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Synthesis and Resistance to in Vitro Proteolysis of Transglutaminase Cross-Linked Phaseolin, the Major Storage Protein from *Phaseolus vulgaris*

LOREDANA MARINIELLO,*,†,‡ CONCETTA VALERIA L. GIOSAFATTO,†,§ PROSPERO DI PIERRO,§ ANGELA SORRENTINO,‡ AND RAFFAELE PORTA§

Department of Food Science and School of Biotechnological Sciences, University of Naples "Federico II", Parco Gussone, 80055 Portici, Naples, Italy

The ability of phaseolin to act as an acyl donor and acceptor substrate of transglutaminase was studied by using an enzyme isolated from *Streptoverticillium mobarense*. Phaseolin, a trimeric storage protein from *Phaseolus vulgaris* L., was shown to possess both glutamine and lysine residues reactive for the enzyme. The extent of transglutaminase-catalyzed cross-linking has been studied in function of both incubation time and enzyme concentration. Native- and SDS-PAGE demonstrated that phaseolin is intra- and intermolecularly cross-linked by transglutaminase and gives rise to different polymers as well as to modified forms of the protein having a similar molecular weight but lower Stokes radius if compared to unmodified phaseolin. Cross-linked phaseolin was found to be more resistant to proteolytic cleavage than the unmodified counterpart, as demonstrated by in vitro trypsin and pepsin digestion experiments. This behavior could suggest novel possible uses of the transglutaminase-modified phaseolin.

KEYWORDS: Enzymatic modification; phaseolin; proteolysis resistance; transglutaminase

INTRODUCTION

Transglutaminase (protein: glutamine γ-glutamyl-transferase, EC 2.3.2.13, TG) catalyzes acyl transfer reactions between γ-carboxyamide groups of glutamine (acyl donors) and the ϵ-amino group of lysines (acyl acceptors) in proteins, leading to inter- and intramolecular cross-linking. The most studied microbial TG was isolated from the culture medium of *Streptoverticillium* sp. S-8112 (*1*), which has been identified as a variant of *Streptoverticillium mobaraense* and also is known as *Streptomyces mobaraensis* (*2*). Previous investigations demonstrated that the enzyme is able to cross-link several proteins of different origins including legume globulins, such as the 11S globulin from soybean (*3*), and milk proteins, such as α-lactalbumin and β-lactoalbumin, as well as other albumins thus changing, in most cases, their functional properties such as solubility, gelation, and emulsion formation and stabilization (*4*). In the present study, we investigated the possibility of using microbial TG as a biotechnological tool to modify phaseolin, the major storage protein in the cotyledons of the bean *Phaseolus vulgaris*. Phaseolin has structural properties very similar to those of the 11S globulins from soybean and other legumes. This globulin belongs to the vicilins, or the 7S class of legume storage proteins, and in the common bean, it represents up to about 50% of the total seed proteins (*5*). Phaseolin is a trimeric high mannose glycosylated protein of about 150 kDa, containing almost identical monomers with a molecular mass ranging from 45 to 51 kDa and isoelectric points from 5.6 to 5.8 (*6*).

Since storage proteins are an important nutritional component of plant seeds, they may represent a substantial source for the human diet. However, heat denaturation of phaseolin is essential to make this protein susceptible to proteolytic cleavage during digestion. In fact, due to its compact structure, unheated native phaseolin is protease resistant, such a property being correlated to a plant defense mechanism against pest insect attacks (*7*). Some reports refer to the detrimental effect of vicilins from different legume seeds as a direct consequence of their low digestibility by insect midgut proteinases together with their capability of binding the chitin containing membrane structures occurring in the midgut of bruchids (*8*, *9*). Therefore, these studies stimulated further investigations to find out natural pesticides from plants (*10*).

In the present paper, we demonstrate the ability of phaseolin to act as both an acyl acceptor and an acyl donor substrate of TG, and we report data on the in vitro increased resistance of the TG-modified form(s) of such vicilin to proteinases.

MATERIALS AND METHODS

Materials. Microbial TG (Activa WM), derived from the culture of *Streptoverticillium* sp., was supplied by Ajinomoto Co. The enzyme was prepared dissolving the commercial preparation (containing 1%

* Corresponding author. Tel.: +39-081-2539470. Fax: +39-081-2539473. E-mail: loredana.mariniello@unina.it.
† These authors contributed equally to this work.
‡ School of Biotechnological Sciences.
§ Department of Food Science.

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TG and 99% maltodextrins) in distilled water. The specific activity of the enzyme was 92 u/g Activa WM. Estimation of enzymatic activity was carried out by a colorimetric hydroxamate test described by Ajinomoto and modified according to Pasternack (*11*).

Phaseolus vulgaris L. beans were purchased from a local supermarket. Chemicals for electrophoresis were from Biorad. Trypsin, pepsin, and all other reagents were purchased from Sigma Chemical Company.

Methods. Preparation of Phaseolin. Phaseolin was isolated by the ascorbate-NaCl procedure described by Sun and Hall (*12*). The isolation procedure was repeated twice to achieve maximum extraction of phaseolin. The precipitate was resuspended in a known volume of 0.5 M NaCl and then lyophilized.

Protein Determination. Protein determination was carried out by the Biorad Protein Assay, using bovine serum albumin as a standard (*13*).

Phaseolin Cross-Linking by TG. A total of 200 μg of phaseolin was incubated at 37 °C for 60 min (except for the time-course studies) with different amounts of TG in the presence of 80 mM Tris-HCl (pH 7.5) buffer (final volume 200 μL). The cross-linking reaction was stopped by adding 28.5 μL of sample buffer (15 mM Tris-HCl, pH 6.8, containing 0.5% (w/v) SDS, 2.5% (v/v) glycerol, 200 mM 2-β-mercaptoethanol, and 0.003% (w/v) bromophenol blue) and heating for 5 min in a boiling water bath to inactivate the enzyme. For native experiments, the sample buffer without denaturing agents was used, and the samples promptly were analyzed after buffer addition. Control samples were prepared by incubating the assay mixture in the absence of TG. The extent of the reaction was determined by following the formation of phaseolin cross-linked products and the disappearance of either the native protein or its monomer by PAGE performed in the absence or presence of SDS.

PAGE. SDS-PAGE analyses were carried out under reducing conditions on slab gels (5% stacking and 12% separating gels) as described by Laemmli (*14*). Electrophoresis was performed at constant current (40 mA for 1 h and 70 mA for another 2 h), and the proteins were stained with Coomassie Brilliant Blue R250. Biorad Precision Protein Standards were used as molecular weight markers.

Native-PAGE was performed at alkaline pH under nondenaturing conditions. Stacking and separating gels were at 4 and 8% acrylamide, respectively. Buffer solutions were 40 mM Tris-HCl pH 8.8 for separating gel and 13 mM Tris-HCl pH 6.8 for stacking gel, whereas the electrode reservoir solution contained 25 mM Tris and 192 mM glycine (pH 8.3). Gels were stained using Coomassie Brilliant Blue R250.

Structure Computer Modeling. The computer modeling of the phaseolin structure was performed with the Swiss-Model server (*15*, *16*). The predicted structure was viewed and displayed by DeepView (Swiss Pdb Viewer).

Phaseolin Digestions. A total of 200 μg of phaseolin was incubated with 36.8 mu of TG for 60 min at 37 °C in the presence of 80 mM Tris-HCl (pH 7.5) buffer (final volume 100 μL). Control samples were prepared by incubating the assay mixture in the absence of TG. At the end of incubation, the samples were subjected to trypsin and pepsin treatments. The same buffer system (80 mM Tris-HCl, pH 7.5) utilized for the TG reaction was used for trypsin digestion, while for pepsin digestion, samples were adjusted to pH 2 by adding HCl to a final concentration of 60 mM. All the proteolytic reactions were performed at 37 °C with a enzyme/phaseolin ratio of 1:100 in an assay volume of 200 μL. At specific times, the digestion reactions were stopped by adding 28.5 μL of sample buffer and by heating the tubes for 5 min before electrophoresis. A total of 20 μL of each sample was finally analyzed by 12% SDS-PAGE, and the gels were Coomassie stained. Densitometry analysis of protein bands was performed by using the software Quantity One (Biorad, version 4.2.1).

RESULTS AND DISCUSSION

Phaseolin is a trimeric glycoprotein, the monomer of which contains 27 glutamine and 25 lysine residues (*17*) available as possible acyl donor and acceptor reactive sites, respectively, for TG.

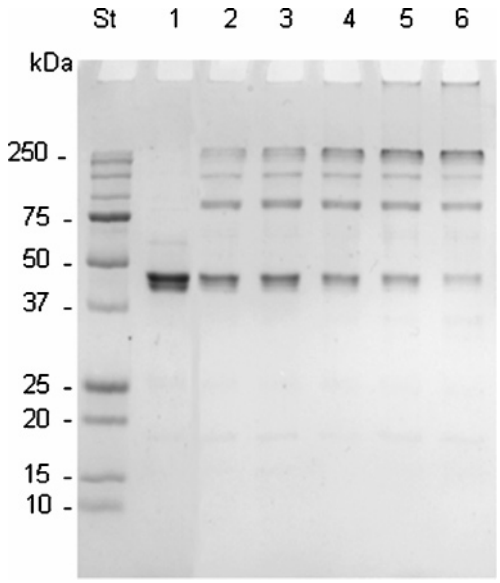

Figure 1. Cross-linking of phaseolin subjected to TG treatment. Phaseolin (200 μg) was incubated at 37 °C for 60 min (final volume, 200 μL) in the presence of increasing amounts (3 mu, lane 2; 4.6 mu, lane 3; 9.2 mu, lane 4; 18.4 mu, lane 5; and 36.8 mu, lane 6) of TG. Control was simultaneously run in the absence of TG (lane 1). The reactions were stopped by sample buffer addition. The resulting samples were boiled for 5 min, and 20 μL of each sample was then analyzed by 12% SDS-PAGE. Proteins were visualized by Coomassie staining. St: molecular weight standard (Biorad, Precision Plus Protein Dual Color Standards).

In the attempt of verifying whether purified phaseolin is able to act as a TG substrate, in vitro cross-linking assays were performed. Hence, phaseolin was treated with increasing amounts of enzyme, and at the end of incubation, an aliquot of the reaction mixture was analyzed by SDS-PAGE followed by Coomassie staining.

The extent of cross-linking was evaluated observing the decrease of the intensity of protein bands at about 50 kDa together with the appearance of new protein bands at higher molecular weights and the accumulation of protein polymer(s) at the top of the gel. As shown in **Figure 1**, a clear attenuation of the phaseolin monomer bands occurs when TG was added to the reaction mixture, with a concurrent formation of new protein bands at higher molecular weights. This result suggests that phaseolin monomers are endowed with both glutamine and lysine TG reactive residues. Moreover, when the highest amount of the enzyme was used, the polymerization of phaseolin seemed to be greatly enhanced since an almost complete disappearance of phaseolin monomers, together with a concomitant formation of polymer(s) unable to penetrate the stacking gel, was observed.

Figure 2 shows the effect of the reaction time on the extent of phaseolin cross-linking. An evident rate of polymerization was observed after only 5 min of incubation with the appearance of a faint protein band at about 100 kDa that indicates the formation of a dimeric cross-linked form of phaseolin. Furthermore, by increasing the incubation time, a progressive increase in the amount of phaseolin polymers with higher molecular weights was observed.

To establish whether TG catalyzes the formation of cross-links inside a single phaseolin monomer (intrasubunit cross-links), and/or between different monomer chains (intersubunit cross-links), and/or between different phaseolin molecules (intermolecular cross-links), native-PAGE experiments were carried out. In **Figure 3**, the native-PAGE (8%) of phaseolin

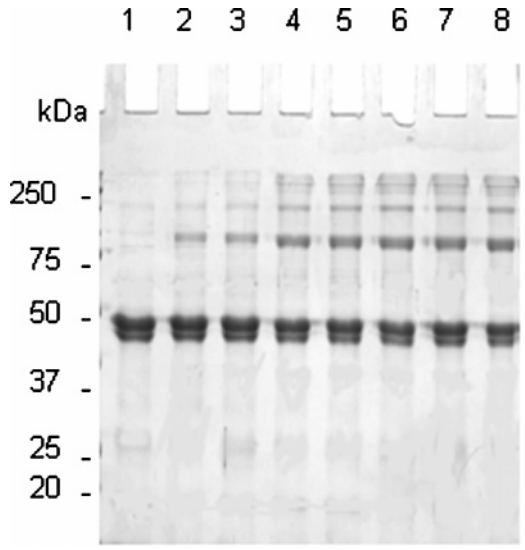

Figure 2. Time-dependent cross-linking of phaseolin. Phaseolin (200 μg) was treated with TG (9.2 mu) at 37 °C (final volume, 200 μL) for different times (5 min, lane 2; 10 min, lane 3; 20 min, lane 4; 40 min, lane 5; 60 min, lane 6; 90 min, lane 7; and 120 min, lane 8). Control (lane 1) was simultaneously run for 120 min in the absence of TG. The reactions were stopped by sample buffer addition. The samples were then boiled for 5 min, and 20 μL of each sample was finally analyzed by 12% SDS-PAGE. Proteins were visualized by Coomassie staining.

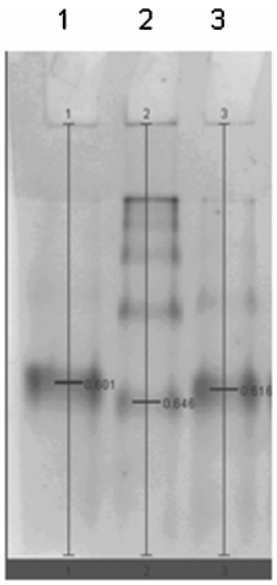

Figure 3. Native-PAGE (8%) of unmodified and TG-modified phaseolin. Phaseolin (200 μg) was incubated at 37 °C for 60 min (final volume, 200 μL) with two different amounts of TG (36.8 mu, lane 2 and 3 mu, lane 3). Control (lane 1) was simultaneously run in the absence of TG. At the end of incubation, the native gel sample buffer was added, and 20 μL of each sample was loaded on the gel. The gel was then Coomassie stained, and the relative front was calculated by using Quantitative One (version 4.2.1) software.

incubated in the absence or presence of different amounts of TG is reported. The mobility of the band corresponding to native phaseolin varied differently depending on the amount of TG used, as demonstrated by the relative front (R_f) calculation. The slight shift of the protein band that migrated faster with respect to unmodified phaseolin is probably due to the changes in its hydrodynamic size resulting from intrasubunit and/or intersubunit cross-links. It is well-known that the R_f value mostly depends on both net charge and Stokes radius of the molecule. Since all the molecules possessed in this case a negative charge (native-PAGE was carried out at pH 8.3), the differences in mobility were mainly due to their Stokes radius value, which should be lower when the TG-catalyzed intra-subunit isopeptide bonds occurred. Conversely, when higher amounts of TG were used, protein bands with a lower mobility than that of unmodified phaseolin were observed on the electrophoretic pattern. These bands possessing an increased Stokes radius value should correspond to different phaseolin molecular forms by containing TG-catalyzed intersubunit and intermolecular cross-links. Moreover, we verified by computer modeling of the phaseolin (PDB: 6n9l), using Swiss Pdb Viewer (*15*, *16*), that several glutamine and lysine residues are externally exposed being available to produce, in the presence of TG, both intersubunit and intermolecular cross-links. For example, lysine in position 68 is close to glutamine 34 and glutamine 65, as well as lysine 51 being near to glutamine 53.

It is well-known that several proteases have no effect in digesting native phaseolin because of the compact structure of such *P. vulgaris* storage protein. Since different investigations have demonstrated that TG-mediated structural modifications of various peptides and proteins change their biological properties (*18*−*23*), we were stimulated to test whether TG treated phaseolin was a better or worse substrate for both trypsin and pepsin. The different susceptibility of phaseolin to various proteases has been widely reported in the literature (*24*−*26*). Pepsin and trypsin have been described to be partially and differently effective to cleave phaseolin, probably because of the compact structure of this protein. In fact, preliminary moist heat treatment of phaseolin, disrupting tertiary and quaternary structures of the trimer, significantly facilitates its proteolysis. Thus, proteolytic assays were carried out by incubating TG-modified phaseolin at different times in the presence of either trypsin or pepsin with a protease/phaseolin ratio of 1:100. The samples were then analyzed by SDS-PAGE, and the TG-modified phaseolin resistance to the proteolytic cleavage was evaluated by comparing the intensity of the protein bands to the one of the control samples containing unmodified phaseolin. The results shown in **Figure 4** demonstrate that unmodified phaseolin was differently susceptible to trypsin (panel **A**) and pepsin (panel **B**) digestions. In fact, whereas pepsin treatment was able to exert a significant proteolysis only after 30 min of incubation, the phaseolin integrity was remarkably compromised by trypsin just following 3 min.

As far as the TG-modified forms of phaseolin, the latter seems to be less sensitive to proteolytic attack, indicating that the introduction of isopeptide bonds confers an increased resistance to the protein digestion by both trypsin and pepsin. In particular, densitometry analysis indicates that, regarding trypsin digestion, a significant resistance to proteolysis is still observed after 15 min of incubation. In fact, TG-modified phaseolin forms represent about 48% of the control (lane 2). On the contrary, pepsin treatment does not affect cross-linked phaseolin forms that show a consistent resistance to proteolysis even after 60 min of digestion. Furthermore, it is worth noting that the band corresponding to the phaseolin fragment with a molecular weight of about 22 kDa is present only when unmodified phaseolin was subjected to trypsin digestion (panel **A**, odd numbered lanes). The formation of this fragment could be prevented in TG treated samples because the involvement of specific lysines in isopeptide bonds would make such residues inaccessible for trypsin. The low digestibility of native phaseolin has been correlated to the detrimental effect that this vicinil-like protein exerts (*8*, *9*) on the development of *Callosobruchus maculates*

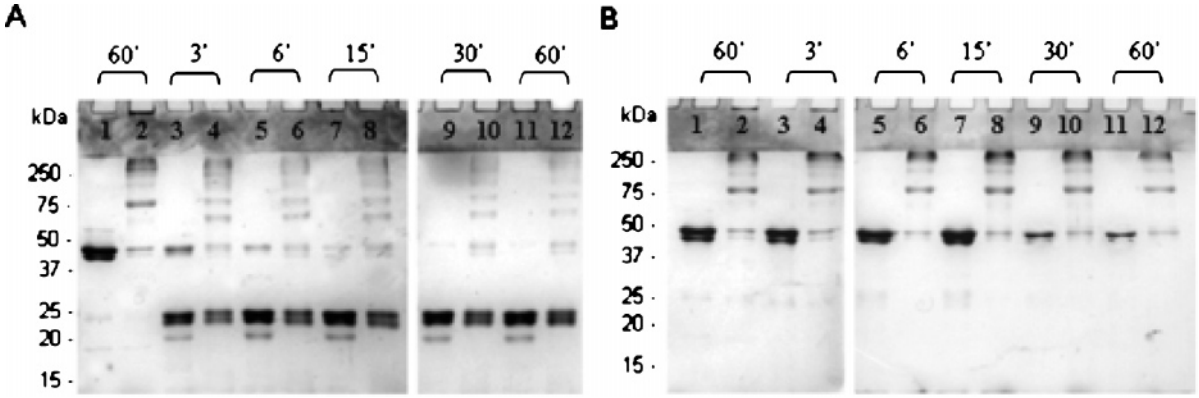

Figure 4. Effect of proteases on TG-modified phaseolin. Time-course of trypsin (**A**) and pepsin (**B**) digestions of unmodified phaseolin (odd numbered lanes) and of phaseolin previously treated with TG (even numbered lanes). Lanes 1 and 2 of both panels represent controls carried out in the absence of proteases.

larvae, a pest insect also known as cowpea weevil. Our findings showing that the TG-catalyzed introduction of cross-links into phaseolin molecules provokes the formation of molecular forms of the protein more resistant to both pepsin and trypsin suggest a possible application of TG-modified phaseolin to protect cultures from insect attacks. Moreover, the capability of TG-modified forms of phaseolin to resist proteolytic degradation makes these molecules possible candidates for being a support for colon specific drug delivery since proteins have been so far considered ineffective for the latter purpose (*27*). TG cross-linked phaseolin, possessing a better resistance to proteinases with respect to the unmodified protein and additional pockets inside its molecule, could be used to entrap specific drugs that should be time-released in the colon. However, further studies devoted to assess swelling properties of the hypothesized colon drug delivery carriers, as well as their susceptibility to microbial degradation, should be performed in the future.

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