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Graspases—a Special Group of Serine Proteases of the Chymotrypsin Family That Has Lost a Conserved Active Site Disulfide Bond

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Abstract—In this report we propose a new approach to classification of serine proteases of the chymotrypsin family. Comparative structure–function analysis has revealed two main groups of proteases: a group of trypsin-like enzymes and graspases (granule-associated proteases). The most important structural peculiarity of graspases is the absence of conservative "active site" disulfide bond Cys191–Cys220. The residue at position 226 in the S1-subsite of graspases is responsible for substrate specificity, whereas the residue crucial for specificity in classical serine proteases is located at position 189. We distinguish three types of graspases on the base of their substrate specificity: 1) *chymozymes* prefer uncharged substrates and contain an uncharged residue at position 226; 2) *duozymes* possess dual trypsin-like and chymotrypsin-like specificity and contain Asp or Glu at 226; 3) *aspartases* hydrolyze Asp-containing substrates and contain Arg residue at 226. The correctness of the proposed classification was confirmed by phylogenic analysis.

Key words: serine protease, classification, substrate specificity, graspase, phylogeny

In this report we propose a new approach to classification of serine proteases of the chymotrypsin family, which was developed during our investigation of bovine duodenal proteases (duodenases) [1-5]. Analyzing structure–function properties of duodenases and other proteases we concluded that duodenases and the vast majority of proteases produced mainly by immune system cells (cathepsin G, chymases, granzymes) form a special group within the chymotrypsin family. Taking into account the fact that these enzymes are stored within cells in secretory granules we propose the name graspase (granule associated protease) for them [6].

It should be noted that during the last 20 years graspases have been under intensive investigation by leading world laboratories due to the importance of their proposal biological role: participation in immune reactions and apoptosis [7-9]. This leads to the rapid growth of information concerning these enzymes, discovery of novel representatives of graspase group, and the need for correct interpretation of the data obtained. These reasons gave rise to the necessity of a new convenient classification of graspases instead of the existing classification based on tissue of origin or function. The proposed classification is based on some structural peculiarities and enzymatic properties of the proteases. The correctness of our classification was confirmed by phylogenic analysis.

MATERIALS AND METHODS

Amino acid sequences of 53 proteins (numbers correspond to those in the figure) were extracted from GenBank nucleotide sequence data bank, their accession numbers are given in parenthesis: 1) sheep MCP 1 (Y14654); 2) sheep MCP 3 (Y13462); 3) bovine duodenase (AF198965); 4) mouse cathepsin G (NM_007800); 5) human cathepsin G (NM_001911); 6) rat MCP 8 (NM_101598); 7) rat GLP II (X68657); 8) rat granzyme J (U72143); 9) mouse MCP 8 (X78545); 10) human granzyme H (M57888); 11) human granzyme B (NM_004131); 12) rat GLP III (X76996); 13) rat GLP I (X66693); 14) rat NKP 1 (M34097); 15) mouse granzyme B (NM_013542); 16) mouse granzyme C (NM_010371); 17) rat NKP 4 (U57062); 18) mouse granzyme D

Abbreviations: MCP) mast cell protease; NKP) natural killer protease; GLP) granzyme-like protein; HLE) human leukocyte elastase (medullasin); PRN 3) human leukocyte proteinase 3 (myeloblastin).

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(J03255); 19) mouse granzyme E (NM_010373); 20) mouse granzyme G (NM_010375); 21) rat NKP 7 (U57063); 22) mouse granzyme F (NM_010374); 23) mouse MCP 4 (AY007569); 24) mouse myonase (AB051900); 25) rat MCP 1 (U67915); 26) gerbil MCP 1 (D45173); 27) hamster MCP 1 (D85517); 28) mouse MCP 2 (NM_008571); 29) mouse MCP 1 (NM_008570); 30) rat MCP 2 (J02712); 31) mouse MCP 9 (AY007568); 32) human chymase (XM_007317); 33) monkey chymase (U38521); 34) dog chymase (U89607); 35) sheep MCP 2 (Y08133); 36) rat MCP 3 (D38495); 37) mouse MCP 5 (M73759); 38) gerbil MCP 2 (D45174); 39) hamster MCP 2 (AB007622); 40) human medullasin (HLE); 41) human myeloblastin (PRN 3) (NM_002777); 42) mouse granzyme K (NM_008196); 43) rat granzyme K (NM_017119); 44) human granzyme K (XM_003782); 45) human granzyme A (XM_003652); 46) mouse granzyme A (NM_010370); 47) mouse granzyme M (AB015728); 48) rat granzyme M (L05175); 49) human granzyme M (NM_005317); 50) bovine tryptase (X94982); 51) bovine chymotrypsinogen A (P00776); 52) bovine trypsinogen (X54703); 53) bovine elastase 1 (M80838).

The protein alignment and phylogenic tree reconstruction were performed using Hein's method [10] supplied by the MegAlign program (DNASTAR Inc., USA). The overall strategy of the method is to use pairwise distances, which can be calculated by traditional methods, based on quick *k*-tuple pairwise alignments. Then an initial distance tree is made, sequences are aligned following this tree, and finally the tree is rearranged to maximize evolutionary parsimony.

RESULTS AND DISCUSSION

Graspases lost the ancient disulfide bridge near the active site. Classic serine proteases of the chymotrypsin family (trypsin, chymotrypsin, elastases, blood coagulation and fibrinolysis factors, etc.) are known to contain three conserved disulfide bonds: Cys42–Cys58, Cys168–Cys182, and Cys191–Cys220 (chymotrypsinogen A numbering system is used throughout). The disulfide bond Cys191–Cys220 resides in the vicinity of the enzyme active site and takes part in maintaining the architecture of the substrate-binding pocket [11]. This disulfide bridge is one of the ancient ones and is important for the catalytic activities of serine proteases. Elimination of this "active site" disulfide bond by mutagenesis leads to a dramatic alteration of the catalytic properties of trypsin and chymotrypsin. The mutants are much less effective and specific, compared with the native enzymes, mainly due to drastic increasing in K_m value [12]. This fact points to the need for the disulfide bond Cys191–Cys220 for high catalytic activity of the enzymes.

However, there are a large number of enzymes of the chymotrypsin family (about 40) which do not possess an "active site" disulfide bond and nevertheless display high catalytic activity. These are leukocyte cathepsin G, mast cell chymases, many granzymes of cytotoxic lymphocytes [13, 14], duodenase of bovine Brunner's gland [3], and myonase of mouse myocytes [15]. The absence of the highly conservative disulfide bond Cys191–Cys220 in the vicinity of active site and a number of other structural differences (which are discussed below) distinguish the above-mentioned enzymes from classic proteases of the chymotrypsin family. This allowed us to separate them into a special group designated by us as graspases [6]. Graspases possess six cysteine residues that are conserved among all members of the group. The residues form three disulfide bonds. Two of them (Cys42–Cys58 and Cys168–Cys182) are present both in the classic serine protease molecules and the graspase molecules. Disulfide bond Cys136–Cys201 is characteristic only of graspases. In graspase molecules, positions 191 and 220 corresponding to "active site" disulfide bridge of classic serine proteases are occupied by residues other than Cys.

Thus, in the chymotrypsin family we distinguish two main groups of proteases: the trypsin group and the graspase group. Representatives of the trypsin group contain a disulfide bond in the vicinity of the active site whereas graspases do not contain such a bond.

Key specificity-determining residues of the S1 subsite in enzymes of the trypsin group and the graspases. The substrate-binding pocket of serine proteases belonging to the chymotrypsin family is formed by residues 189-228. Three of them, namely 189, 216, and 226 which belong to the S1-subsite (nomenclature of Shechter and Berger [16]) are the most crucial for the primary specificity of the protease. Trypsin group proteases such as blood clotting and fibrinolysis factors [17]; complement factors C1s, C1r, B, D [18-20]; enteropeptidase [21], kallikrein [22, 23], contain a negatively charged Asp residue at position 189 and Gly residues at both positions 216 and 226 and possess trypsin-like specificity. As demonstrated using X-ray data for trypsin, the residue at position 189 is the most important for the primary substrate specificity of the protease [24]. This residue makes electrostatic interactions with the positively charged P1 residue of substrate or inhibitor. The presence of Gly residues at positions 216 and 226 provides access of substrate bulky lysine or arginine residues to Asp189 which is positioned at the bottom of the S1 subsite. Substitution of Ala for Gly residues at positions 216 and 226 in the trypsin molecule leads to dramatic deterioration of its catalytic properties [25].

The absence of the negative charge at position 189 leads to a global change in protease specificity—loss of trypsin-like properties. Such proteases as chymotrypsin [26], elastases [11], granzyme M [27], having uncharged residues at 189, cleave uncharged substrates. One exception is known—in bovine tryptase residue 189 is

uncharged, but the enzyme possesses trypsin-like specificity [28]. Amino acid side chains at positions 216 and 226 play an additional role in modulating the specificity profile of serine proteases (elastases [11], crab collagenase [29], granzyme M [30]) (Table 1).

It must be emphasized that the substrate specificity of a certain protease depends on numerous factors. Enzyme catalytic properties are influenced by structural peculiarities of both substrate-binding pocket and of some distant fragments of the polypeptide chain. Although these fragments do not contact directly with the substrate residue, they may influence the conformation of the substrate binding pocket [31]. Nevertheless, the residue at position 189 remains of primary importance for specificity of trypsin group proteases.

A different situation is observed in the case of graspases. Many authors describing catalytic properties of the proteases from hemopoietic cells (chymases, some granzymes) underline their unusual specificity. The characteristic of graspases is their more pronounced secondary specificity, i.e., susceptibility to residues distant from the cleavage bond in comparison with trypsin, chymotrypsin, and elastase. Graspases usually do not cleave the single amino acid or dipeptide derivatives of *p*-nitroanilide or 7-aminomethylcoumarin. The enzymes show a strict discrimination among substrates, preferring extended peptide derivatives and demonstrating pronounced selectivity towards these substrates [32-34]. The primary specificity of graspases is also varied. Some of them (e.g., mast cell chymases [31], mouse myonase [15], and human granzyme H [33]) possess chymotrypsin-like specificity, cleaving hydrophobic substrates. Such proteases as bovine duodenase [2], human cathepsin G [35], and sheep mast cell protease 1 [36] demonstrate unusual dual specificity combining trypsin- and chymotrypsin-like activity. Some graspases (mainly granzymes B from different species [8]) are Asp-specific. It is interesting to note that residue 189, which is crucial for specificity of trypsin group enzymes, in graspases is uncharged. This raises the question: what determines graspase specificity?

Study of granzyme B from human T-lymphocytes revealed the crucial role of Arg226 for its specificity towards aspartate containing substrates [37]. By analogy with granzyme B, a determining role for residue 226 was proposed in dual specific graspases (duodenase [3, 38], human cathepsin G [39], sheep mast cell protease 1 [40]). These enzymes contain a negatively charged residue at 226 (Asp or Glu) that seems to be responsible for the trypsin-like component of their specificity. The crystal structure of human cathepsin G in complex with phosphonate inhibitor [39] and native bovine duodenase [38] showed that the carboxylate group of residue 226 in both enzymes is accessible to substrate. Graspases that are specific toward hydrophobic substrates (chymases, myonase, and granzyme H) have an uncharged residue at position 226. Thus, on analyzing all known structures of graspases we observed strict correlation between the nature of residue 226 and substrate specificity of the enzyme. Therefore, a crucial role in graspase P1-specificity is apparently played by the residue at position 226, but not 189 as was described for trypsin group enzymes. This may be caused by loss of the "active site" disulfide bond that changes the properties of the substrate-binding pocket of graspases.

Based on the nature of residue 226, and the corresponding enzymic substrate specificity, we divided the graspases into three subgroups (Table 1).

1. Chymozymes—graspases with an uncharged residue at position 226 and displaying chymotrypsin-like specificity. This subgroup is represented by mast cell chymases of different origin, human granzyme H from cytotoxic T-lymphocytes, and mouse myonase. Mouse granzymes C-G, rat natural killer proteases 4 and 7 (rNKP 4,7), mouse cathepsin G structurally belong to the group, but their specificity has not been experimentally determined.

2. Duozymes—graspases having a negative charged residue at position 226 and possessing dual, trypsin-like and chymotrypsin-like specificity. This subgroup is represented by human cathepsin G, bovine duodenase, sheep mast cell protease 1 (sMCP 1), and presumably rat granzyme-like protein III (rGLP III) (the enzyme is known as an oligonucleotide sequence).

3. Aspartases—graspases with Arg at position 226. These enzymes cleave peptide bonds carboxyterminally to Asp residues; this group includes cytotoxic T-cell granzymes B (human, mouse), rat natural killer protease 1 (rNKP 1), and presumably (the enzymes are known only as oligonucleotide sequence) mast cell protease 8 (mouse, rat) (MCP 8), rat granzyme J, and rat granzyme-like proteins I and II (rGLP I, rGLP II).

Thus, within the chymotrypsin family we revealed two main groups of proteases which differ in architecture of their substrate-binding pockets and location of residue crucial for enzyme specificity: the trypsin and the graspase groups.

Besides the differences discussed above (presence/absence of "active site" disulfide bond and different specificity determinant), trypsin group enzyme and graspase precursors are distinct in terms of the length of their activation peptides. While propetides of all graspases are dipeptides, the activation peptides of the trypsin group proteases are of variable size. The main differences between graspases (cathepsin G group) and the trypsin group and characteristic representatives of the groups are summarized in Table 2.

Among proteases of the chymotrypsin family, there are two proteases combining properties of the graspases and enzymes from the trypsin group. The first one, crab collagenase, having the "active site" disulfide bond (Cys191–Cys220), characteristic of enzymes of the trypsin group, exhibits an alternative localization of negative charge in the S1 subsite compared to trypsin (Table

Table 1. S1-subsite of trypsin group and graspase group proteases

Group	Enzymes	S1-subsite residues*						P1-specificity
		189	191	195	216	220	226	
Trypsin group	trypsin and trypsin-like proteases	Asp	Cys	Ser	Gly	Cys	Gly	Arg, Lys
	chymotrypsins	Ser	Cys	Ser	Gly	Cys	Gly	Tyr, Phe, Trp, Leu
							Ala	
	elastases	Ser	Cys	Ser	Val	Cys	Thr	Ala, Val, Ile, Leu
							Asp	
	granzymes M	Ala	Cys	Ser	Ser	Cys	Pro	Met
							Thr	
	crab collagenase	Gly	Cys	Ser	Gly	Cys	Asp	Arg, Lys, Ala, Trp, Phe, Tyr
Graspase group								
chymozymes	chymases; myonase; human granzyme H; mouse granzymes C-G**; mouse cathepsin G**; rNKP 4, 7**	Ser	Phe		Gly	Ala	Ala	Tyr, Phe, Trp, Leu
		Asn	Tyr	Ser	Val	Gly	Gly	
		Thr	Gly		Tyr	Thr	Gln	
		Ala	Ala		Ala		Ser	
			Ser				Ile	
			Thr					
duozymes	duodenase; sMCP 1; human cathepsin G; rGLP III**	Asn	Phe	Ser	Gly	Gly	Asp	Arg, Lys,Tyr, Phe, Leu
		Ala					Glu	
aspartases	granzymes B; rat granzyme J**; rNKP 1; rGLP I, II**; mMCP 8**	Ala	Phe	Ser	Gly	Gly	Arg	Asp
		Thr	Gly		Arg	Trp		
			Ala					

* Residues of the most importance for primary specificity (see text) and catalytically active Ser195.
** Enzyme specificity has not been experimentally determined.

1). The trypsin-like component of collagenase "triple" specificity (trypsin-, chymotrypsin- and elastase-like), as with duozymes, is determined by the presence of a negative charge at position 226 [29]. The second enzyme, sheep mast cell protease 3 (sMCP 3), for which only cDNA nucleotide sequence is known [40], is the single graspase containing a negative charge (aspartate residue) at position 189, like trypsin. It is difficult to predict the substrate specificity of this protease. It might be able to bind basic residues by interaction with Asp189 in a fashion similar to trypsin. In this case sMCP 3 will be an exception among graspases, having the specificity determinant at position 189. Alternatively, sMCP 3, having a bulky amino acid side chain at 226 (Asn), may possess elastase-like specificity. Further investigations of sMCP 3 will be required to resolve this question.

Table 2. Main differences between enzymes of trypsin and granzyme groups and characteristic representatives of the groups

Characteristic features	Trypsin group	Graspase group
Conservative disulfide bonds	Cys42–Cys58 Cys168–Cys182 Cys191–Cys220	Cys42–Cys58 Cys168–Cys182 Cys136–Cys201
Specificity determinant	residue 189	residue 226
Activation peptide	variable	dipeptide
Representatives	pancreatic proteases, enteropeptidase, factors of coagulation, fibrinolysis and complement, granzymes A, K, M, tryptases, crab collagenase	chymases, granzymes B, C, F-H, J, duodenase, cathepsin G, sMCP 1

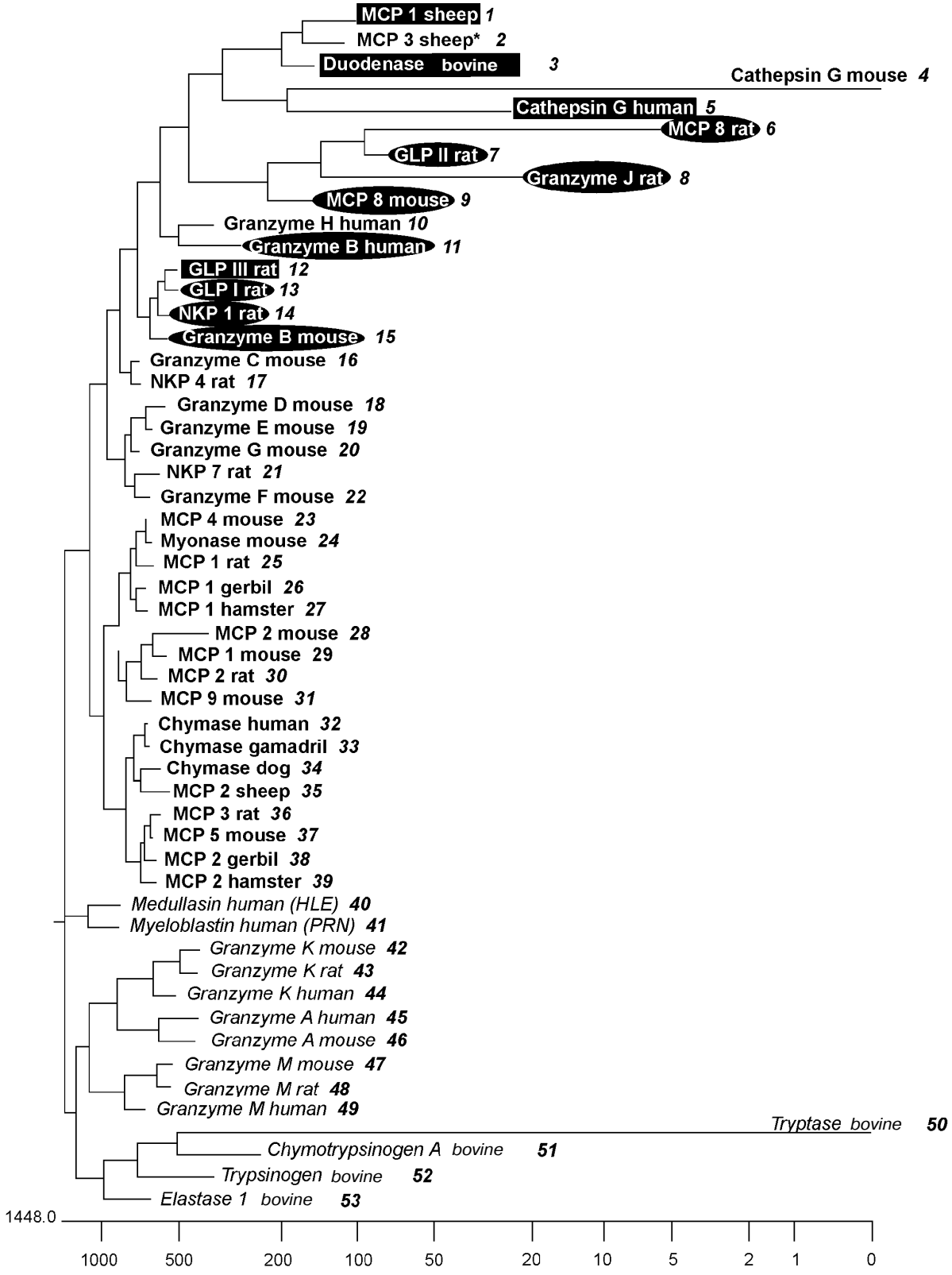
Phylogenic analysis of enzymes of the chymotrypsin family. To give more detailed analysis of graspases and their relation to proteases of the trypsin group, we have constructed a phylogenetic tree for the enzymes, including almost all known graspases and some typical representatives of the trypsin group (the figure). The tree demonstrates that proteases of the chymotrypsin family are divided between two main branches, leading to graspases (proteins 1-39) and trypsin group enzymes (proteins 42-53). Besides the two main branches, there is a third branch producing leukocyte proteases (proteins 40, 41). The leukocyte proteases—medullasin (human leukocyte protease, HLE) and myeloblastin (human leukocyte protease 3, PRN 3)—are somewhat intermediate between graspases and trypsin group enzymes: whilst having the conserved disulfide bond in their active site (Cys191–Cys220) as do trypsin group enzymes, the leukocyte proteases possess an alternate geometry of their primary specificity pocket similar to some graspases (duozymes). These proteases, despite being an intermediate link between graspases and the trypsin group, should seemingly be classified as proteases of the trypsin group, based on their structural and enzymatic properties. Indeed, the presence of a negative charge at position 226 in the S1 subsite of the enzymes does not impart any trypsin-like properties to the proteases as occurs with duozymes. Medullasin and myeloblastin possess elastase-like specificity [11, 41], i.e., residue 226 only modulates the specificity of these proteases by hampering access of bulky P1-residues into the substrate-binding pocket.

Considering the two main branches, we can see the trypsin group branch generates cluster of granzymes A, K, and M (proteins 42-49) and cluster of pancreatic enzymes and mast cell tryptase (proteins 50-53). Thus, the phylogeny of the enzymes examined here is evidence of granzymes A, K, and M belonging to the trypsin group in contrast to other granzymes (B-H and J) which are graspases. It is of interest that mast cell tryptase has common pedigree lineage with pancreatic enzymes, but not with granzymes A, K, and M, although tryptase has like the granzymes hemopoietic cell origin.

Graspases in their turn are also split into two main clusters (the figure). The first, the granzyme cluster (proteins 1-22), comprises granzymes B-G, J, H and granzyme-like proteins (GLP); natural killer proteases (NKP); cathepsin G; duodenase; some mast cell proteases (MCP) different from chymases and tryptases. The second, chymase cluster, which is represented by all chymases and myonase, branches into α- and β-chymase groups (proteins 32-39 and 23-31, correspondingly) described in [13]. In general, our results are in agreement with chymase and granzyme phylogeny described in [13, 42].

In general, our classification of graspases based on their substrate specificity (chymozymes, duozymes, and aspartases) correlates with the phylogeny of graspases. The three proposed specificity groups are not randomly superimposed on the phylogenetic tree, but are consistent with its branching pattern. It is of interest that whereas all chymases are chymozymes, enzymes of granzyme cluster, being descendants of one ancestral gene, are distributed among three specificity groups: duozymes (proteins 1, 3, 5, 12), aspartases (proteins 6-9, 11, 13-15), and chymozymes (proteins 4, 10, 16-21) (the figure).

The positioning on the tree of some graspases (proteins 2, 4, 10, 12) is in obvious contradiction with their specificity. For example, sheep mast cell protease 3 (sMCP 3) and mouse cathepsin G are found among duozymes although the proteases do not have a negative charge at position 226, which crucially determines duozyme specificity. Additionally, chymozyme human



Phylogenetic tree showing the evolutionary relationships for 53 aligned mammalian serine proteases of the chymotrypsin family. Graspase specificity subgroups are marked (proteins 1-39): duozymes, white letters in black rectangles; aspartases, white letters in black ovals; chymozymes, bold letters. Protein 2 marked with an asterisk cannot be attributed to any specific group (see text). Proteases of the trypsin group (proteins 40-53) are shown in italic

granzyme H and rat granzyme-like protein III (GLP III), predicted to be a duozyme, are positioned among the aspartases. These cases may be instances where the structural divergence of proteins combines with convergence of functional properties. An enzyme that shares the highest level of total structural identity with a certain specificity group of enzymes, nonetheless may possess an alternate specificity similar to that of a different group (functional convergence), due to selective mutation in the substrate-binding region.

In some articles concerning proteases from hemopoietic cells, such designation of enzymes can be found as chymase family, granzyme family or hemopoietic serine proteases, usually noting conservative N-terminal sequence motifs (IIGG, residues 1-4; PHSRPYMA, residues 9-16) and origin from certain type of cells. However, these indications are not sufficient to ascribe an enzyme to a definite group. For example, granzyme A and granzyme B, having the same conservative sequence motifs and the same cell origin, nevertheless belong to different groups. In the granzyme A molecule, like in trypsin, the "active site" disulfide bridge (Cys191–Cys220) is present, and Asp189 is the specificity-determining residue; therefore, granzyme A belongs to the trypsin group, whereas granzyme B is a graspase (the disulfide bridge is absent in its active site) with a crucial specificity-determining Arg226 residue. Thus, proteases structurally similar to graspases, but possessing an S1 subsite analogous to that of trypsin—granzyme A (human, mouse), granzyme K (human, rat, mouse), granzyme M (human, rat, mouse), leukocyte protease 3 (human)—belong to the trypsin group.

Confusing definitions may also appear in the literature, for example: "granzyme H is a chymase" [30]. The authors use the term "chymase" to underline chymotrypsin-like specificity of the enzyme, whereas the term is usually used to designate a mast cell protease that cleaves hydrophobic substrates. According to our classification, this should read: "granzyme H is a chymozyme (a graspase with chymotrypsin-like specificity)".

So far about 40 enzymes (all of mammalian origin) that belong to the graspase group are known. The majority of graspases are enzymes produced by hemopoietic cells (lymphocytes, leukocytes, natural killers, mast cells). These proteases participate in the immune response, inflammatory reactions, and apoptosis. In addition, there are two graspases that have alternative tissue origins—murine myonase and bovine duodenase. Myonase was found in myocytes where the enzyme presumably regulates the degeneration–regeneration of muscle [15]. Duodenase was observed to be synthesized in epitheliocytes of duodenal glands [43] and the role of duodenase was proposed to be activation of enteropeptidase, a key enzyme of the activation cascade of digestive hydrolases [44].

For a significant proportion of recently discovered tissue serine proteases, only DNA nucleotide sequence is available, and oligonucleotide sequences encoding new serine proteases are still being discovered. We believe that the proposed classification will help to correlate new enzymes with one of the two groups of the chymotrypsin family described here (trypsin group and graspases) and to predict primary substrate specificity based on the structure of the enzyme substrate binding region.

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