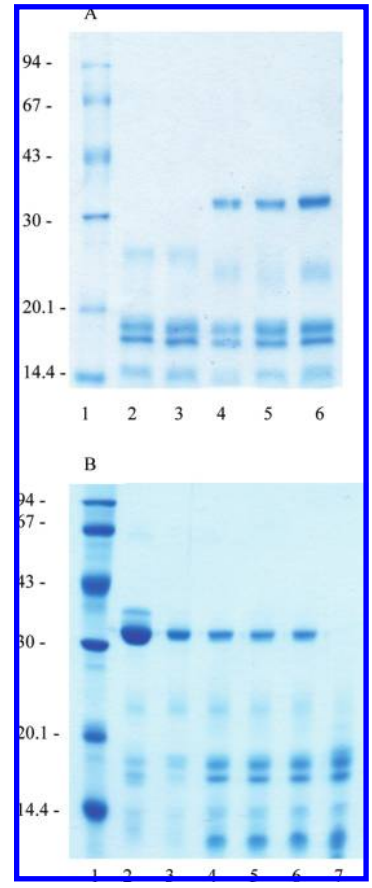

Figure 4. EDTA enhances and $CaCl_2$ gives resistance to PHA hydrolysis by trypsin. (**A**) SDS electrophoregram of the influence of EDTA concentration: lane 1, protein standards; lanes 2–5, PHA hydrolyzed by trypsin for 48 h in buffers with different concentrations of EDTA (500 μM, 100 μM, 5 μM, and 500 nM, respectively); lane 6, PHA hydrolyzed by trypsin in standard buffer. (B) SDS electrophoreogram of the influence of $CaCl_2$: lane 1, protein standards; lanes 2–5, PHA hydrolyzed by trypsin for 48 h in buffers with different concentrations of $CaCl_2$ (20 mM, 500 μM, 5 μM, 500 nM, respectively); lane 6, PHA hydrolyzed by trypsin in standard buffer; lane 7, PHA hydrolyzed by trypsin in buffer−EDTA-2. The M_r values of standard proteins (in kDa) are shown on the left for both electrophoreograms. The conditions of electrophoresis are detailed under Materials and Methods.

Influence of EDTA on PHA Hydrolysis. Although decreases of total protein during the hydrolysis of PHA by trypsin are similar both in standard buffer and in buffer−EDTA-2, the SDS electrophoresis showed that in the presence of EDTA the hydrolysis of the initial PHA band is greatly enhanced. We checked at which concentration of EDTA takes place the switch between these two mechanisms. As can be seen from **Figure 4A** at both 500 and 100 μM EDTA the initial PHA band is completely hydrolyzed after 48 h, whereas already at 5 μM EDTA it is present, although it is not as intense as in the buffer without EDTA. Therefore, it might be concluded that the more quickly Ca^{2+} are removed from the PHA molecule, the more susceptible it becomes to trypsin proteolysis.

Influence of $CaCl_2$ on PHA Hydrolysis. SDS electrophoresis showed that the reduction of $CaCl_2$ concentration in assay buffer enhances PHA hydrolysis and at a concentration of 5 μM the

hydrolysis is similar to that performed in buffer without $CaCl_2$ (**Figure 4B**). As in the case with EDTA, these results also indicate the same conclusion—the more quickly Ca^{2+} are removed from the PHA molecule, the more susceptible it becomes to trypsin proteolysis.

DISCUSSION

Our results show for the first time that Ca^{2+} gives resistance to the PHA molecule to trypsin proteolysis. In the presence of Ca^{2+} only 10% of protein is hydrolyzed, whereas in the absence of Ca^{2+} protein hydrolysis reaches 75% in 48 h. The prolonged hydrolysis used in this work permitted us to detect a gradual decrease of residual protein. We observed that the cleavage of PHA by trypsin is enhanced in the presence of EDTA. Thus, the hydrolysis of PHA by trypsin differs from the action of trypsin on phaseolin (**Figure 1** and ref *24*). In contrast to phaseolin, PHA is profoundly hydrolyzed by trypsin. However, this proteolysis seems to be regulated by the calcium ions, which give resistance to the PHA molecule to trypsin proteolysis. During trypsin hydrolysis the native subunit(s) of PHA disappear and a group of fragments with M_r in the range from 25.2 to 10.2 kDa are formed. Stable fragments are formed during the hydrolysis both in the absence and in the presence of EDTA. After 48 h of hydrolysis, there are four fragments in the PHA sample hydrolyzed by trypsin in buffer−EDTA-2 and five fragments in the PHA sample hydrolyzed by trypsin in standard buffer. The cleavage of PHA subunits does not break up its quaternary structure. During the first hour of the hydrolysis the PHA molecule is modified, giving PHA-T, which has a M_r of 114 kDa. This corresponds to the splitting off of 7% of the M_r of native PHA. The PHA-T is afterward hydrolyzed completely without change in its M_r.

Many lectins are localized within the cell where they may be present in considerable amounts. They react with storage proteins, and their affinity to the species' own storage proteins is far greater than that to foreign proteins (*2*). This leads to the assumption that they complex proteins and thus transform them into a compact, insoluble state that renders them easier to store than a soluble state would (*32*). Because lectins are deposited in considerable amounts in the seeds, their role as storage proteins is considered as evident (*2*). Proteolysis of seed storage proteins, under the action of both endogenous and exogenous proteinases, takes place in two stages, which are distinctive by mechanism (*26*). At the first stage, which is relatively short, takes place a fast decrease of protein. Then, at the second stage, the dependence of the logarithm of protein concentration versus time of hydrolysis becomes linear, which is characteristic of a pseudo-first-order reaction. Non-co-operative proteolysis take place at the first stage; meanwhile, the kinetics of the second stage is determined by the cleavage of the modified protein either by the co-operative mechanism only or by the mixed-type proteolysis. The results presented in this work show that PHA hydrolysis by trypsin take place in two stages:

1. At the beginning, the native PHA molecule is modified by the non-co-operative mechanism of hydrolysis (**Figure 2**), which results in the formation of PHA-T (**Figure 3**). The modification of PHA by limited proteolysis only might have some important consequences. The lectins' quaternary structure relates to their activity (*7*), and thus PHA-T is expected to preserve the activity of PHA, which might explain the results observed in the experiments with the feeding of PHA to experimental animals that showed that PHA interacted with and was endocytozed by the epithelial cells of the intestine (*10*).

2. Subsequently, PHA-T is hydrolyzed almost completely by parallel co-operative proteolysis (**Figures 2** and **3**).

Thus, the proteolysis of PHA by trypsin seems to follow the basic mechanisms of the proteolysis of seed storage proteins (*26*). This mechanism differs from the proteolysis of phaseolin by both exogenous (*24*) and endogenous proteases (*25*).

Our results show that trypsin performs a thorough hydrolysis of PHA. However, this hydrolysis is prevented by the calcium ions (**Figures 1–3**). In the presence of $CaCl_2$ 90% of PHA remains unhydrolyzed and only two minor fragments are formed. The native PHA subunits remain almost intact during the hydrolysis, and the native tetramer does not change its M_r either. These results looked somewhat puzzling because it is known that the trypsin reaction buffer is required to contain 20 mM $CaCl_2$ for maximal activity and stability of the protease (*33*). Ca^{2+} binds to a high-affinity calcium-binding site, and in its absence trypsin autodegradation quickly occurs. Because the PHA is a metalloprotein that contains bound Ca^{2+} in its structure, it might be that in the absence of $CaCl_2$ from the buffer PHA loses some of the bound Ca^{2+}, and this results in conformational changes that render PHA susceptible to trypsin proteolysis.

The PHA-L structure was determined to a 2.8 Å resolution by Hamelryck et al. (*20*). PHA is a member of a large family of the legume lectins that have homologous secondary and tertiary structure of the subunits (*7*). All legume lectins are metalloproteins. They possess two bound metal ions per monomer. One ion is Ca^{2+} and another one is a transition metal ion (mainly Mn^{2+}). These metals are essential for correct folding of the carbohydrate-binding site. The structure of the metal-binding site in the structures of legume lectins is highly conserved. The Ca^{2+} ion in PHA is coordinated by Leu-126, Asp-124, Asn-128, and Asp-132 (*20*). PHA is composed from two polypeptides, PHA-E and PHA-L, that have 254 and 252 amino acid residues, respectively (*34*). Taking into account the sizes of the fragments of the first group (17–16 kDa) formed as the result of the PHA hydrolysis by trypsin (**Figure 3**), it is evident that trypsin cleaves PHA subunits in the middle. On the other hand, the amino acid residues that coordinate calcium ion in the metal-binding site also lie approximately in the middle of the polypeptide (*20*). A Lys residue is present in this region in the sequences of both PHA-E and PHA-L. Probably demetallization of PHA makes this region susceptible to proteolysis.

Although at present no structure of demetallized PHA is solved, it is known that demetallization induces conformational changes in the metal-binding site of lectins (*7*). The three-dimensional structure of demetallized concanavalin A (Con A) showed large structural differences between the native and the metal-free lectin in the metal-binding region. The demetallization invokes a series of conformational changes in the protein backbone initiated mainly by the loss of the calcium ion. No calcium-binding site is present. The metal-free ("unlocked") and metal-bound ("locked") conformations of Con A are in equilibrium, and calcium stabilizes the locked conformation. The binding of calcium during Con A remetallization induces an all-or-nothing conformational process resembling protein folding, however, with a slower time constant (*7*). It has been shown that digestion of native Con A is slow and incomplete (*35*), whereas demetallized Con A becomes susceptible to proteolysis (*36*). This might explain the results of PHA hydrolysis by trypsin. In the absence of Ca^{2+} some parts of the PHA molecule, mainly the loops that form the calcium-binding site, change conformations and perhaps this renders these sites accessible and susceptible to proteolytic attack. However, the specific changes of PHA structure that determine the specific cleavage of PHA by trypsin in the absence of Ca^{2+} remain to be

established. Further studies on the structure of either demetallized PHA or PHA-T are needed to identify the location of the cleavage sites that become available to the action of trypsin after the PHA molecule loses Ca^{2+}.

During the co-operative proteolysis the splitting of one peptide bond induces the unfolding of the polypeptide chain of the protein, resulting in a dramatic increase in the rate of the subsequent hydrolysis (*37*). The parts of the polypeptide chain that are likely to be accessible to proteolytic attack are exposed loops within the domains and/or the linking regions of polypeptide chain between domains. These regions could be present in the native protein or arise from conformational changes generated during unfolding of the protein. It seems that Ca^{2+} prevents the modification of PHA by co-operative proteolysis, which seems likely to occur in the region of the calcium-binding site in the PHA molecule, and this gives PHA resistance to trypsin proteolysis.

Because lectins are found in many plant species and in many different organs of plants, it is believed that they play fundamental biological roles in plants. Lectins are synthesized during seed development together with seed storage proteins, and during germination and seedling growth, they both are broken down to provide amino acids for the growing seedling (*3*). The abundant presence of lectins in storage organs led to the suggestion that plants accumulate a part of their nitrogen reserves in the form of lectins, which are used as passive-defense mechanisms against predators (*8*). The toxicity of lectins to animals is due to the interaction of lectins with the glycoproteins of the epithelial cells along the digestive tract. This interaction is sensitive to the structure of the interacting molecules and, consequently, the lectins must preserve their native structure during the passage through the digestive tract in order to interact with epithelial cells. Experiments on animals fed purified PHA have shown that this lectin is highly resistant to gut proteases and that PHA reaches the intestine, where it binds to brush border cells and consequently is rapidly endocytozed (*10*). The final effect on animals is so severe that they are very reluctant to consume a PHA-containing diet (*8*). The regulatory effect of Ca^{2+} on PHA proteolysis by trypsin might explain these findings. It is known that calcium is absorbed in the small intestine (*38*), so when consumed as part of food it will be present all the way through the digestive tract of animals. Seeds of legumes contain all 15 of the essential minerals (*1*), and the concentration of calcium in seeds of the common bean is high in comparison to other elements (*39*). The high concentration of calcium in bean seeds might prevent PHA hydrolysis in the animal's digestive tract, as indeed it has been found to be the case in the experiments on animals fed bean-containing diets (*10*).

Although the biological roles played by lectins in vivo are not well established, their in vitro applications in medical studies and as useful tools for the investigations of carbohydrates is well documented (*2, 6*). Lectins also have attracted attention for their potential usage for pharmacological purposes. In recent years there has been a resurgence of interest in carbohydrate-based therapeutics that involve, among others, delivering and targeting of drugs to specific sites of action (diseased cells) via carbohydrate−lectin interactions (*40*). In contrast to the mucoadhesive polymers, lectins specifically recognize receptor-like structures of the cell membrane and therefore bind directly to the epithelial cells. Furthermore, receptor-mediated bioadhesion, as in the case of lectins, is not only restricted to mere binding but may subsequently trigger the active transport of large molecules by vesicular transport processes. Therefore, lectin-mediated bioadhesion bears the potential for the controlled delivery of diverse biopharmaceuticals at relevant biological barriers, such as the epithelia of the intestine (*40*). Biorecognition between lectin-mediated drug delivery systems and glycosylated structures in the intestine can be exploited for improved therapy, and it is expected that lectin−carrier systems might improve the absorption and probably bioavailability of poorly absorbable drugs. However, there are factors that adversely influence the feasibility of this concept such as the toxicity of plant lectins and their intestinal stability (*41*). The protection of lectin against degradation by the proteolytic enzymes of the digestive tract is one topic related to this issue. In lectin-mediated drug delivery system the preservation of the native conformation of the lectin molecule is of primary importance for its interaction with receptors. Because PHA hydrolysis by trypsin, one of the main proteases secreted by the pancreas in the intestine (*33*), is Ca^{2+} dependent, this may be of great importance for these types of drug delivery systems. Perhaps supplementing these types of drugs with calcium ions could prevent unwanted lectin proteolysis.

Another issue connected to the resistance of lectins to proteolysis is the quality of protein source for food. Digestibility of proteins, the content of indispensable amino acids, and bioavailability of amino acids are basic parameters in determining the quality of a protein source for food. Digestibility of protein in traditional diets from developing countries is considerably lower compared to that of developed countries—54–78 versus 88–94% (*14*). For this effect on the diets of developing countries, which are based on less refined cereals and legumes as major sources of protein, are responsible the presence of less digestible protein fractions, high levels of insoluble fiber, and antinutritional factors. Food and feed products may contain a number of antinutritional factors that may adversely affect protein digestibility and consequently amino acid availability. These antinutritional factors may occur naturally, such as hemagglutinins in legumes, or may be formed during heat/alkaline processing of protein products (*14*). The presence of high levels of antinutritional factors from soybeans, kidney beans, or other legumes can cause up to 50% reductions in protein digestibility in rats and pigs. Of course, the proteins used as supplements in foodstuffs for humans are heat processed. However, not all naturally occurring antinutritional factors are inactivated by heat. For example, PHA can resist mild cooking, preserving its toxic properties, and can cause maladsorption and irritations in the digestive tract (*13*). Perhaps the fact that PHA hydrolysis by trypsin is regulated by Ca^{2+} might help in modulating nutritional diets.

Because PHA is toxic to monogastric animals and because it is present in common bean in substantial amounts, it was attempted to develop a breeding program to remove PHA from bean seeds (*22*). However, PHA, alongside phaseolin, is also catabolized during common bean germination and seedling growth. In seeds germinated for 7 days a decrease of the PHA content by 85% has been observed, and it has been shown by SDS-PAGE that degradation of both PHA subunits occurs during germination (*42*). It also has been shown that an endogenous cysteine proteinase hydrolyzed PHA in vitro (*4*). It was showed that during a 2 h hydrolysis the PHA-E subunit is cleaved by this protease into fragments with M_r of 20 and 15 kDa and subsequently into low molecular weight fragments, whereas the PHA-L subunit was found to be resistant to the action of this protease. It should be noted that these experiments were carried out in a buffer that did not contain either $CaCl_2$ or EDTA. Therefore, they might be compared to our results obtained in standard buffer that show that some initial PHA subunit is not cleaved even after 48 h of hydrolysis. Under the action of trypsin no fragments with similar M_r values were

observed, indicating that there are differences between the action of these two proteinases. Perhaps experiments with purified PHA-E and PHA-L subunits could give more information about the particulars of their individual susceptibilities to protelysis.

It is interesting to note that the inhibitory action of EDTA on erythrocyte agglutination by lectins was observed long ago by Tunis (*43*), and this evidently is caused by demetalliszation. In a recent study it has been shown that EDTA facilitates the digestion of metalloproteins that precedes analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (*44*). Because EDTA is a synthetic chemical compound, it is reasonable to wonder whether in vivo demetallization has any physiological significance. However, there exist natural compounds that are known to chelate cations. For example, in plants phytic acid is a good chelator of cations (*45*).

Regulation of cellular processes by limited proteolysis is a recurrent motive involved in every aspect of organism functions. The finding that PHA hydrolysis by trypsin is Ca^{2+}-dependent might indicate a significant physiological role of this type of regulated proteolysis. The processes of storage protein turnover during seed maturation and germination are tightly connected with plant vacuoles. The vacuoles of plant cells are multifunctional organelles that are central to plant development and play an essential role in plant cells in protein dynamics and proteolysis. They contain a high percentage of hydrolytic enzymes and lectins (*46*), and the central vacuole contains also most of the water-soluble Ca^{2+} (*47*). In lytic vacuoles takes place immediate protein degradation, whereas mobilization of storage proteins during seed germination and seedling growth take place in protein storage vacuoles. Transformation of lytic vacuole during early seed development into protein storage vacuole and merging of emptying protein storage vacuoles and their transformation into a few or a single central lytic vacuole during germination are well-known. Stored proteins are protected against degradation during deposition by different mechanisms that must be overcome at the onset of germination (*46*). Whether Ca^{2+} also plays a role in this process remains to be seen.

In summary, results of this work show for the first time that Ca^{2+} gives resistance to the native PHA molecule to trypsin proteolysis. This dependence is probably due to the stabilization of PHA structure by Ca^{2+}. In the absence of Ca^{2+} trypsin performs a deep proteolysis of PHA. To the best of our knowledge there are no reports on the influence of Ca^{2+} on PHA proteolysis by other, including endogenous, proteases. It remains to be established whether this mechanism of lectin proteolysis is unique to PHA proteolysis by trypsin or is also valid for its proteolysis by other proteases. Certainly, more research is needed in the future to understand the relationships between PHA toxicity and the degree of its resistance to (trypsin) proteolysis.

ABBREVIATIONS USED

Con A, concanavalin A; EDTA, ethylenediaminetetraacetic acid; M_r, molecular mass; PHA, phytohemagglutinin; TCA, trichloroacetic acid.

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