PHASEOLIN SUBJECTED TO LIMITED PROTEOLYSIS BY PAPAIN-LIKE PROTEINASE: PRIMARY STRUCTURE AND CRYSTALLIZATION

Vitalie ROTARI, Krisna PRAK[*], Tatiana STEPURINA, Diana MORARI, Natalia LAPTEVA, Bunzo MIKAMI[*], Shigeru UTSUMI[*], Karl WILSON[**], Andrei SHUTOV

Laboratory of Plant Biochemistry
[*]Kyoto University, Japan
[**]State University of New York at Binghamton, USA

Fazeolina, globulina de rezervă 7S din cotiledoanele semințelor de *Phaseolus vulgaris* a fost supusă proteolizei limitate cu proteinaza papainică endogenă PP purificată din cotiledoanele plantulelor de fasole de 6 zile. Digestul a fost separat în două fracții: prima cu masă moleculară mare (fazeolina-PP care reține structura cuaternară), iar a doua cu masă moleculară mică (peptide eliberate). Structura primară completă a fazeolinei-PP a fost descrisă în baza rezultatelor combinate a mass-spectrometriei peptidelor eliberate şi a secvenării amino-terminale a fazeolinei-PP. Au fost obținute câteva tipuri de cristale ale fazeolinei-PP. Cel mai bun cristal cu rezoluția difracției razelor X de 5,5 Å a fost găsit că aparține grupei spațiale $P6_322$, care diferă de grupa spațială a cristalelor fazeolinei intacte. Este discutat rolul posibil al proteolizei limitate a fazeolinei induse de PP la degradarea ei *in vivo*.

Papain-like proteinase PP (emb|CAB17074, trivial name CPPh [1]) and Asn-specific legumain LP (emb|CAB17078, trivial name LLP [2]) are responsible for degradation of the 7S globulin phaseolin in cotyledons of *Phaseolus vulgaris* seedlings [3]. Individually, both enzymes catalyze *in vitro* only limited proteolysis of phaseolin generating stable high-molecular-mass products phaseolin-PP and phaseolin-LP, respectively [1,3]. *In vivo*, the LP-induced limited proteolysis was found to be a prerequisite for further unlimited (cooperative [4]) proteolysis of phaseolin that is catalyzed by PP [3]. It remains unclear whether the PP-induced limited proteolysis of phaseolin-LP formed *in vivo* [2,5] is a second prerequisite for its unlimited degradation. In contrast to *in vitro* experiments [3], up to now no indications on the *in vivo* occurrence of the PP-induced limited proteolysis of phaseolin-LP have been observed [2,5]. Two alternative suggestions were made to explain this phenomenon [5]: *first*, phaseolin-LP itself is susceptible enough to be quickly removed by cooperative proteolysis omitting any additional limited proteolysis, and *second*, the PP-induced limited proteolysis is an obligatory step for phaseolin-LP unlimited degradation; however, the product of the sequential LP- and PP-induced limited proteolysis is highly susceptible, quickly removed by cooperative proteolysis and therefore cannot be detected.

Aiming to choose between these two suggestions, we estimated here whether limited proteolysis of phaseolin by PP is extensive and therefore might be necessary for formation of a protein substrate susceptible for unlimited breakdown.

Materials and Methods

Phaseolin was isolated from *P. vulgaris* seeds according to Schlesier et al. [6]. Papain-like proteinase PP was isolated as described previously [1]. Bz-Phe-Val-Arg-*p*-nitroanilide (Bachem, Germany) was used for determination of PP activity as described earlier [3]. The enzyme activity was expressed in terms of mU (the amount of enzyme releasing 1 nmol of *p*-nitroaniline per min at 30°C). To prepare phaseolin-PP, equal volumes of 2 % (w/v) protein and enzyme (200 mU/ml) solutions were mixed and incubated in previously described conditions [1] for 2 h at 30° C. To stop the reaction, E-64 (L-3-carboxy-2,3-trans-epoxypropionyl-leucylamido[4-guanidino]butane) was added to final concentration 10 μM. The released peptides were separated from phaseolin-PP using ultrafiltration (10 kD cut-off Centricon, Millipore), further purified by gel filtration on a Sephadex G-15 column equilibrated with 0.1 N acetic acid, and desiccated in a rotor evaporator.

The peptide preparation was subjected to mass spectrometry using an Applied Biosystems QStar XL hybrid quadrupole time-of-flight mass spectrometer. The peptides were separated on a Michrom Magic C18AQ 5 μm resin column (0.075 x 150 mm) with a 15 % (v/v) to 65 % gradient of 98 % acetonitrile in 0.1% formic acid + 0.01% trifluoracetiuc acid, and introduced into the spectrometer with a nanospray ionization source.

For crystallization experiments, phaseolin-PP was isolated from the summary digest by gel filtration using HiLoad 16/60, Superdex 200 prep grad (GE Healthcare) column equilibrated with 0.4 M NaCl, 0.1 M imidazol pH 7.0, 0.02 % NaN_3, and concentrated to 10 mg/ml using a VIVASPIN 20 5,000 MWCO PES (VIVASCIENCE, UK). Crystallization screens were performed using crystallization screening kits 82009 Crystallization Basic (Sigma I) and 70437 Crystallization Extension (Sigma II) from SIGMA, USA, and Wizard I and Wizard II from Emerald BioSystems, USA, with 1 µl crystallization reagent and 1 µl protein sample in an Intelli-Plate 96 well crystallization plate (USA) at 293 K. Several potentially successful conditions were identified and optimized by varying the protein concentration, type of buffer, and the use of additives. The crystals were prepared by the hanging drop vapor diffusion method in a 24 well plate and stored at 293 K. Optimal condition for crystal growth was obtained by mixing 3 µl protein sample (26.2 mg/ml) with 3 µl reservoir solution containing 35 % 2-methyl-2,4-pentanediol, 0.2 M $MgCl_2$, 0.1 M Tris-HCl, pH 8.0. Crystal suitable for X-ray analysis grew within 2 weeks and was approximately 0.8 mm in length. A portion of the crystal was placed in a cold nitrogen gas stream at 100 K. X-ray diffraction images were collected using a Jupiter210 area detector (Rigaku, Tokyo, Japan) with synchrotron radiation at a wavelength of 1.0 Å at the BL-38B1 station of SPring-8 (Hyogo, Japan). The images were processed to a resolution of 5.5 Å.

Results

In combination with previously analyzed phaseolin-PP N-terminal sequences [1], the results of mass spectrometry of released peptides led to definition of changes in primary structures of both the α- and β-subunits of phaseolin subjected to PP attack (Fig.1). Multiple cleavages (not all shown in the figure) generate the detachment of C-terminal region that is disordered [7] and thus structurally inessential. Unexpectedly, a wide central region covering the disordered inter-domain linker, helix 4 and strand J' (also a half of the helix 3 that can be predicted for α-phaseolin structure) was found to be detached in phaseolin-PP (Fig.1,2). The molecular mass of phaseolin-PP trimer anticipated from its primary structure shown in Fig. 1 and the masses of the attached glycan moieties [8] (126.6 kD) is in good accordance with the results of direct molecular mass determinations using gradient-pore gel electrophoresis (128 kD [1]).

Alterations of phaseolin primary structure generated by PP cannot overcome its insusceptibility to further cooperative proteolysis [1,3]. However, in accordance with the first suggestion (see above), phaseolin becomes susceptible when summary PP- and LP-induced limited proteolysis happens and the detached central region is extended (see Fig.1).

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α 1 tslr⇓EE//SKFEEI NRVLF↓eeegqqeegqqegvivn↓idseqieels↓khaks↓ssrk↓shs⇓KQDN⇑TIGN 230
α 1 tslr⇓EE//SKFEEI↓nrvlf aeegqqe-----gvivn↓idseqieels↓khaks↓ssrk↓sls⇓KQDN⇑TIGN 224
β 1 tslr⇓EE//SKFEEI NRVLF↓eeegqqe-----gvivn↓idseqikels↓khaks↓ssrk↓sls⇓KQDN⇑TIGN 224
                  h3                      J'       h4                    A'

α 369 ALDGKDVLGLTFSGSGEEVMKLINKQSGSYFVDGHHHQQEQQK↓gshqqeqqkgrk↓gafvy 412 embCAA26718
α 363 ALDGKDVLGLTFSGSGEEVMKLINKQSGSYFVDGHHHQQEQQK↓gshqqeqqkgrk↓gafvy 406 embCAA36853
β 363 ALDGKDVLGLTFSGSGDEVMKLINKQSGSYFVDAHHHQQEQQK↓---------grk↓gafvy 397 pdb|2PHL
          h2        h3          J'
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Fig.1. Primary structures of α- and β-subunits of phaseolin-PP (capitals). Low case letters, sequence regions cleaved off by PP. Limits of peptides identified by mass spectrometry (*lower case italics*) are shown by arrows (↓). The cleavages generated by PP (⇓) and LP (⇑) were identified by N-terminal sequencing of respective polypeptide fragments [1,2]. Sequence numbering corresponds to mature phaseolin subunits. Secondary structures of β-phaseolin (see also Fig.2) are underlined.

Formation of several kinds of phaseolin-PP crystals was observed. The X-ray diffraction of the best crystal (Fig.3) turned out too low to build phaseolin-PP model. However, it was found that the phaseolin-PP crystal belongs to space group different from those of crystals obtained for intact β-phaseolin homo-trimers [7]. A certain alteration of phaseolin 3 D structure can be supposed because of the removal of polypeptide region that forms strand J' and the helix 4 of the N-terminal domain of phaseolin (Fig.1,2).

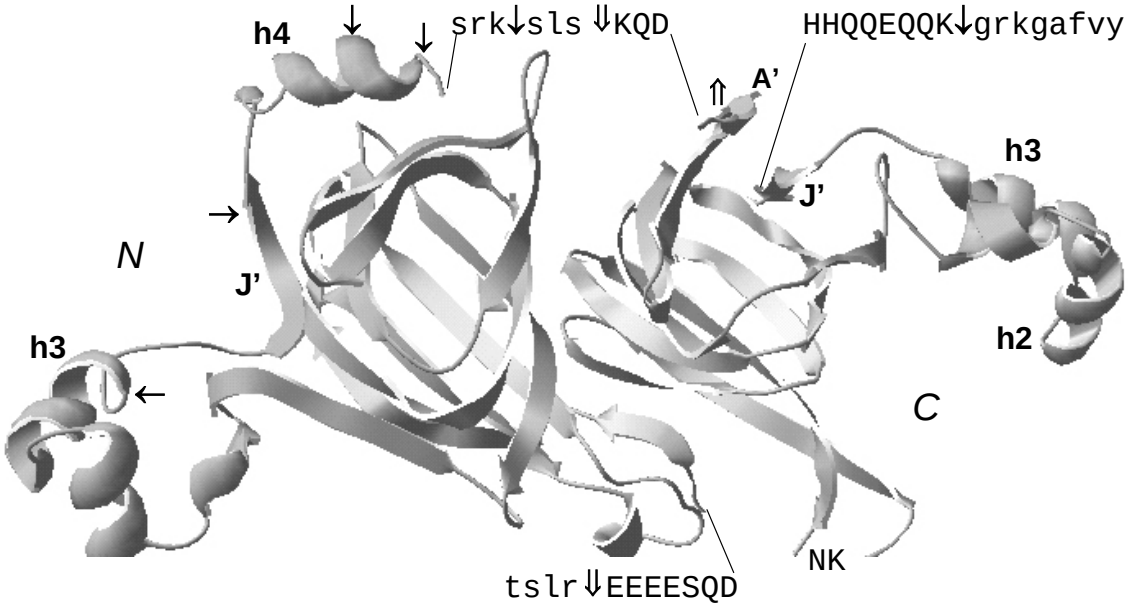

Fig.2. Structure of phaseolin β-subunit pdb|2PHL [7] and polypeptide cleavages found here and previously [1,2]. *N* and *C*, N- and C-terminal domains, respectively. Secondary structures and cleavage points are shown in accordance with Fig.1. The ribbon diagram generated with the Swiss-Pdb Viewer program (http://www.expasy.org/spdv/). Is supplemented with sequences of four disordered regions (N- and C-termini, central region and C-domain EF loop.

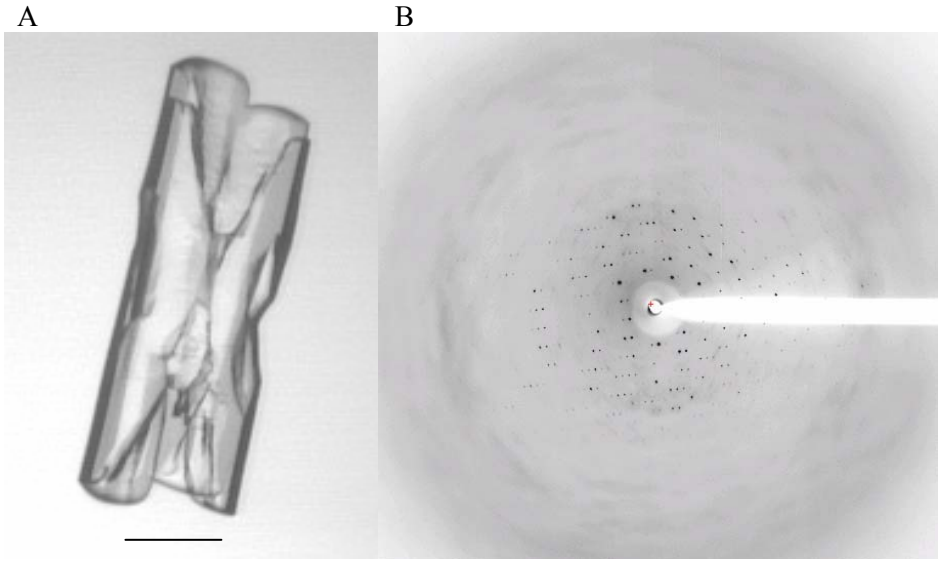

Fig.3. Phaseolin-PP crystal (A) and X-ray diffraction picture (B). The crystal belongs to space group $P6_322$ and contain one phaseolin-PP subunit per asymmetric unit with the unit cell parameters a = b = 101.36 Å, c = 144.15 Å, the angle $\alpha = \beta = 90°$, $\gamma = 120°$. The bar corresponds to 0.2 mm distance.

Discussion

It cannot be excluded that the PP-induced limited proteolysis brings about a certain alteration of phaseolin 3 D structure. However, up to now there are no direct experimental proofs that the PP-induced limited proteolysis of phaseolin (native or phaseolin-LP) happens *in vivo*.

Comparable activities of PP and LP are present in cotyledons of *P. vulgaris* seedlings simultaneously [9]; both phaseolin-PP and phaseolin-LP are stable [1,3]. Nevertheless, only phaseolin-LP (rather than phaseolin-

PP) was found *in vivo* [2,5]. Thus, phaseolin-LP is the major (or even single) natural substrate of PP. When phaseolin-LP is attacked by PP *in vitro*, limited proteolysis occurs quickly enough to be exhausted long before complete unlimited degradation of the substrate [3]. Therefore, it was tempting to suggest that the product of the sequential limited proteolysis (phaseolin-LP→PP) is the only substrate of PP that can be degraded *in vivo* via a cooperative mechanism [5]. Meantime, no direct indications for the *in vivo* formation of phaseolin-LP→PP were found.

The following formal extremes might explain the observed difference between the *in vitro* and *in vivo* processes.

1. In contrast to the *in vitro* experiments, the PP-induced limited proteolysis of the native phaseolin and phaseolin-LP *in vivo* does not happen at all (or occurs slowly); phaseolin-LP is degraded rapidly by PP directly without observing any additional limited proteolysis. Different conformational states of both the native phaseolin and phaseolin-LP *in vivo* and *in vitro* can be speculated to explain their inaccessibility (or low susceptibility) to PP-induced limited proteolysis. For instance, the *in vitro* disordered regions (N- and C-termini and the inter-domain region [7]) *in situ* might be ordered or masked and therefore stable. Remarkably, phaseolin-LP→PP is formed when seedling cotyledons are homogenized in the absence of cysteine proteinase inhibitors [5].

2. Formation of phaseolin-LP→PP is a limiting stage of the unlimited proteolysis *in vivo* as well as *in vitro*. *In vivo* the PP-induced limited proteolysis of phaseolin-LP does occur but essentially slower than that *in vitro* (see the above reasons). Thus, the rate of the unlimited proteolysis is relatively high, and only non-detectable remnants of phaseolin-LP→PP are present in seedling cotyledons.

The suggested susceptibility of phaseolin-LP→PP (rather than phaseolin-LP) to unlimited proteolysis can be tested for experimentally using a third enzyme as a structural probe. Pepsin is an excellent candidate for this purpose because the native phaseolin is almost completely inaccessible to its action (neither unlimited nor practically any limited proteolysis was observed [10].

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