ORIGINAL RESEARCH

Molecular Life

doi: 10.26600/MolLife.1.3.2.2019.

Molecular evolution, structure and activation of legumains, asparagine-specific proteinases

Andrei Shutov[1]*, Irina Kakhovskaya[1], Karl Wilson[2]

[1]*State University of Moldova, MD-2009 Chişinău, Moldova*
[2]*State University of New York at Binghamton, NY 13902-6000 Binghamton, NY, USA*

(Received 26 March 2019; accepted 5 November 2019)

Abstract

Evolutionary analysis of eukaryote legumains was conducted using sequences of their precursors composed of Asparaginyl EndoPeptidase (AEP) domain, Legumain Stabilization and Activity Modulation (LSAM) domain, and Activation Peptide (AP) in between. Three basic clusters were observed: ancient (from Parabasalia to Stramenopiles), plant and animal legumains. Early described mechanisms of auto-maturation of plant and animal legumains were compared in relation to their crystal structures and models. In both cases the auto-maturation starts from cleavage of Asn/Asp-flanked peptide bonds at central (plants) or C-terminal (animals) parts of the AP peptide resulting in detachment of the LSAM domain. It is finalized by cleavages of additional Asn/Asp-flanked bonds inside the AP peptide region that proximately block the active site Asn, His and Cys residues. In this way the active site triad becomes released, and complete activation of plant legumains occurs. Possibly, these additional cleavages might be essential for activation of animal legumains as well.

Keywords: legumain, evolution, gene exon/intron structure, tertiary structure, activation

INTRODUCTION

Legumains (family C13, clan EC 3.4.22.34) are Asn/Asp-specific proteinases characteristic both of prokaryotes and eukaryotes [1]. The first legumain (proteinase B of unique Asn specificity) was isolated from germinating vetch *Vicia sativa* seeds [2]. Further on, legumains from other plants [3] and from the human parasite *Schistosoma mansoni* [4] were characterized. It was shown in early investigations that plant legumains are involved in storage globulin degradation [5, 6] and maturation [7]. Recently, the involvement of animal and plant legumains in manifold other physiological functions has been summarized in the revue by Dall and Brandstetter [8]. Depending on pH and other environment conditions, legumains reveal not only endoproteinase, but also asparaginyl carboxypeptidase and protein ligase or cyclase activities [8].

The tertiary structure of the inactive precursors of animal and plant legumains comprise a short N-terminal propeptide, Asparaginyl EndoPeptidase (AEP) domain, Activation Peptide (AP) and Legumain Stabilization and Activity Modulation (LSAM) domain [8] (Fig. 1). Auto-maturation of animal and plant legumain precursors consists of removal of the N-terminal propeptide and several cleavages inside the AP peptide (Fig. 1) resulting in complete detachment of the LSAM domain [8].

Amino acid sequences of eukaryote legumains are highly conserved [1]. It seems likely that their structures are organized similar to those shown above. In contrast, prokaryote legumain sequences are truncated C-terminally; thus, they lack both AP and LSAM regions. Therefore, evolutionary analysis of a limited set both of prokaryote and eukaryote legumains available in 2012 [1] was restricted to the AEP sequence region.

At present, thousands of full length sequences of eukaryote legumain precursors as well as tertiary

*Corresponding author (Moldova, MD-2009 Chişinău, Mateevici str. 60, State University of Moldova, fax: (37)322-244+248; E-mail: shutovandrei@yahoo.com

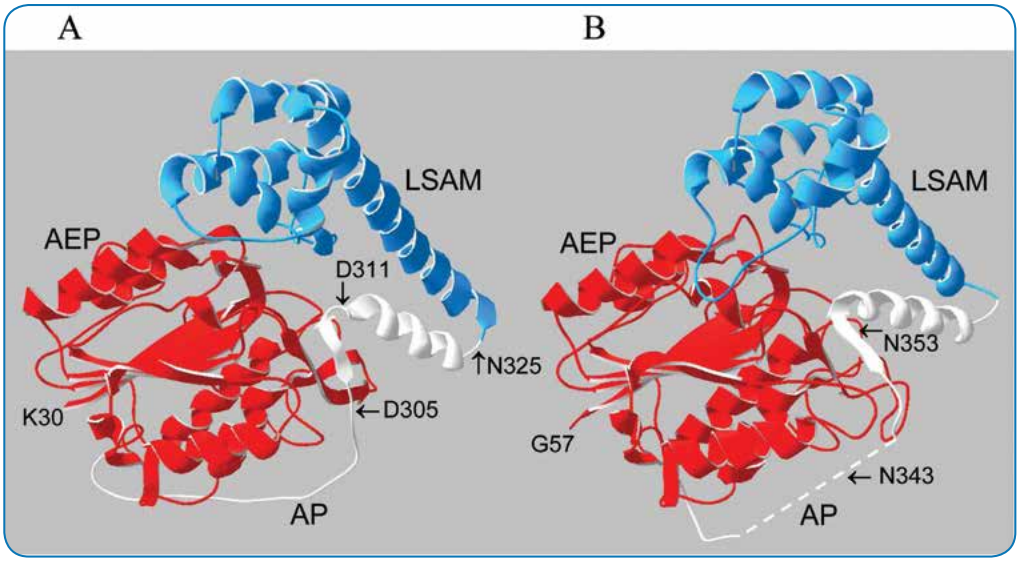

Fig. 1. Ribbon diagrams of tertiary structures of legumain precursors from *Mus musculus* 4nok [9] (A) and *Arabidopsis thaliana* 5nij [10] (B). The structures are superimposed but shown separately. Sequence numbering here and further corresponds to that used in the original papers. AEP, active endopeptidase domain, AP, activation peptide, LSAM, legumain stabilization and activity modulation domain. Arrows correspond to Asn- and Asp-flanked cleavage points detectable during auto-maturation of legumain precursors. Dotted line corresponds to the region disordered in 5nij crystal structure.

structures of several animal and plant legumains are available. Therefore, analysis of the evolutionary pathway of eukaryote legumains using entire amino acid sequences of their inactive precursors was conducted in this investigation. In addition, mechanisms of activation of plant and animal legumains based on their crystal structures and models were compared.

MATERIALS AND METHODS

We used the following programs for data analysis: 1) BLAST available from the site http://www.ncbi.nih.gov/ for searching of amino acid sequences homologous to plant/animal legumains; 2) Clustal Omega for sequence alignments; 3) TREECON [11] for evolutionary sequence analysis; 4) DeepView/Swiss-Pdb Viewer for analyzing of 3D protein structures, contouring of ribbon diagrams and calculation of a root-mean-square deviation (RMSD) of C^{α} atoms of superimposed legumain structures. 5) Swiss-Model [12] for modeling of protein 3D structures. The surface area an amino acid residue accessible to the solvent (ASA) in the crystal and model structures of legumains, expressed as $Å^2$, was calculated using the program http://cib.cf.ocha.ac.jp/bitool/ASA/. The relative ASA value of an X residue in legumains was expressed in percent of its accessibility in the GXG tripeptide.

To provide statistically significant topology of legumain evolutionary trees we used the following strategy. 1) Sequence region selected covers almost entire sequences of eukaryote legumain precursors; the region is bordered with conserved Trp and Cys residues; 2) The selection of sequence set (most critical point) was based on the analysis of statistical certainty of sequence clustering and branching of clusters. Each cluster is represented by two or more sequences; 3) Legumains from *Trichomonas vaginalis*, estimated as most ancient organism among eukaryotes [13], were used as outgroups.

RESULTS AND DISCUSSION

Legumain evolution. The evolutionary tree of eukaryote legumains rooted with *Trichomonas* sequences contains three basic clusters (Fig. 2): the ancient group of Alveolata and Stramenopiles legumains, Plant and Metazoa legumains. Only single legumain sequences from species taxonomically relevant to the ancient group are available: abi13175 (Haptophyceae), egd80967 (Choanoflagellida) and xp_014156698 (Ichthyosporea). The first sequence is close to the ancient group, but two other sequences seem to be rather advanced.

Legumain sequences within plant cluster strongly follow the evolutionary pathway of species: green algae (Chlorophyta), mosses, seed plants (Fig. 2). Exon/intron structures of legumain genes from land plants are almost identical and close to that of *Klebsormidium nitens* legumain (green alga, Charophyta) (Table 1). In contrast, position of introns in Chlorophyta legumain genes is completely different. Additionally, long variable inserts outside the AEP domain are specifically characteristic of amino acid sequences only from Chlorophyta legumain species. These observations both are in line with the plant tree of life, estimated Chlorophyta as most ancient green algae [14].

Collection of Metazoa sequences composed of legumains from the four basic groups (Platyhelminthes, Ecdysozoa, Cnidaria and Chordata, Fig. 2) is most rich. Legumains sequences from *Schistosoma*, estimated as one of the most primitive organisms among metazoans [15], occupy expected ancestral position in the cluster. Legumains from Ecdysozoa cluster strongly follow evolutionary pathway of species. Thus, Nematode and Targidrade legumain sequences represent sister groups, and all other sequences (from Crustaceans to Ticks) belong to Arthropoda species [16]. Additionally, the evolutionary pathway of Chordata legumains is classic (from the most ancient Lancelets to Mammalians).

All the five available sequences of Cnidaria legumains belong to Hexacorallia taxon. Two Cnidarian sequences selected for the tree construction occupy position between Ecdysozoa and Chordata legumain clusters (Fig. 2). When other three Cnidarian sequences (pfx15979, xp_015747571 and xp_020618825) are separately involved in the tree construction, they form common cluster with Branchiostoma legumains (data not shown). This observation supports the tree topology (Cnidarians between Ecdysozoa and Chordata clusters) shown in Fig. 2.

Evolutionary position of available Lophotrochozoa legumain sequences not included in the tree (for instance, owf50347, Mollusca; aiz77505, Annelida; xp_013394313, Brachiopoda) is indefinite. According to the data of molecular-based metazoan phylogeny, Lophotrochozoans and Ecdysozoans are directly evolutionarily related [15]. However, positions of Lophotrochozoa sequences in the tree are highly different and low statistically supported (data not shown). Possibly, the problem might be solved when the collection of

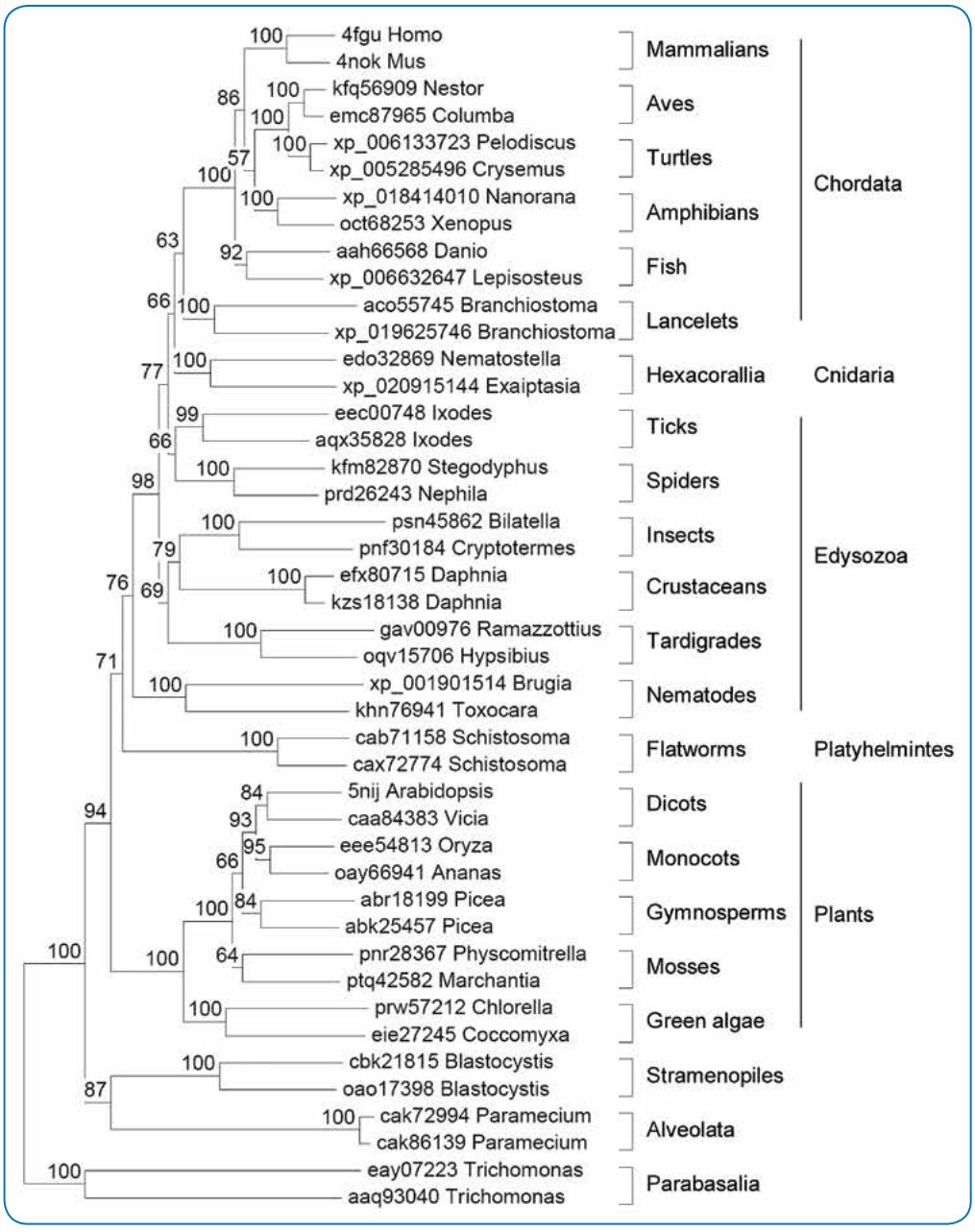

Fig. 2. Evolutionary tree of eukaryote legumains. In total, 484 positions of aligned amino acid sequences covered AEP, AP and LSAM regions were used for the tree construction. Numbers along branches refer to bootstrap values (% from 1000 replicates). Parabasalia legumains were used as outgroups.

Table 1. Number of introns matching in position/phase in plant and animal legumain genes

Viridiplantae		
caa21203	8 (8)	Arabidopsis
eee54813	7 (8)	Oryza
efj15688*	7 (8)	Club moss
ptq42582	7 (8)	Moss
gaq89237*	5 (8)	Green alga
Chordata		
eaw81501	12 (12)	Homo
rmb98804	12 (12)	Aves
kfq56909	12 (12)	Turtle
oct68253	12 (12)	Amphibia
awp16104	12 (12)	Fish
een60572	11 (12)	Lancelet
Others		
edo32869	11 (14)	Cnidaria
owf50347	11 (12)	Lophotrochozoa
prd26243	10 (12)	Ecdysozoa
edv29220*	10 (12)	Placozoa
ccd59168	10 (11)	Platyhelmintes

In Table 1: Total number of introns is shown in brackets. Single available sequences mentioned in the text are indicated by asterisks.

Lophotrochozoa legumain sequences will be enriched. The only single legumain sequences from Deuterostomia species taxonomically relevant both to Ecdysozoa and Lophotrochozoa [15] are available: xp_022105316 (Echinodermata) and xp_006822143 (Hemichordata). Both sequences occupy expected position in the tree but only under a low statistical support (data not shown).

In general, the evolutionary pathway of eukaryote legumains analyzed on the basis of their amino acid sequences (Fig. 2) is in line (although with several remarkable exceptions) with exon/intron structures of their genes (Table 1). Similar to plants (see above), most of animal legumain genes (from Flatworms to Mammalians including Lophotrochozoa and Placozoa genes) contain introns of identical positions/phases. Meantime, positions of introns in plant and animal legumain genes are different (with a single exception discussed below). Similar to legumain genes from the ancient green algae (Chlorophyta cluster), the number and position of introns in genes of the ancestral legumain group (from Parabasalia to Stramenopiles) is different from those of most plant and animal legumain genes.

Remarkably, the position/phase of intron 1 (see Fig.3) in the overwhelming majority both of plant and animal legumain genes (Table 1) are identical. Intriguingly, intron 1 of human [17] and mouse [18] legumain genes contains p53-binding sites involved in positive regulation of gene expression at the transcriptional level.

Structures and activation of plant and animal legumains. Amino acid sequences of plant and animal legumains are highly conserved in the AEP domain (an RMSD of 0.63 Å over 259 C^{α} atoms of *M. musculus* 4nok and *A. thaliana* 5nij legumains) and variable in the N-terminal part of the AP peptide (Fig. 3). Analysis of 4nok and 5nij prolegumain structures reveals that only

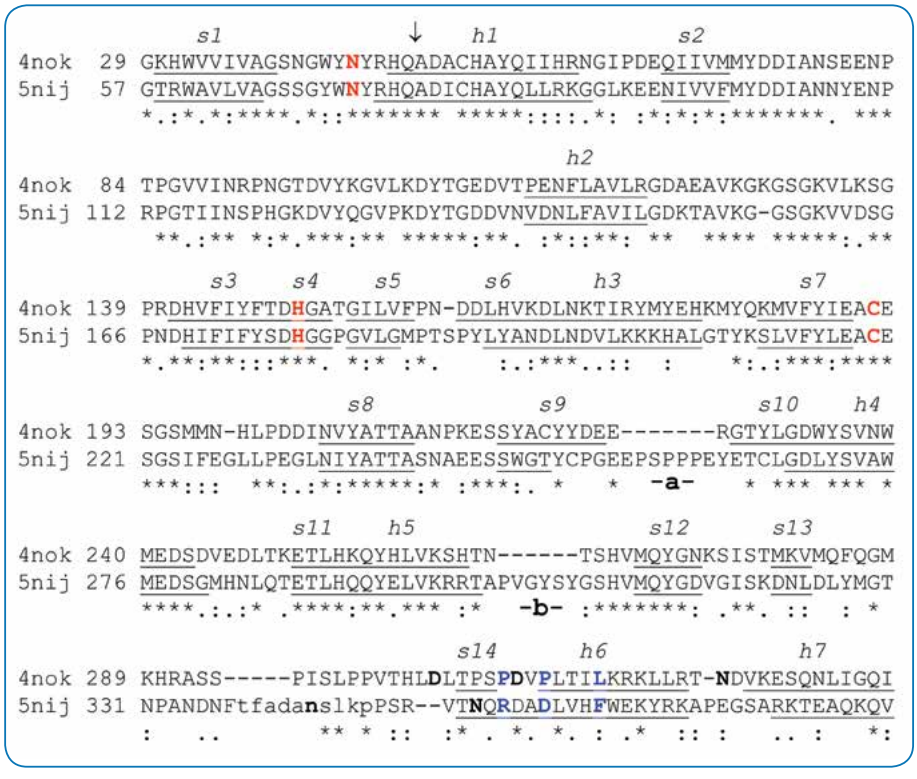

Fig. 3. Amino acid sequences of legumains from *M. musculus* 4nok [9] and *A. thaliana* 5nij [10] exemplify primary structures of eukaryote legumains. Aligned sequence region is restricted to the AEP domain (β-strands *s1-s13* and α-helices *h1-h5*) and the activation peptide AP containing β-strand *s14* and α-helix *h6*. Low case letters indicate the region disordered in 5nij crystal structure. The AEP domain contains two inserts: a, characteristic of the ancient group (Alveolata, Stramenopiles) and plants; b, present only in plant sequences. Residues in bold correspond to the active site triad Asn, Hys and Cys (red), to Asn/Asp-flanked auto-cleavage points detectable during maturation of 4nok and 5nij precursors (black) and to residues that proximately block the active site triad in prolegumain structures (blue; see also Fig. 4). An arrow indicates the intron 1 position characteristic of the overwhelming majority both of plant and animal legumain genes.

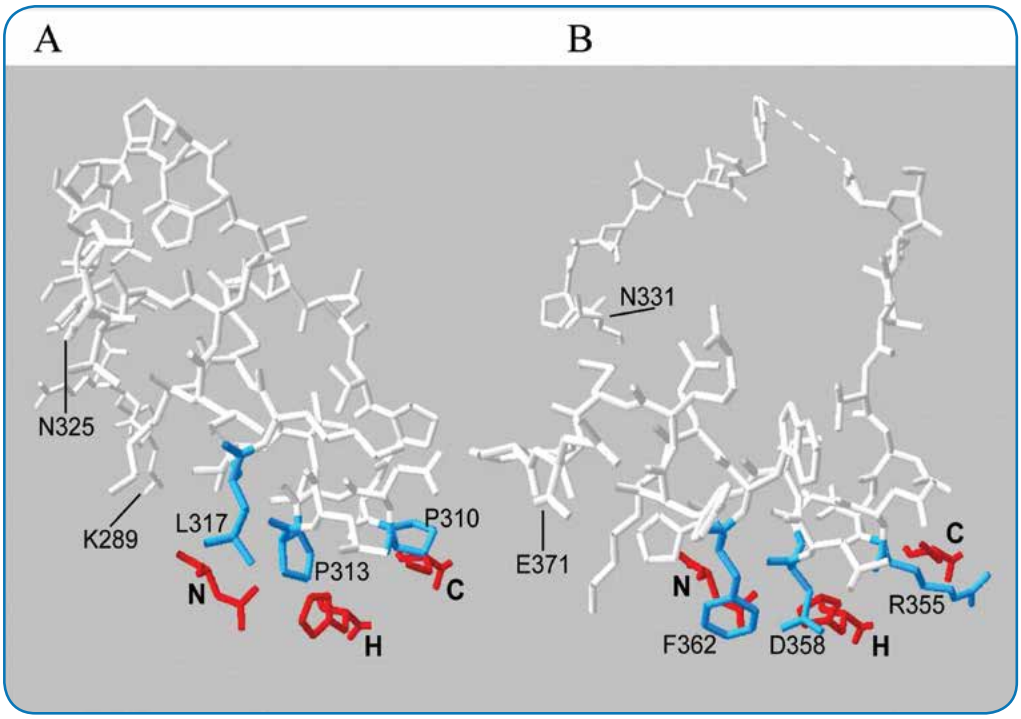

Fig. 4. Activation peptide AP of legumain precursors from *M. musculus* 4nok [9] (A) and *A. thaliana* 5nij [10] (B). Blue, amino acid residues that proximately block the active site triad (residues Asn, His and Cys shown in red). The structures are superimposed but shown separately. Dotted line corresponds to the region disordered in 5nij crystal structure.

the C-terminal structurally organized parts of the AP peptides (β-strand *s14* and α-helix *h6*) are responsible for blocking of the active site Asn, His and Cys residues. Moreover, it seems likely that in both structures only three residues matching in sequence and structural alignments proximately block the active site triad (Fig. 4).

In general, activation both of animal (4nok, *M. musculus* [9] and 4fgu, *Homo sapience* [19] and plant (5nij, *A. thaliana* [10]) legumains that occurs at low pH values consists of release of the active site Asn, His and Cys residues and complete removal of the LSAM domain. However, the detailed analysis of the animal and plant

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                    s12       s13                                    s14
aaq93040      244 SHVLSFGDMKLAKLPLSTFLLNAEPEEVNNED-------SGDSENSVENGAST
cbk21815      258 SHASRYGDVSFESDLIGEYVGYPEEKFNYDHQ-------------SSVAWDS
prw57212      284 SHVMRFGTLGIAEEVAAEFMGYGNTGAKKQAAAT----NGAAPSWAPQGALPQ
xp_024395066  213 SHVLEFGDLKMKPEELDQYLGYDPANENVTGPIFLREYLAIRLGGVEERHINQ
eee54813      318 SHVMEYGSLELNAHHVFMYMGSNPANDNATFV-------EDNSLPSFSRAVNQ
5nij          309 SHVMQYGDVGISKDNLDLYMGTNPANDNFtfa-------danslkpPSRVTNQ
cax72774      264 SHVQRYGDKKMGKLYLSEFQGSRKKASTEHD----------EPPMKPKDSIPS
efx80715      302 SHVQEYGDLTIGKMKVGEFQGKGKAPMASNG---------RKRVSPLLDAVPS
prd26243      274 SHVQEYGDMSIAKMHVSEFQGRKKSEPIVV----------PKVEYDAVRS
xp_020915144  270 SHVMKYGDMSFENEAVDDFQGDPSAVSRPMIK-----INSANVPEPEFDAVPA
oct68253      265 SHVMQYGNRTISQMKVNQFQGNVKITSTPVY----------LEPVKHMDLTPS
4nok          267 SHVMQYGNKSISTMKVMQFQGMKHRASSPIS----------LPPVTHLDLTPS
                  **.  :*   :       :

                        h6                    h7
aaq93040      290 HVAALEYLQRRLKETT-SKEEANAIKGQIEHEVQRRARSDK  Parabasalia
cbk21815      297 RDAKFLFLLYKYQHTTGS--EKAKWEKLYLEEMSLRQQIDR  Stramenopiles
prw57212      284 RDADLAHLWHKFASAPEGP-AKAAALSQLSAETSARSRVDA  Green alga
xp_024395066  266 RDADLVHYWHRYHKSKVGSTAKAEAELDLMRILSHRMYIDK  Moss
eee54813      364 RDADLVYFWQKYRKLPESSPEKNEARKQLLEMMAHRSHVDN  Oryza
5nij          355 RDADLVHFWEKYRKAPEGSARKTEAQKQVLEAMSHRLHIDN  Arabidopsis
cax72774      308 RDIPLHTLHRRIMMANN-MNDKNLLMKILGLKLKRRDLIKD  Flatworm
efx80715      346 GDVPLEILRHKLRKMNS-SPESAEIQRKIRGIEKKRQHLKD  Crustacea
prd26243      314 RDVPIEIVKRKYHKSNT-VEDQTALLKKLNKMLRNRKFLAQ  Spider
xp_020915144  318 PDVPIAILEHRLKAAKD-PEEKQFIEQKLEKEIKNRRKIQD  Cnidaria
oct68253      308 PDVPLAILKRKLMATND-ILQARAIVREIKAHQEAKQLIKE  Amphibia
4nok          310 PDVPLTILKRKLLRTND-VKESQNLIGQIQQFLDARHVIEK  Mammalia
                    :     :                        :
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Fig. 5. Aligned sequences of selected legumains inside the AP region and adjoin areas of β-strands *s12/s13* and α-helix *h7*. Low case letters correspond to the region disordered in 5nij crystal structure. Asn/Asp residues in P1 position of peptide bonds cleaved during maturation of *A. thaliana* [10] and *M. musculus* [9] legumains are shown in red. Accessibility to the solvent of other Asn/Asp residues in legumain precursors: from 50 to 100%, black; <50%, blue. Following crystal structures were used as templates for 3D structure modeling: *A. thaliana* 5nij for Parabasalia, Stramenopiles and plant legumains; *M. musculus* 4nok for animal legumains.

legumain sequences and structures we conducted reveal certain differences.

Sequences and structures of animal legumains are highly conserved (an RMSD of 0.96 Å over 394 C^{α} atoms of 4nok and 4fgu structures). Therefore, below we used sequence numbering of *M. musculus* legumain (Fig. 3) to simplify further description of the auto-cleavage events shown experimentally for both animal legumains.

According to our measurements, the conserved Asn325 residue between α-helices *h6* and *h7* (Fig. 3) in the animal legumain structures is most accessible to the solvent within the AP structure (ASA ~100%). Thus, maturation of the animal legumains starts from cleavage of respective Asn-flanked peptide bond [9, 19]. Further cleavages of Asp305/Asp311-flanked bonds at both sides of the β-strand *s14* (Fig. 3) occur. It is tempting to conclude that cleavages of all the three Asn/Asp-flanked bonds shown experimentally for both *H. sapience* and *M. musculus* legumains [9, 19] are necessary for their activation due to release of the active site triad.

However, complete activation of *H. sapience* legumain was observed when Asp305/Asp311 residues are substituted [19]. Therefore, it was concluded that following the cleavage of the Asn-flanked bond between α-helices *h6* and *h7* subsequent activation of *H. sapience* and other animal legumains is only resulted from their conformational rearrangement induced by pH lowering, and thus independently of the cleavage at Asp305 or Asp309 [8]. Nevertheless, it should be noted in this context that both Asp305/Asp309 residues are highly conserved among animal legumain sequences (from Flatworms to Mammalians, Fig. 5) and therefore should be functionally important. It seems probable, that the activation of animal legumains results both from cleavages at Asp305 and Asp309 (shown experimentally [9, 19]) and conformational changes within AEP/AP structure [8, 19].

Similar to animal legumains, the loop between α-helices *h6* and *h7* in the structure of *A. thaliana* legumain 5nij [10] reveals enhanced accessibility to the solvent (ASA >90%) but lack Asn or Asp residues (Fig. 3). It seems likely that 5nij maturation starts from cleavage of experimentally detected [10] Asn343-flanked bond inside disordered AP region (ASA 85% we obtained for a local modeling of the region). Further cleavage of the Asn353-flanked bond [10] finalizes the 5nij activation. It should be noted, that the Asn353 residue is of a low accessibility to the solvent, and thus of a low susceptibility to auto-cleavage in the original 5nij structure. Probably, cleavage of this bond occurs due to a local alteration of the AP structure after the first cleavage at Asn343.

In general, mechanism of activation of other plant and even Parabasalia and Stramenopiles legumains seems to be similar to that of *A. thaliana* legumain (Fig. 5). Indeed, they all contain Asn/Asp residues of enhanced accessibility to the solvent and thus probable susceptibility to auto-cleavage inside central variable part of predicted AP peptide. They all lack Asn/Asp residues within a loop between predicted α-helices *h6* and *h7*. Additionally, they all contain Asn/Asp residues matching with those essential for maturation of either *A. thaliana* [10] or *M. musculus* [9] legumains (Fig. 5).

In summary, activation of both *M. musculus* and *A. thaliana* legumains comprises two steps: first, cleavage of the most susceptible Asn-flanked bonds in the original structures of legumain precursors resulted in local alteration of the AP structures, and second, cleavage of Asn/Asp-flanked bonds either surrounding or inside sequence segments that block the active site triad (Fig. 3, 4). The second step seems to be either obligatory (*A. thaliana*) or accessory (*H. sapience*) for the active site release in both legumains.

Eukaryote legumain sequences are conserved enough for dependable description of their evolutionary pathway (Fig. 2) and reliable modeling of their structures even outside the active AEP domain (Fig. 5). These analyses both reveal clear difference in structures and maturation mechanisms between ancient (from Parabasalians to plants) and more advanced (from Flatworms to Mammalians) legumains.

Author contributions: analysis, A. Shutov, I. Kakhovskaya; writing: K. Wilson, A. Shutov
Conflicts of interest: None declared.

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