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LIMITED PROTEOLYSIS OF OAT 11S GLOBULIN BY PAPAIN

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Limited proteolysis was found to occur during papain proteolysis of the oat 11S globulin. The initial limited proteolysis is extremely quick and consists of an N-terminal and C-terminal truncation of α-chains and C-terminal truncation of β-chains. Further on, the α-chain remnants are cleaved into similarly sized N-terminal and C-terminal fragments. Similar pattern of the globulin limited proteolysis was observed during germination of the oat seeds. Functional role of limited proteolysis in degradation of seed 11S globulins is discussed.

Keywords: *limited proteolysis, seed storage globulin, protein structure, germination.*

PROTEOLIZA LIMITATĂ A GLOBULINEI 11S DIN OVĂZ DE PAPAINĂ

Proteoliza limitată a fost găsită în timpul proteolizei globulinei 11S de ovăz de papainã. Proteoliza inițială este extrem de rapidă şi constă dintr-o trunchiere N-terminală şi C-terminală a lanțului α şi din trunchierile C-terminale ale lanțului β. În continuare, resturile lanțului α sunt scindate în fragmente N-terminale şi C-terminale de dimensiuni similare. Modul similar de proteoliză limitată a globulinei a fost observat în timpul germinării semințelor de ovăz. Este discutat rolul funcțional al proteolizei limitate în degradarea globulinei 11S din semințe de ovăz.

Cuvinte-cheie: *proteoliză limitată, globulină de rezervă, structura proteinelor, germinare.*

Conserved core structure of legumins, two-domain seed storage 11S globulins, in both the domains consists of a jelly-roll β-barrel of eight antiparallel β-strands *BCDEFGHI* conjoined with a group of α-helices [10]. Three extended hydrophilic disordered regions are specific of legumin sequences, namely a loop between β-strands *E* and *F* and a β-barrel/α-helix junction (both inside the N-terminal domain), and the inter-domain linker. The extended regions are exposed on a surface of legumin molecule and therefore exhibit an enhanced susceptibility to proteolytic attack. It was suggested that genuine legumins acquired these extensions as specific target sites for limited proteolysis during seed germination and *in vitro* [5]. Quick mobilization of a susceptible part of protein reserves prior to their massive degradation might be a simple explanation for the functional role of legumin limited proteolysis.

In this report, limited proteolysis of oat seed 11S globulin (OG) by papain as a model enzyme is studied.

Materials and Methods

The OG was isolated from dry oat seeds (*Avena sativa* L.) as described by Mikola and Jones [3] with minor modifications. The meal was three times washed (1:25, w/v) with 0.05 M Tris-HCl buffer, pH 8.0, and the OG was extracted from the pellet (1:5, w/v) with 1 M NaCl, 0.02% (w/v) NaN_3, 1 mM EDTA, 10 mM 2-merkaptoethanol (ME). Oat seeds were germinated in dark at 25°C for 8 days and the OG modified by *in vivo* limited proteolysis was isolated as described above. Papain from papaya latex was purchased from Sigma Life Science (St. Louis, MO, USA).

For the OG digestion, the reaction mixture containing 5 mg/ml substrate and either 0.1 mg/ml or 0.01 mg/ml papain (enzyme/substrate ratio 1:50 or 1:500, w/w) in Standard Buffer (0.037 M phosphate/citrate buffer, pH 6.2, adjusted with NaCl to the ionic strength 1.0, 0.02% (w/v) NaN_3, 1 mM EDTA, 10 mM ME) was incubated for 11 h at 30° C. To follow the time course of the digestion, aliquots of the incubation mixture were sampled from time to time and the reaction was stopped by addition of trichloroacetic acid (TCA) to a final concentration 5% (w/v). In some cases the reaction was stopped by addition of *trans*-epoxysuccinnyl-L-leucylamido-(4-guanidino) butane (E-64) from Sigma, Life Science, to final concentration 10 μM and the protein samples were purified from low-molecular mass products by gel filtration. For gel filtration, a column (0.9 x 70 cm) of Sephacryl S-300 High Resolution (Pharmacia Biotech, Uppsala, Sweden) equilibrated with Standard Buffer was used.

SDS-PAGE was carried out in 15% (w/v) gels by the method of Laemmli [2]. Prior to electrophoresis, the residual TCA in precipitated protein samples was removed by washing with acetone, the pellets dried, and dissolved in sample buffer without or with ME under standard conditions. Protein samples purified by gel

filtration were also analyzed by SDS-PAGE. Molecular weight markers from Fermentas Life Sciences (Vilnius, Lithuania) were used. The electropherograms were scanned (ImageScanner III, GE Healthcare) and analyzed quantitatively using Phoretix 1D Gel Analysis v.5.10 software.

Swiss-Model program [1] was used for modeling of the OG 3D structure. The accessibility of a given amino acid residue (X) in the OG structural model and in crystal structures of soybean 11S globulins, defined as the percentage of its surface accessibility to the solvent in the extended pentapeptide GGXGG, was calculated using the program Deep View/Swiss-Pdb Viewer. This program also was used for construction of structural alignments and contouring of ribbon diagrams.

Results and Discussion

Modeling of OG Structure. The OG is a hetero-hexamer formed due a chance combination of a large number of different subunits [4]. Like in other 11S globulins, the subunit polypeptides of the OG precursor (pro-OG) undergo posttranslational site-specific cleavage that generates α- and β-chains (N-terminal and C-terminal domains, respectively) connected with a disulfide bond. Six full-length subunit sequences of OG are available in databases. The sequence gb|AAA32720 estimated as most characteristic of OG subunits was used as a target for modeling of its 3D structure.

All secondary structure elements characteristic of *Prunus dulcis* legumin pdb|3FZ3 chain C used as a best template are present in the model (Fig.1). Additionally, among 11 identical residues involved in formation and stabilization of similarly organized trimers of 11S and 7S seed storage globulins [10] only a single one tolerates conserved substitution in the OG sequences. As expected, amino acid residues of an enhanced accessibility to the solvent were found located in the OG structural model within the two extended regions of the N-terminal domain (the *E'F'* loop and the β-barrel/α-helix junction). In addition, five residues are specifically accessible in the model (Fig.1).

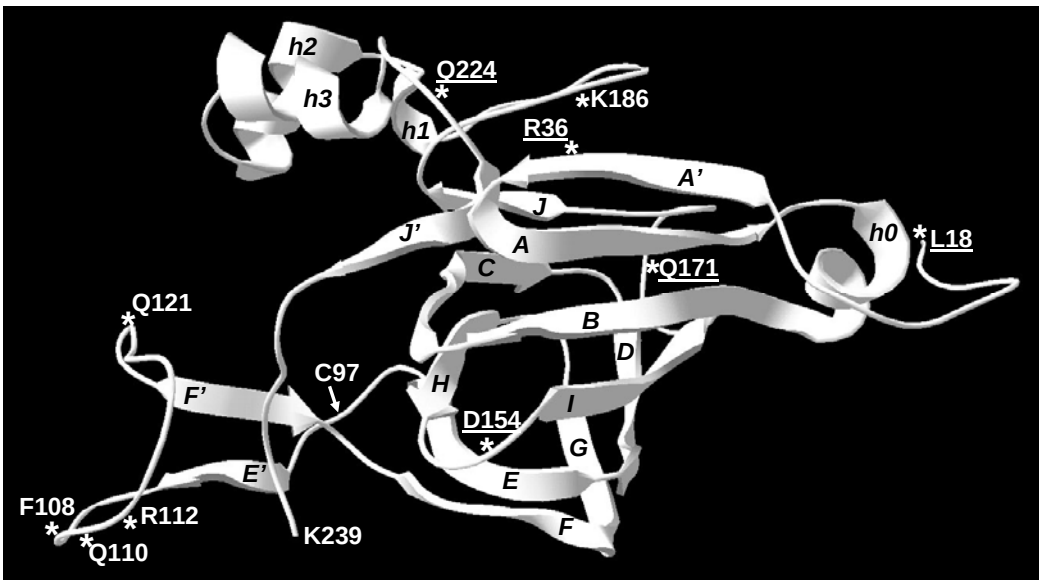

Fig.1. Ribbon diagram of the OG gb|AAA32720 structural model. The diagram is restricted to the N-terminal domain (α-chain) sequence lacking the inter-domain linker. An arrow indicates Cys residue involved in disulfide bridging of α- and β-chains. Asterisks denote residues of enhanced (≥60%) accessibilities to the solvent. Accessible residues specific of the OG structure are underlined.

General description of the OG limited proteolysis. In non-reducing conditions, SDS-PAGE of the intact OG (Fig.2, lane 1) revealed a heterogeneous band αβ of disulfide bonded α- and β-chains. The upper minor bands probably correspond to the OG disulfide polymers. The α- and β-chains are separated from each other in reducing conditions (lane 4). The major part of intact α-chains are sized similarly (31.7-33.6 kD, lane 4). They probably correspond to uniformly sized (32.9-33.9 kD) α-chains from four OG subunits (CAA35631, CAA52764, CAA54153 and AAA32720). Minor α-chain bands of lower molecular masses (25.2-28.0 kD, lane 4) probably correspond to small OG subunits (like CAA54152 that lacks the α-chain C-terminal extension).

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The presence of a cultivar-specific subunit CAA52763 of large (36.4 kD) α-chain [9] was not detected. Sequences of all six β-chains of the available intact subunit sequences are sized almost identically (22.7-22.9 kD), although the apparent molecular masses of β-chains were found to be slightly lower (21.6-22.7 kD, lane 4).

The OG limited proteolysis starts from formation of bands α' (25.1-25.5 kD) and β' (19.8 kD) of truncated α- and β-chains, respectively (Fig.2, lanes 5,6). During formation of OG-P, the final product of limited proteolysis, the upper part of the band α' disappears and the bands of fragments Fn (13.8 kD) and Fc (11.2 kD) are formed. The band Fn detectable in both reducing and non-reducing conditions (lanes 3 and 7, respectively) was found in SDS-PAGE patterns of the OG-P preparations purified from low-molecular-mass products by the gel filtration. Hence, the respective polypeptide is retained in the OG-P structure due to non-covalent interactions. Conversely, the band Fc is detectable only in reducing conditions (lane 7). Thus, it is covalently bound with the chain β' and form the band Fcβ' in non-reducing conditions (lane 3). Finally, the upper band denoted α'β' (lane 3) can be identified as covalently bound pair of non-cleaved lower part of α' chain and β' chain.

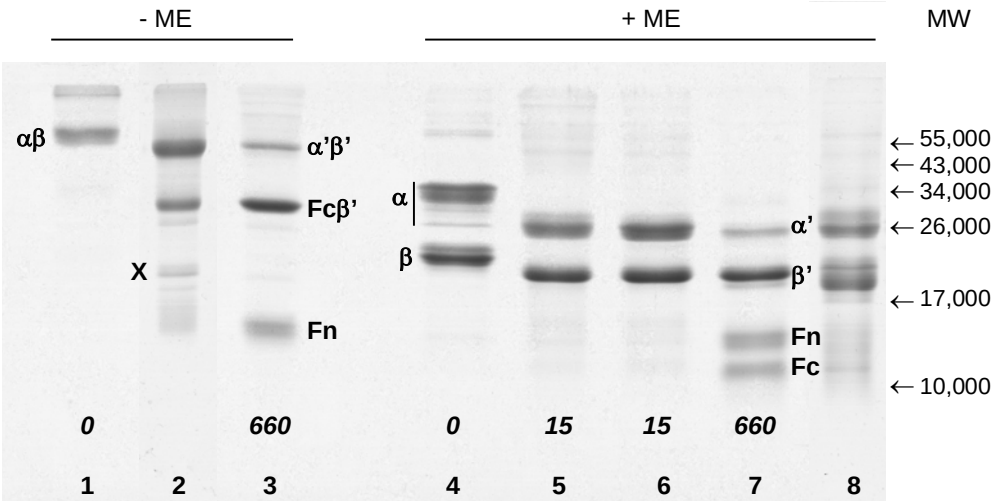

Fig.2. SDS-PAGE in non-reducing and reducing conditions of intact OG and OG remnants after proteolysis *in vivo* (eight days of germination, lanes 2 and 8) and by papain. Time of the reaction *in vitro* is shown in italics (min). Enzyme/substrate ratio (w/w): 1:50 (lanes 3,6,7) and 1:500 (lane 5). Numbers at the right side correspond to molecular weights (MW) of the markers. See Fig.3 and text for nomenclature of the initial polypeptides and their fragments. The SDS-PAGE patterns before and after gel filtration are identical.

The OG-P-like protein remnants are formed during germination of oat seeds (Fig.2). Formation of α'β'-like and Fcβ'-like bands is clear (lane 2). The Fn-like and Fc-like fragments are also detectable (the latter only in reducing conditions, lane 8). Certain differences in degradation patterns *in vivo* and *in vitro* should be noted. Cleavage of α'-like chain into Fc-like and Fn-like fragments is slow and the Fn-like fragment is heterogeneous. Finally, the origin of a minor band X (lane 2) that looks as a negligible contamination in the OG-P SDS-PAGE pattern (lane 3) remains unclear.

Modeling of the OG-P primary structure. The OG-P primary structure can be deduced using the above experimental data and OG structural model, and known degradation patterns of 11S globulins [5,8,11]. Following logical assumptions are basic.

First, Cys residue involved in formation of the α/β inter-chain disulfide bond belongs to the extreme N-terminal part of β-chains. Hence, the retention of this bond in the OG-P structure (Fig.2, lane 3, band Fcβ') indicates that the formation of the band β' (lanes 5,6) is due to C-terminal truncation of the intact β-chain. *Second*, the extended C-terminal sequence of α-chains (the disordered hypervariable region HVR) is known as most susceptible to proteolytic attack in 11S globulins. Hence, the formation of the band α' (lanes 5,6) should be due to C-terminal truncation of the α-chains. *Third*, covalently and non-covalently bound fragments Fc and Fn of the α' chain (N-terminal domain) belong to its N-terminal half (containing Cys97 residue involved in α/β chain bridging, Fig.1) and C-terminal half, respectively. The *E'F'* loop as a connection between the domain halves is largely accessible to the solvent in the OG model (Fig.1) usually is susceptible to limited proteolysis in 11S globulins and therefore should be a target for papain attack that generated the fragments Fc and Fn.

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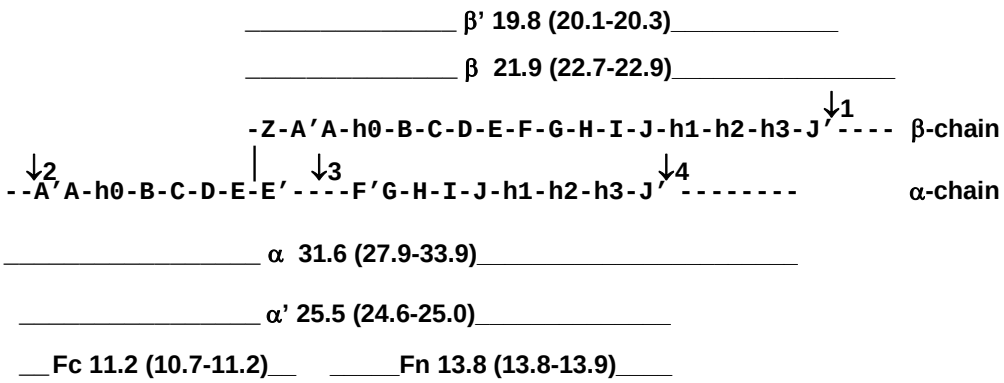

Fig.3. Modeling of the OG-P primary structure. Deduced cleavage points are shown by arrows (see also Fig.4). Numbers correspond to molecular mass values (kD) from SDS-PAGE data. Numbers in brackets correspond to molecular masses calculated from predicted fragment sequences of the five OG subunit sequences available from databases. A vertical line indicates position of the disulfide bond between α and β-chains.

Further specification of the OG-P primary structure shown in Fig.3 is supported by following information. All the four cleavage points tentatively predicted (Fig.4) are located inside extended (points 3,4) or short (points 1,2) regions that are disordered in all the hitherto known legumin structures (for instance, 1FXZ, 2EVX and 3KGL in addition to 3FZ3); these regions are potentially susceptible to limited proteolysis [5,8]. Two of the disordered regions (the α-chain N-terminus and especially the *E'F'* loop) are bordered with residues of an enhanced accessibility to the solvent (Fig.1), which has been shown to coincide with an enhanced susceptibility to proteolytic attack [13]. When presumed polypeptides of the OG-P derived from all the five available OG subunits are bordered with residues that correspond to primary specificity of papain (Fig.4), their molecular masses are close to the apparent molecular mass values of the fragments β', α', Fc and Fn (Fig.3). Non-cleaved part of the chain α' might correspond to the subunits like CAA54153 or CAA54152 that are poor in residues potentially susceptible to papain attack inside the *EF* loop.

```
                          A'       A        h0       B        C        D        E
Gly  9 qNECQIQKLNALKPDNRIESEGGLIETWNPNNKPFQCAGVALSRCTLNRNALRRPSYTNGPQEIYIQQGKGIFGM 83
Oat 18 LRGCKFDRLQAFEPLRQVRSQAGITEYFDEQNEQFRCAGVSVIRRVIEPQGLLLPQYHNAPGLVYILQGRGFTGL 92
       ↑  * ...* *. * .. *..*. * . * *.****.. * .. .* * * *.* .** **.*. *.

                          F      G        H        I        J
Gly 84 IYPGCPST:RHQKIYNFREGDLIAVPTGVAWWMYNNEDTPVVAVSIIDTNSLENQLDQMPRRFYLAGNQEQEFLK 175
Oat 93 TFPGCPAT:EHQRVHHIKQGDVVALPAGIVHWCYNDGDAPIVAVYVFDVNNNANQLEPRQKEFLLAGNNKREQ-- 189
        .****.*↑ **.. ....**..*.*.*. * **. *.*.*** ..* *. ***. . * **** .*

                 h1       h2       h3       J'                 Z        A'
Gly 176 YQQ:GGSILSGFTLEFLEHAFSVDKQIAKNLQGE:KGAIVTVKGGLSVIKP:ICTMRLRHNIGQTSSPDIYNPQA 319
Oat 190 -QF-GQNIFSGFSVQLLSEALGISQQAAQKIQSQ:RGEIIRVSQGLQFLKP:FCSLEARQNIENPKRADTYNPRA 321
         * * .*.***....* *... * * ..*.. .* *. * ** . ↑  .*.. *.**    .* ***.*

            A        h0       B        C        D        E        F       G
Gly 320 GSVTTATSLDFPALSWLRLSAEFGSLRKNAMFVPHYNLNANSIIYALNGRALIQVVNCNGERVFDGELQEGRVLI 394
Oat 322 GRITHLNSKNFPTLNLVQMSATRVNLYQNAILSPYWNINAHSVMHMIQGRARVQVVNNHGQTVFNDILRRGQLLI 396
        * .*   * .**.*. ...**  .* **.. * *.**.*... . *** .**** .*. **. *. *..**

                 H        I        J       h1      h2       h3       J'
Gly 395 VPQNFVVAARSQSDNFEYVSFKTNDTPMIGTLAGANSLLNALPEEVIQHTFNLKSQQARQIKNNNPFKFLVPPQE 469
Oat 397 IPQHYVVLKKAERGEGCQYISFKTTPNSMVSYIAGKTSILRALPVDVLANAYRISRQESQNLKNNRGEEFGAFTpk 471
        .**..** ... .  .*.****  .*.. .**  *.* *** .*. ... . *... .***   *    ↑
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Fig.4. Structural alignment of domain sequences of soybean glycinin pdb|1FXZ (chain C) and OG subunit AAA32720 (structural model). Sequence numbering corresponds to mature proteins. Low case letters indicate terminal residues lacking in the crystal structure and model. Colons designate positions of sequence segments disordered in 1FXZ structure and respective residues in the OG sequence. Arrows indicate deduced cleavage points (Fig.3). Residues of an enhanced accessibility to the solvent (≥50%) compared in Fig.6 are in bold.

Functional role of limited proteolysis in 11S globulin mobilization. Limited proteolysis of 11S globulins is restricted to rapid mobilization of only a part of the globulin molecule. The massive degradation of 11S globulins occurs by a co-operative mechanism that is slower but consists of a deep on-by-one cleavage of the substrate molecules [6,12]. Depending on conformational state of 11S globulins, their native molecules might be either accessible or inaccessible to the co-operative proteolysis. In the first case, limited and co-operative processes occur independently from each other, in parallel, from the very beginning of reaction [14,15]. In the second case, the onset of the co-operative proteolysis is delayed and the process starts only after a certain alteration of the 11S globulin structure generated by preceding limited proteolysis [7].

Limited proteolysis of the oat 11S globulin is restricted to removal of terminal disordered regions from α- and β-chains and to cleavages inside the disordered region between the β-strands *E* and *F*. It seems hardly probable that respective alterations of the oat 11S globulin primary structure are coincided with any essential alterations of its conformation. Therefore, it can be hypothesized *a priori* that the oat 11S globulin mobilized during seed germination should primordially be susceptible to the co-operative proteolysis irrespective of limited proteolysis. Indeed, the hypothesis is supported experimentally in this report.

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