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BASIC 7S GLOBULIN FROM SOYBEAN SEEDS AS A MEMBER OF A SUPERFAMILY OF PLANT XYLANASE INHIBITORS AND ASPARTIC PROTEINASES

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Globulina de bază 7S prezentă în semințele uscate de soia (Bg) şi eliberată din semințe prin imersiunea în apă fierbinte (BgH) a fost izolată şi comparată folosind cromatografia pe SP Sefaroza şi SDS-PAGE. Au fost identificate cel puțin patru tipuri de subunități Bg care se deosebesc dupa mărime. A fost demonstrat că BgH practic nu se deosebeşte de Bg nici prin comportarea cromotografică, nici prin componență şi raportul subunităților. A fost urmărită calea evolutivă a Bg ca membru al superfamiliei de inhibitori xilanaseci ai plantelor şi ai proteinazelor aspartice.

Basic 7S globulin (Bg) immunologically and structurally distinct from 7S and 11S seed storage globulins has been detected in soybean *Glycine max* seeds long time ago [1-3]. Recently, Bg was described as a dimer of subunits [4]. Each Bg subunit synthesized as a pro-polypeptide undergoes proteolytic processing into disulfide linked (27 kD) and (16 kD) chains, and glycosylation. Small amount of Bg precursor was found non-cleaved in mature Bg preparations [5]. Bg-like proteins might be widely distributed in legume species [6-8].

Bg was found in the plasma membranes and the middle lamella of the cell wall on the side facing to the intercellular space [9]. When immersed in hot water, mature soybean seeds release large amounts of Bg [7]. Bg forms complexes with insulin and insulin-like growth factors [10-12] and binds leginsulin, a hormone-like 4-kD peptide isolated from the radicles of germinating soybean seeds and characterized [4,13,14]. Bg has protein kinase activity [15] that is stimulated by leginsulin [13], suggesting that Bg is a part of the plant cellular signal transduction system [14].

At least four copies of Bg genes were detected in soybean genome [16]. Two cDNA sequences (Bg1, BAA03681 and Bg2, BAB91077) close to each other are available, and two kinds of Bg polypeptides are detectable using SDS-PAGE [3]. Non-dissociated Bg was shown heterogeneous chromatographically [5]. The N-terminal sequence of Bg released in hot water was found identical to Bg1 [7].

Bg accounts for about 3% of total protein in mature soybean seed [6]. It contains high amounts of Met and Cys, which are deficient in soybean storage globulins.

In this paper we analyzed polypeptide composition of Bg isolated from dry seeds and released from seeds in hot water. Evolutionary pathway of Bg as a member of a superfamily of functionally distinct proteins was followed.

Materials and Methods

Soybean seeds (*Glycine max* [L.] Merr., cv. Licurici, Moldova) were used. Bg preparations from cotyledons of dry seeds were isolated as described earlier (Shutov et al. 2006) with minor modifications. Defatted soybean meal (10 g) was washed tree times with water (1:70, w/v) and the pellet was extracted with 0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 0.02% NaN_3 (Buffer A). The extract was filtered through a layer of DEAE Sepharose CL-6 B equilibrated with Buffer A for almost complete removal of contaminating proteins. The filtrate was adjusted to pH 4.5 with 10% acetic acid and loaded onto a column (1.6 x 2.5 cm) of SP Sepharose equilibrated with 0.1 M acetic buffer, pH 4.5, 0.1 M NaCl, 0.02% NaN_3 (Buffer B). After removal of non-adsorbed fraction, Bg was eluted with a linear gradient of NaCl concentration in Buffer B (from 0.1 M to 0.5 M, total elution volume 150 ml). In separate experiments, 10 g of mature seeds were soaked in 100 ml of hot (60°C) water for 1.5 h. The released Bg was precipitated via cooling of surrounding water, the precipitate was dissolved in Buffer 2 and subjected to SP Sepharose chromatography as described above. Chromatographic materials were obtained from Pharmacia. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was

performed according to Laemmli [17] in 15% gels in reducing conditions. Polypeptides separated by SDS-PAGE were blotted onto PVDF membranes (Millipore) and subjected to N-terminal sequencing.

BLAST searches were conducted for amino acid sequences related to Bg. The CLUSTAL program was used for alignment of amino acid sequences. The evolutionary tree was constructed using TREECON program package [18].

Results and Discussion

Polypeptide composition of Bg isolated from dry seeds (BgD). A high degree of BgD heterogeneity was observed when SP Sepharose chromatography fractions were analyzed by SDS-PAGE (Fig.1). At least three kinds of α-chains (~27 kD) different in their mobility (bands α1-α3) were found partially separated along the elution profile composed of large peak 1 (~90% of total BgD) and small peak 2. Minor bands of α-chains (α' and α'') also were found. Heterogeneity of the β-chains (~16 kD) is less obvious. A γ-polypeptide that presumably corresponds to non-cleaved α/β precursor is accumulated specifically in the right slope of the peak I. N-terminal sequences of α1 (VPIPQH), β (STIVGS) and γ (VPIPQH) polypeptides from the left slope of the peak 1 (Fig.1, lane 2) were found identical to Bg2 (BAB91077) sequence. The α2 polypeptide might belong to unknown kind of BgD, and the lower band α3 (peak 2) probably corresponds to the α-chain of Bg1 (BAA03681), which is 0.8 kD smaller than the α-chain of Bg2.

Native BgD exists as a dimer [4] probably formed by chance combination of four kinds of differently charged major subunits, namely α1β, α2β, γ and α3β (enumerated in order of their elution, Fig. 1). Whereas homo-dimers $(\alpha1\beta)_2$ and $(\alpha3\beta)_2$ are preferentially present in the extreme parts of the elution profile (peak 1, left slope, and peak 2, respectively), hetero-dimers (α1β)(α2β) and (α1β)γ/(α2β)γ are eluted in central part of peak 1 and its right slope, respectively.

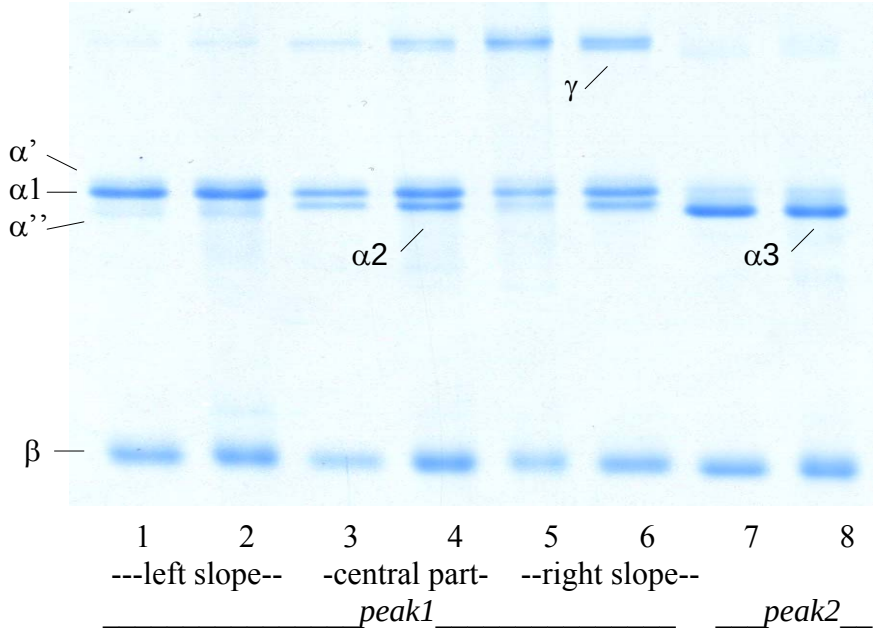

Fig.1. Polypeptide composition of Bg released in hot water (BgH) and isolated from dry seeds (BgD). SDS-PAGE of fractions along the elution profile during SP-Sepharose chromatography are shown. To optimize separation of Bg α-chains, we used gels of bisacrylamide/acrylamide ratio 1:100.

Polypeptide composition of Bg released in hot water (BgH) as well as the chromatography elution profile are closely similar to those observed for Bg isolated from dry seeds; The only difference is smaller proportion of γ polypeptide in BgH preparation (Fig.1). These results contradict with those of Hirano *et al*. [7] who found specific release of only Bg1 in hot water. The source of this contradiction remains unclear.

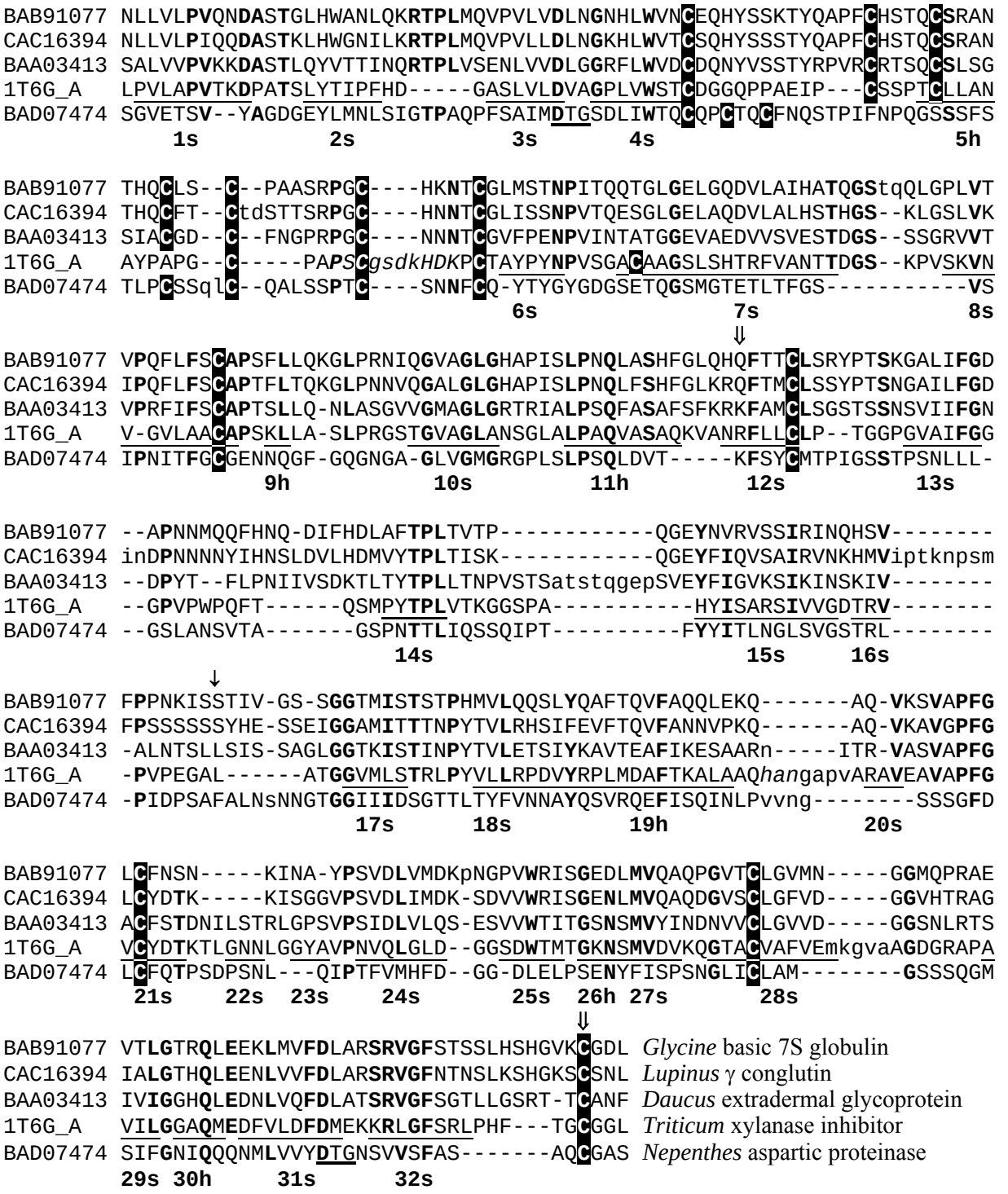

Fig.2. Basic 7S globulin (Bg) as a member of a superfamily of plant xylanase inhibitors and aspartic proteinases. Selected representatives of the superfamily are shown from multiply aligned sequences analyzed by the evolutionary tree (Fig.3). Residues identical at least in four sequences are printed in bold. All Cys residues are black shaded. Low case letters, inserts peculiar in individual sequences and specific for clusters, including that of aspartic proteinases from *Nepenthes* and related enzymes from *Arabidopsis* (AAP21262) and *Oryza* (CAD40873). Secondary structures of *Triticum* xylanase inhibitor (1T6G.pdb) are underlined and numbered (s, β-strands, h, α-helices). Italics, disordered regions, see Fig.4. An arrow indicates processing site of the Bg precursor. Symbol ⇓ indicate position of Cys residues involved in formation of the inter-chain disulfide bond in Bg and conglutin γ. DTG residues underlined in sequence of *Nepenthes* aspartic proteinase correspond to active site triads.

Evolutionary pathway of Bg. BLAST searches revealed three groups of proteins related to Bg (Fig.2): conglutin γ from *Lupinus*, xylanase inhibitors from dicots and monocots, and pepsin A-like plant aspartic proteinases. Because of ancient origin and widespread distribution of commonly rooted pepsin A-like enzymes in different kingdoms it seems obvious that an aspartic proteinase rather than any other protein can be considered to be a suitable outgroup for construction of a rooted evolutionary tree. An aspartic proteinase from *Nepenthes distillatoria* was selected as the best characterized enzyme [19,20]. We restricted analyzed sequence set only to well-characterized proteins. Remarkably, these proteins are similarly sized and reveal homology along almost total-length sequences. Therefore, extensive sequence information can be obtained from ~400 alignment positions (Fig.2). Highly conserved positions emphasized in Fig.2 (especially, Cys residues) are helpful to align distantly related groups of aspartic proteinases and other proteins. The resulting evolutionary tree (Fig.3) is stable. The tree topology is the same when different alignment regions are examined separately or when the analysis is restricted to the sum of conserved regions. Below we provide summary information on proteins related to Bg.

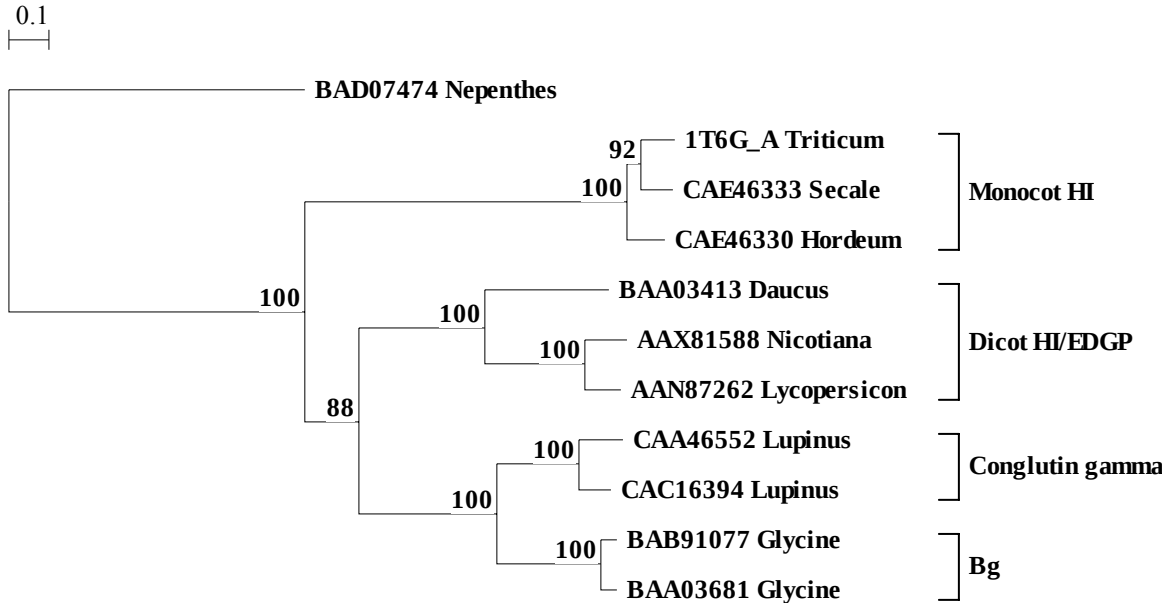

Fig.3. Putative evolutionary pathway of Bg.
In total, 398 alignment positions of aligned amino acid sequences of Bg and related proteins have been used for the tree construction. Sequence region selected for analysis (Fig.2) covers almost total sequences excluding variable N-/C-termini and short inserts peculiar in individual sequences and specific for clusters (see low case letters in Fig.2). Numbers along branches refer to bootstrap values (% from 100 replicates). Aspartic proteinase from *Nepenthes distillatoria* has been used as an outgroup. HI, xylanase inhibitors; EDGP, extradermal glycoproteins.

Conglutin γ (C(), a closest relative of Bg (>60 % identities, Fig.2), has been purified from mature Lupinus angustifolius [21] and Lupinus albus [22] seeds. Similarly to Bg, C(subunits are synthesized as precursors [23] that undergo processing into disulfide linked (- and (- chains and glycosylation. L. albus C(has extensively been characterized at molecular level [24-26]. Depending on pH, it exists either as (/(protomer or as a tetramer [27]. C(accounts for about 5 % of total protein in mature L. albus seed [22].

A certain functional relation of C(and Bg can be suggested. As well as Bg, L. albus C(binds insulin [28] and is located in the extracellular spaces of seedling cotyledons [29,30]. When L. albus seeds are immersed in hot water, de novo synthesized portion of C(is secreted in surrounding water [31]. The expression of L. angustifolius C(gene is accompanied with the expression of leginsulin-like homolog [32]. Thus, as well as in soybean, in lupin seeds both components of the putative signal transduction pathway are present.

Xylanase inhibitors (XI), closest sequence relatives both of Bg and C(, are a part of the network of plant defenses against microbial pathogens [33]. The inhibition of microbial xylanases by XI is a direct consequence of a high affinity association of the two proteins. XIs are single-chain glycoproteins sized similarly to Bg/C(precursors. Daucus carota extracellular dermal glycoprotein (EDGP) recently proved to inhibit xylanase [34]

is specific to the epidermis and other dermal tissues [35]. As well as Bg, EDGP are localized in the plasma membranes and middle lamella of cell walls. D. carota EDGP is closely related to well-characterized XIs from Lycopersicon esculentum [36] and Nicotiana langsdorffii [37]. XIs from dicots exhibit strict conservation of six Cys residue pairs, which have been proved to form disulfide bridges in EDGP molecule [38]. All these Cys residues are present in Bg (Fig.2) suggesting similar folding both of known dicot XIs and Bg.

Cereal XIs exemplified by *Triticum aestivum* inhibitor TAXI-I and its close relatives from *Secale cereale* and *Hordeum vulgare* [39] are of special interest because of solution of crystal structure of TAXI-I {pdb|1T6G) [40]. TAXI-I folds as two β-barrel domain protein with a few helical segments and the separate domains are divided by an extended cleft (Fig.4A). As well as in *D. carota* EDGP [38] six pairs of Cys residues form disulfide bridges in TAXI-I structure [40]. However, only three of six Cys pairs are structurally identical in these proteins suggesting difference in folding of N-terminal polypeptide regions of Bg/EDGP and TAXI-I. The tertiary structure of TAXI-I is highly similar to the core structure of aspartic proteinase from fungi *Rhizomucor miehei* (pdb|2RMP). A least-squares fit (Swiss-Pdb Viewer program, http://www.expasy.org/spdbv/) produced an RMSD of 1.47 Å between TAXI-I and the proteinase using 206 C(atoms (Fig 4 B).

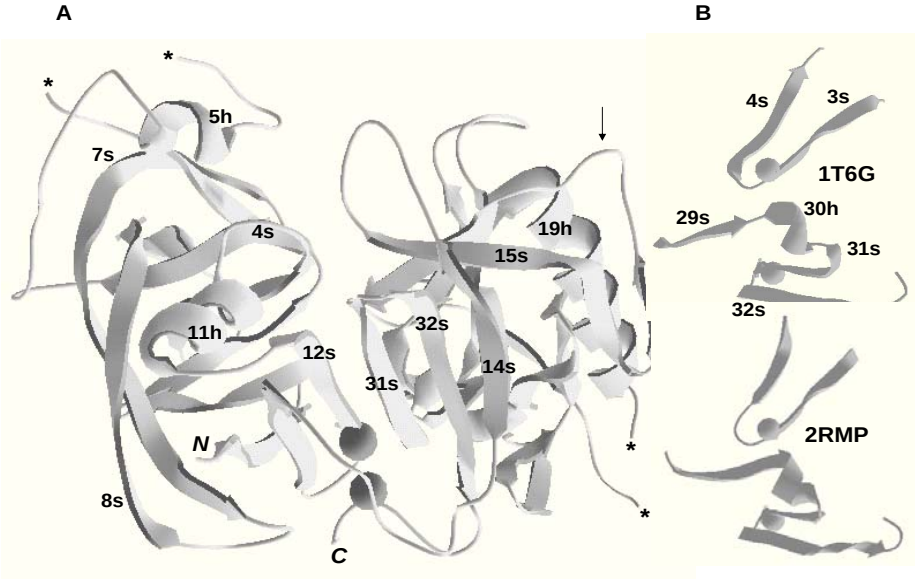

Fig.4. Structure of xylanase inhibitor TAXI-I from *Triticum*.
A. The ribbon diagram generated from 1T6G.pdb with the Swiss-Pdb Viewer program (http://www.expasy.org/spdbv/). Selected secondary structures (s, β-strands, h, α-helices) are numbered in accordance with TAXI-I sequence (see Fig. 1). Limits of disordered regions are shown by asterisks. Cys residues involved in formation of inter-domain disulfide bonds are shown as spheres. *N* and *C*, respective termini of TAXI-I polypeptide. An arrow indicates position of Bg processing site projected to the TAXI-I structure (Fig. 2). B. Ribbon diagrams of TAXI-I and fungal aspartic proteinase 2RMP structural regions surrounding proteinase active site. Spheres, two Asp residues globally conserved in Bg superfamily (Fig. 2) form two active sites in the entire family of aspartic proteinases. The structural regions shown here separately were extracted from superimposed C^{α} traces of TAXI-I and proteinase.

Plant aspartic proteinases share common ancestry with pepsin A but structurally and evolutionary are highly diverged [41,42]. A structure-based sequence alignment of the entire set of aspartic proteinases deposited in the PDB reveals only 23 strictly conserved residues [40]. Nepenthesin (NEP) from *Nepenthes distillatoria* [19,20] is a member of a novel family of plant aspartic proteinases that is only distantly related to plant pepsin-like enzymes of known tertiary structures [43,44]. Sequences of NEP and of its close relatives from *A. thaliana* and *O. sativa* exibit similarity to Bg, Cγ and EDGP, and might be similarly folded (see conservation of most of NEP Cys residues matching with those of EDGP, Fig.2). NEP-like proteinases are suggested widely distributed in plants and appears to be present even in *Chlamidomonas* [19].

The evolutionary pathway of Bg can be described as follows. An ancestral pepsin-like proteinase exemplified by extant NEP probably is formed at an early step of diversification of eukaryotes. The structure of putative progenitor of XIs descends from similarly ancient evolutionary step as suggested by Sansen et al. [40]. Tran-

sition of an aspartic proteinase into XI consisted in lost of proteolytic activity and formation of structural basis for xylanase inhibition via formation of substrate-mimicking contacts [40]. Dicot XIs that gave rise to Bg and Cγ probably reflect further evolutionary step. Common characteristics of Bg/Cγ as XI-related proteins are their capacity to bind specific proteins or peptides and probable involvement in cellular signal transduction system, and their enhanced expression when the plant is exposed to a stressful stimulus such as heat shock.

Functional distinction of dicot XIs and Bg is not obvious. Although carrot EDGP and Bg belong to different plant orders, they are highly similar to each other suggesting similar folding. They both are dermal proteins that bind insulin [12] and leginsulin [34]. Thus, dicot XIs might be involved both in plant's defense and signal transduction systems. In this context, it should be mentioned that XI-like activity of Bg and Cγ has never been examined.

A deficiency in sulfur-containing amino acids is a common problem for soybean and other crop legume seeds. Possibly, increased expression of Bg genes that encode protein rich both in Cys and Met can partially recover the deficiency. It is difficult to predict consequences of such an over-expression because of Bg activity as a protein kinase. However, it should be remind presence of Cγ, a close Bg structural and probably functional relative, in protein storage vacuoles of *L. angustifolius* seeds. Sorting of Bg into protein bodies probably is achievable. If so, tolerance of soybean seeds for relatively large amounts of Bg is can be suggested.

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