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Destabilase from the medicinal leech is a representative of a novel family of lysozymes

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Abstract

Intrinsic lysozyme-like activity was demonstrated for destabilase from the medicinal leech supported by (1) high specific lysozyme activity of the highly purified destabilase, (2) specific inhibition of the lysozyme-like activity by anti-destabilase antibodies, and (3) appreciable lysozyme-like activity in insect cells infected with recombinant baculoviruses carrying cDNAs encoding different isoforms of destabilase. Several isoforms of destabilase constitute a protein family at least two members of which are characterized by lysozyme activity. The corresponding gene family implies an ancient evolutionary history of the genes although the function(s) of various lysozymes in the leech remains unclear. Differences in primary structures of the destabilase family members and members of known lysozyme families allow one to assign the former to a new family of lysozymes. New proteins homologous to destabilase were recently described for *Caenorhabditis elegans* and bivalve mollusks suggesting that the new lysozyme family can be widely distributed among invertebrates. It remains to be investigated whether the two enzymatic activities (isopeptidase and lysozyme-like) are attributes of one and the same protein.

Keywords: Destabilase gene family; Lysozyme activity; Baculovirus expression; Antibodies to destabilase; Medicinal leech

1. Introduction

Earlier Baskova et al. detected in the medicinal leech salivary gland secretion a novel enzymatic endo-ε-(γ-Glu)-Lys isopeptidase activity splitting isopeptide bonds formed between glutamine γ-carboxamide and ε-lysine amino groups by transglutaminase [1,2]. The corresponding enzyme was called destabilase. The complete amino acid sequence of the purified 12.3 kDa protein exhibiting this activity as well as the sequences of three homologous cDNAs (Ds1, Ds2 and Ds3) related to the protein were determined [3,4].

The results suggested the existence of a gene family containing at least three members. Mature 115 or 116 amino acids long proteins of the family each comprising 14 highly conserved Cys-residues were formed from precursors containing specific leader

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peptides. One of the cDNAs was cloned in a baculovirus expression vector, and isopeptidase activity of destabilase has been detected in the infected insect cells [3]. This led us to a conclusion that isopeptidase activity is an inherent property of the proteins of the family.

A search for analogues in available data bases [5] revealed a clear homology between residues 2–16 of destabilase and positions 4–19 of a lysozyme from *Asterias rubens* [3,6]. This homology prompted us to test whether destabilase possesses also lysozyme activity. Here we present the data demonstrating the ability of destabilase to digest lysozyme substrates

2. Materials and methods

2.1. Purification and analysis of destabilase

Preparation of destabilase from the medicinal leech and D-D dimer monomerization assay were done as described [3]. Lysozyme-like activity of destabilase was analyzed according to [7] as follows: 40 μl of *Micrococcus lysodeikticus* bacterial walls suspension (ICN Biomedicals, USA) in 0.02 M Tris-HCl buffer, pH 7.4, was mixed with 710 μl of the buffer and 50 μl of one of the test samples (0.5 μg native destabilase, or 0.5 μg hen egg white lysozyme, or 250–500 μg of the total protein extracted from insect cells infected with recombinant or wild type baculoviruses). Inhibition of lysozyme activity by anti-destabilase antibodies was assayed by adding the antibodies to the above mixture. An initial apparent optical density of the mixture was 0.6–0.9 at a wavelength of 450 nm (A_{450}). The mixture was incubated for 2 h at 25°C with stirring. Decrease in the optical density of the mixture at 450 nm was used as a measure of lysozyme-like activity. One unit of lysozyme-like activity corresponded to a 0.001 A_{450} decrement per minute. Hen egg white lysozyme (Sigma) was used as a positive control.

All the chemicals were of analytical grade.

2.2. Escherichia coli expression system

Several different *E. coli* expression systems were tested in attempts to produce the recombinant destabilase. In all the cases the recombinant protein was expressed at high levels in the form of inclusion bodies. Attempts to detach the protein from the insoluble bodies and then to refold it into an enzymatically active form with the use of concentrated urea or guanidine chloride followed by dialysis against PBS [8] were unsuccessful.

2.3. Yeast expression systems

cDNA of Ds3 isoform was expressed in *Saccharomyces cerevisiae* (strain YBS618 [9]) under the control of Gal1 promoter essentially as described in [10]. The resulting protein was produced in an insoluble and enzymatically inactive form. Its suspension was used for immunization of rabbits.

2.4. Baculovirus systems

A baculovirus system in *Spodoptera frugiperda* cell line IPLB-Sf 9 was used to express Ds2 and Ds3 genes encoding Ds2 and Ds3 mature enzymes, respectively. Procedures of cloning and analysis of the expression products were as described [11,12].

The Ds2 cDNA was obtained from the total leech cDNA using PCR amplification with primers 1 and 2 (Table 1). Primer 1 included a *Bam*HI site, an ATG initiation codon, and seven codons corresponding to the 5′ terminus of the cDNA encoding the mature protein. Primer 2 contained a *Bgl*II site, a TTA triplet complementary to the TAA termination codon, and a sequence complementary to nucleotides 417–437 of the Ds2 gene coding region. The amplification products were digested with *Bam*HI and *Bgl*II restriction endonucleases and cloned into a pAcCL 29-1 plasmid [13] pre-digested with *Bam*HI. The structure of the resulting recombinant plasmid was confirmed by sequencing. A pAcDs2 plasmid was used as a transfer vector for insertion of the expression construct into the baculovirus genomic DNA.

A transfer vector for the Ds3 form was prepared similarly. In this case the primer for the N-terminal part of the cDNA (primer 3) contained a *Bam*H1 site, an ATG codon, and six triplets corresponding to the 5′-terminus of the mature Ds3 protein. The primer corresponding to the C-terminus of the protein (primer 4) contained a *Bgl*II site, 12 nucleotide residues complementary to the 3′-noncoding part of

Table 1
Primers used to prepare destabilase cDNA constructs for expression in a baculovirus-Sf-cells system

Primer designation	cDNA	Primer 5′–3′ sequence
1	Ds2	TATA*GGATCC*ACC**ATG**CAATTCACT GATTCTTGCCTT
2	Ds2	TATA*AGATCT***TTA**TCTCAAACATTT CTGTACCTT
3	Ds3	TATA*GGATCC*ACC**ATG**ACCGTCCCAT CCGATTGC
4	Ds3	TATA*AGATCT*CTCTTTCTTTCT**TTA**GCCC

*Bam*HI and *Bgl*II restriction endonuclease sites GGATCC and AGATCT are shown in italic, initiation codons and triplets complementary to a TAA stop codon are marked in bold.

the cDNA, a TTA triplet, and a part complementary to nucleotides 428–431 of the Ds3 cDNA [3]. The resulting vector was designated as pAcDs3.

Recombinant viruses were obtained by co-transfection of Sf 9 cells with *Sau*I linearized AcRP23-lacZ virus DNA [12], and pAcDs2 or pAc-Ds3 recombinant transfer vectors as described by Matsuura et al. [13]. Plaques with recombinant viruses (white phenotype) were selected after X-gal staining [12] and used to prepare virus stocks. The recombinant viruses were designated as AcDs2 and AcDs3, respectively. To express the inserted genes Sf 9 cells were infected with AcDs2 or AcDs3 recombinant viruses.

To obtain cell extracts, 100 ml of Sf 9 cell cultures infected with AcDs2 or AcDs3 were incubated for 72 h at 28°C in TC100 medium supplemented with 10% FCS [12]. Cells were collected, washed with PBS, sonicated (3×20 s bursts at a medium power) in 800 μl of 0.15 M NaCl and centrifuged for 10 min at 14 000 rpm in an Eppendorf tube.

The protein content of the cell extracts was analyzed by 12.5% SDS-PAGE according to Laemmli [14].

Expression of destabilase was estimated by immunoblotting with Ds2Ec+leech destabilase (LD) primary antibodies against destabilase (Table 2).

2.5. Antibody production and immunoassays

A rabbit was primarily immunized with 0.5 mg of recombinant destabilase Ds2 isoform produced by *E. coli*. For this purpose the inclusion bodies were dissolved in Laemmli buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v Glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) [14] and purified by SDS-PAGE. Next boosts of immunization were implemented with a mixture of recombinant Ds2 and purified LD. Additional boosts were made with LD with 2 month intervals. The immune serum after the third boost was collected, the corresponding antibodies being denoted as Ds2Ec+LD in Table 2. The obtained antibodies against destabilase are listed and briefly characterized in Table 2.

Ds3y and Ds2Ec antibodies were obtained in a similar way using SDS-PAGE purified recombinant Ds3 isoform from yeast and Ds2 isoform produced in *E. coli* cells, respectively.

Table 2
Characteristics of the anti-destabilase antibodies

Immunization substrate	Animal	Cross-reactivity of antibodies with different substrates				Enzyme inhibition	
		Ds3 y	Ds2 Sf[f]	Ds3 Sf[g]	Ds2 Ec	lysozyme	destabilase
LD[a]	mouse	+	+	+	+	−	−
Ds3y[b]	rabbit	+	+	nd	+	−	−
Peptide[c]	rabbit	+	−	+	nd	−	−
Ds2Ec[d]	rabbit	+	+	nd	+	−	−
Ds2Ec+LD[e]	rabbit	+	+	+	+	+	−

nd, not determined.
[a]Leech destabilase
[b]Recombinant product from the yeast expression system.
[c]The synthetic peptide.
[d]The recombinant Ds2 protein produced in *E. coli* cells.
[e]Mixture of Ds2 recombinant product inclusion bodies and LD.
[f]Ds2 protein produced in insect cells.
[g]Ds3 protein produced in insect cells.

A peptide GRPGGGYQQCTKEK (the synthetic peptide in Table 2) corresponding to amino acids 46–59 of the LD was synthesized and used for rabbit immunization as a conjugate with BSA.

In all subsequent experiments with immunoassays Ds2Ec+LD antibodies were used.

Immunoblotting was performed as described in [15]. The proteins were separated by 12.5% SDS-PAGE according to Laemmli [14] and transferred to nitrocellulose membrane (BA-85, S and S) in a semi-dry transfer unit. The membrane was incubated in blocking buffer (2% non-fat dry milk in PBS) then transferred to 1% BSA with 1:1000 dilution of the immune serum in PBS. To detect immune complexes the membrane was incubated with sheep anti-rabbit peroxidase conjugates in PBS (Sigma). The blots were developed with diaminobenzidine in PBS with 0.03% H_2O_2.

2.6. Protein concentration

Protein concentration in solutions was determined by staining the proteins with Coomassie brilliant blue [16]. Protein in insect cell extracts containing recombinant Ds2 and Ds3 proteins was quantitated by densitometry of immunoblots calibrated with immunoblots containing known concentrations of the LD using the equipment and software of UVP (USA).

Table 3
Relative changes in A_{450} of suspensions of *M. lysodeikticus* bacteria cell walls after incubation with mixtures containing destabilase or hen egg white lysozyme

	X	(Ctrl−X)*	Statistical significance#
Sus[a]	1 (Ctrl)	–	–
Sus+LD[b]	0.7	0.30 ± 0.03	hs
Sus+EWL[c]	0.68	0.32 ± 0.03	s
+ anti-destabilase antibodies			
Sus+Ab[d]	1.04 (Ctrl)	–	–
Sus+LD+Ab	0.99	0.05 ± 0.02	n
Sus+EWL+Ab	0.67	0.33 ± 0.03	s

X: Average values of A_{450}/A_{450}(Sus) ratios calculated from five or six independent measurements. An initial absolute average value of A_{450} for the *M. lysodeikticus* cell walls suspension was 0.7.

* Standard deviations of the average difference (S.D.) indicated.

The statistical significance of (Ctrl−X) values: hs -highly significant, s -significant, n -insignificant (negligible).

[a]Sus: Suspension of *Micrococcus* bacterial walls.

[b]LD: Leech destabilase.

[c]EWL: Egg white lysozyme.

[d]Ab: Anti-destabilase antibodies.

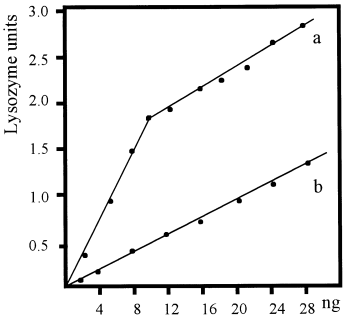

Fig. 1. Lysozyme activity of destabilase (a) and that of hen egg white lysozyme (b) versus protein concentration. Lysozyme activity was measured in units as defined in Section 2.

3. Results

3.1. Lysozyme-like activity of destabilase

The homology between the N-terminal parts of a lysozyme from *A. rubens* and destabilase suggested that destabilase might possess lysozyme activity [3,6]. We tested the lysozyme-like activity of the LD versus that of hen egg lysozyme.

The data in Table 3 show the relative decrease of A_{450} for a standard lysozyme substrate, *M. lysodeikticus* bacteria walls suspension, after treatment with either destabilase or hen egg white lysozyme. The statistical processing of the values for several independent measurements presented in Table 3 allows one to conclude with high statistical certainty that destabilase is characterized by lysozyme-like activity comparable with that of hen egg white lysozyme.

The destabilase lysozyme-like activity depends on the enzyme concentration in a biphasic manner (Fig. 1a) that differs from a linear dependence for hen egg white lysozyme (Fig. 1b). Using the initial part of the curve a specific lysozyme-like activity of destabilase was calculated to be 180 000 units/mg as compared to 41 000 units/mg of hen egg white lysozyme, a member of the c-type lysozyme family. The 'slower' part of the curve in Fig. 1a corresponds to a specific lysozyme-like activity of 43 000 units/mg. The

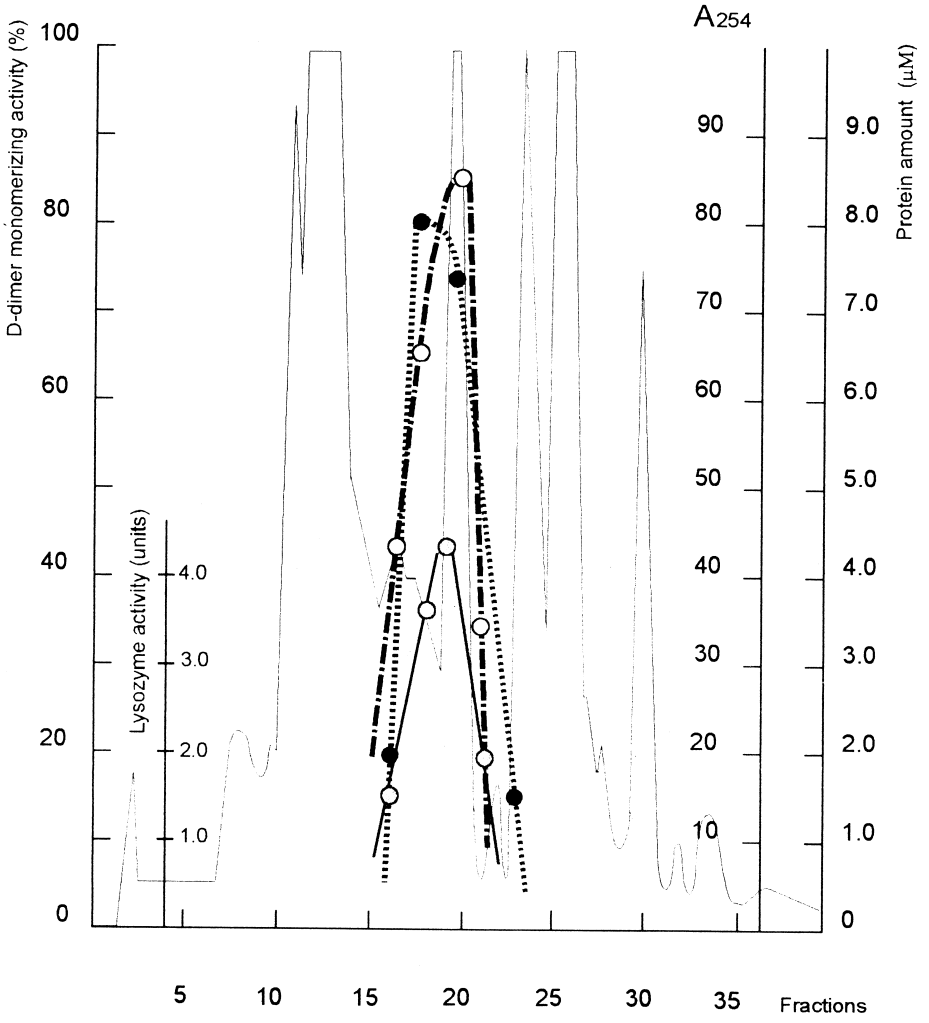

Fig. 2. D-D-dimer monomerizing (isopeptidase) activity as percent of the dimer into monomer transformation (dotted line), lysozyme activity (thick solid line), and protein amount detected by immunoassay with anti-destabilase antibodies (thick line with points) during the final step of destabilase purification procedure (gel filtration through Superose-12 HR, elution with 0.02 M Tris-HCl buffer, pH 7.4). The thin solid line: protein elution profile.

biphasic character of the curve (Fig. 1a) can not be unambiguously explained. It can be due to various factors starting with the enzyme aggregation at higher concentrations and finishing with some inhibitor contamination of the enzyme manifested at certain concentrations [17]. This point will be investigated separately.

One of several different antibodies against destabilase (see below) demonstrated statistically significant inhibition of lysozyme activity (compare rows 'Ctrl+LD' and 'Ctrl+LD+Ab' in Table 3). Therefore, lysozyme-like activity might be an intrinsic property of destabilase.

3.2. The isopeptidase and lysozyme activities comigrate during enzyme purification

Fractions of the medicinal LD eluted from a Superose-12 HR column were tested by ELISA (see Section 2) and for destabilase and lysozyme activities (Fig. 2). Destabilase loaded onto the column was pre-purified and shown to be electrophoretically homogeneous [3]. One can see that only those fractions immunopositive for destabilase possess isopeptidase and lysozyme-like activities. We were unable to separate these activities also by HPLC cation-exchange chromatography (Column Mono S, 'Pharmacia') of

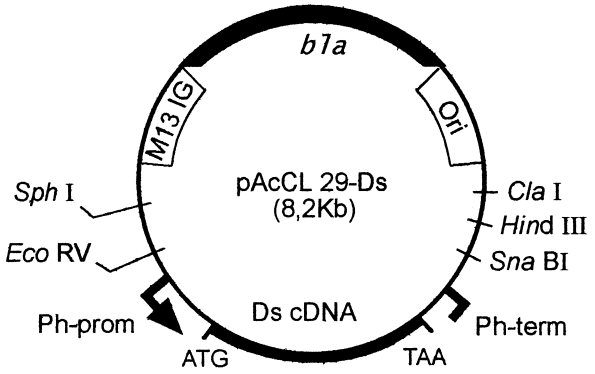

Fig. 3. The schematic views of transfer plasmid obtained as the result of cloning Ds2 and Ds3 cDNAs in the baculovirus expression vector. Positions of the beta-lactamase gene (*bla*), Ds2 or Ds3 isoforms cDNAs (Ds cDNA), ATG: start, TAA: terminating codons, polyhedrin promoter (Ph-prom), and terminator (Ph-term) are indicated.

electrophoretically homogeneous destabilase (data not shown).

3.3. Enzymatic activities in extracts of insect cells infected with recombinant baculoviruses carrying different destabilase cDNAs

Several different heterologous expression systems were tested to express isoforms of destabilase. Active destabilase molecules were produced neither in *E. coli* nor in yeast cells. In all the cases insoluble inclusion bodies were formed, and it was impossible to decompose the bodies into individual protein molecules and then to refold the molecules by known techniques used for similar purposes [8]. This can be explained by a high content of Cys-residues in the destabilase protein. Fourteen Cys residues might covalently cross-link protein molecules in the aggregates by forming intermolecular disulfide crosslinks. Even when a portion of the product was in the soluble fraction, no destabilase or lysozyme-like activity was detected[1].

In our previous paper [3] we described a detectable manifestation of isopeptidase activity in the baculovirus-insect cell system. In continuation of the work with this system we have constructed a number of baculoviruses containing all three destabilase cDNA isoforms under the control of the polyhedrin promoter. Several constructs used for the cDNAs expression are schematically presented in Fig. 3. We have failed to express Ds1 isoform but the insect cells infected with the recombinant virus containing Ds2 or Ds3 cDNAs produced protein(s) immunologically related to destabilase as judged from immunoblotting of the infected cells extracts (Fig. 4). The product was mostly present in an insoluble form, only about 5% of it being soluble.

Irrespective of the cDNA used, the level of the soluble recombinant protein was about 4.5×10^6 molecules per cell, quantitated by immunoassay for Ds2 and Ds3. That is close to the sensitivity threshold of the destabilase detection technique. On the other hand, a much more sensitive lysozyme activity assay allows one to detect 1.1×10^6 molecules for Ds2 and 0.8×10^6 molecules for Ds3.

The data presented in Table 4 demonstrate that the extracts of the insect cells infected with recombinant baculoviruses carrying either Ds2 or Ds3

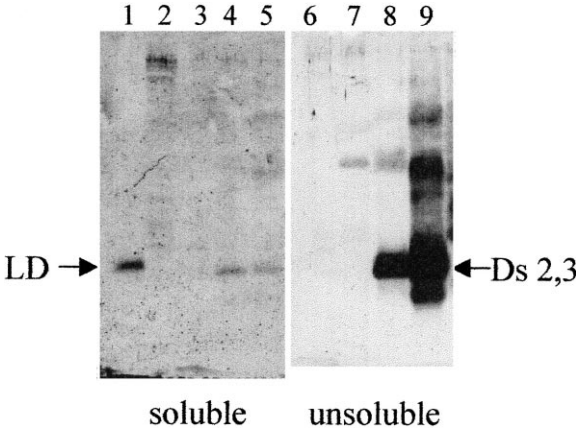

Fig. 4. An immunoblot for lysates of Sf9 cells infected with recombinant viruses. Left: Soluble fraction. Right: Insoluble fraction. Lines: 1 -LD, 2,6 - mock-induced Sf9 cells, 3,7 - Sf9 cells infected with wild type AcNPV (for virus designations see Section 2), 4,8 - Sf9 cells infected with AcDs2, 5,9 - Sf9 cells infected with AcDs3. Arrows show the positions of LD and recombinant Ds2 and Ds3 proteins. Sf 9 cells infected with recombinant or wild type viruses were suspended in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.5, buffer to a final cell concentration of 10^4/μl. The cells were disrupted by either sonication or three freezing-thawing cycles. Soluble and insoluble fractions were separated by centrifugation (14 000 rpm, 5 min.). Protein content of the fractions was determined by Western blot immunoassay with anti-destabilase antibodies.

[1] We have omitted description of our numerous attempts to achieve the expression in these traditional systems, but the results can be mailed to those interested in the expression of this protein. The cDNAs and expression constructs are also available.

Table 4
Lysozyme-like activity in extracts of *S. frugiperda* cells infected with recombinant baculoviruses containing different destabilase cDNAs, and its inhibition by anti-destabilase antibodies

	X	(Ctrl−X)*	Statistical significance#
Ctrl[a]	1.011		
Sf (Ds2)[b]	0.616	0.395 ± 0.012	hs
Sf(Ds2)+Ab[c]	0.741	0.270 ± 0.089	s
Sf(Ds3)[b]	0.723	0.288 ± 0.019	hs
Sf(Ds3)+Ab[c]	0.947	0.064 ± 0.020	n

X: Average values of A_{450}/A_{450}(Sus) ratios calculated from 5 or 6 independent measurements, where A_{450}(Sus) is an initial absolute average value of A_{450} for the *M. lysodeikticus* cell walls suspension + extract from *S. frugiperda* cells.
* Standard deviations of the average difference (S.D.) indicated.
# The statistical significance of (Ctrl−X) values: hs -highly significant, s -significant, n -insignificant (negligible).
[a]An average value of A_{450}/A_{450}(Sus) for four control measurements: (i) suspension of *M. lysodeikticus* cell walls + extract from non-infected *S. frugiperda* (1), (ii) the same as in (i) + anti-destabilase antibodies (1.017), (iii) the same as in (i) except that the extract was from *S. frugiperda* cells infected with non-recombinant baculovirus vector (1.001), (iv) the same as in (iii) + anti-destabilase antibodies (1.028).
[b]Suspension of *Micrococcus* cell walls + extract from *S. frugiperda* cells infected with baculovirus vectors with the inserts of Ds2 or Ds3 cDNAs.
[c]The same as in [b] but + anti-destabilase antibodies, respectively.

cDNAs contain the activity manifesting itself in statistically significant hydrolysis of *Micrococcus* cell walls. It suggests that both Ds2 and Ds3 isoforms possess intrinsic lysozyme activity. The antibodies added to these active cell extracts inhibited the hydrolysis, the inhibition being more pronounced in the case of Ds3, the structure of which is closer to the LD [4].

Knowing the total lysozyme-like activity in the infected insect cells and specific lysozyme activity of the LD, we have calculated that only about 20% of the immunologically detected protein revealed the lysozyme-like activity. This low value can probably be explained by incorrect folding of the protein due to high Cys content.

4. Discussion

The data presented above clearly demonstrate that the lysozyme-like activity is characteristic of destabilase. It is confirmed by: (1) high specific lysozyme activity of the highly purified destabilase; (2) specific inhibition of the lysozyme activity by anti-destabilase antibodies, and (3) significant lysozyme activity in insect cells infected with recombinant baculoviruses carrying cDNAs encoding Ds2 or Ds3 isoforms of destabilase [3,4].

As mentioned above, homology between destabilase and lysozyme from *A. rubens* [3,6] was revealed using available data bases. Moreover, the isoforms of destabilase were distinctly different in their structures from known lysozymes of the c-, g-, and p-families. Therefore we conclude that destabilase belongs to a new family of lysozymes. Recently two more homologous sequences appeared in the data bases. One was found among *Caenorhabditis elegans* genomic sequences [18], and the other belonged to a lysozyme family recently detected in bivalve mollusks [19]. The N-terminal sequences of lysozymes from this family were highly homologous to the N-terminal counterparts of destabilase (Fig. 5). Therefore the new family of lysozymes might be widely distributed among invertebrates. This view is shared by Ito et al. [20], who described homology of lysozyme from *A. rubens* with some lysozymes of invertebrates.

Earlier we have shown that there are at least three different medicinal leech genes *Ds*1–*Ds*3 encoding closely related but still distinctly different proteins with the structures homologous to that of destabilase from the leech [3]. The existence of the gene family suggests the ancient origin of their predecessor and essential functions of the lysozyme/destabilase. It is important that at least two members of the family - Ds2 and Ds3 (which deduced amino acid sequence is practically identical to the enzyme isolated from the leech) - possess lysozyme activity. As it is seen from Table 4 lysozyme activity of Ds2 is some higher than of Ds3. Lysozyme activity per 1 mg protein for Ds2 and Ds3, calculated on the base of protein concentration data presented in Fig. 4, is also higher for Ds2 than for Ds3. That is why it is not surprising that high homology exists between Ds2 and marine bivalve lysozyme, *Tapes japonica* [20] (Fig. 5). It remains to be investigated what functions the lysozymes serve in the leech and why it is necessary to have several isoforms of the enzyme. The Ds3 form was isolated from the secretion of the medicinal leech salivary glands. It is possible that the enzyme is in-

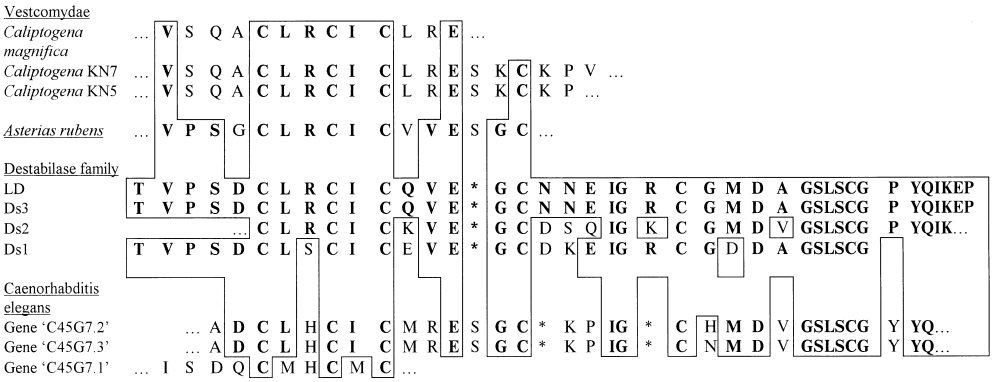

Fig. 5. N-terminal sequences of the destabilase family (LD, Ds1, Ds2 and Ds3) [4]: a comparison with an invertebrate lysozyme family (*A. rubens* [6], three Vesicomydae [19], nematode *C. elegans*, genes C45G7,1–3 [18]), * -means gaps introduced into the sequence to achieve better alignment.

volved in blood conservation in the leech stomach where blood can remain fresh for months.

The finding that destabilase can also specifically split isopeptide bonds in cross-linked fibrin monomer molecules and their fragments [1,2] and dissolve stabilized fibrin in vitro raises the question whether the same protein molecule has two different active centers. Up to now our attempts to separate the two activities using various separation systems were unsuccessful, and the lysozyme activity was always copurified with the isopeptidase activity.

On the other hand, we were unable to systematically reproduce our data on the expression of active destabilase in the baculovirus system [3]. This can be explained by rather low specific activity of the enzyme and therefore low total activity of the soluble protein fraction. Indeed, according to our estimations, the reliable detection of the destabilase activity is only possible for more than 10^8 active molecules of the enzyme per cell. The actually measured soluble protein level was 4.5×10^6 molecules per cell, of them only 20% possessed the lysozyme activity. If the same fraction of the molecules retained destabilase activity then it would be well below the sensitivity threshold of the destabilase detection technique. This is probably the reason why the previously published result [3] on the detection of destabilase activity among Ds2 expression products can be reproduced only occasionally. We were also unable to find antibodies selectively inhibiting the isopeptidase activity of destabilase but leaving the lysozyme activity unaffected. Although these two failures do not disprove our earlier suggestion of intrinsic isopeptidase activity of destabilase, they however dictate the necessity of further studies aimed at decisive evidence that the protein assigned to destabilase and characterized by lysozyme activity has also isopeptidase activity.

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