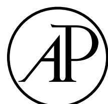

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Multiple forms of medicinal leech destabilase-lysozyme☆

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Abstract

Earlier, three genes Ds1, Ds2, and Ds3 encoding corresponding destabilase-lysozyme isoforms were identified. However only one form of the enzyme encoded by Ds3 gene coincided with the protein CNBr fragments [Mol. Gen. Genet. 253 (1996) 20]. In this work we found by ESI-TOF mass spectrometry that the enzyme preparation consists of at least three forms with molecular masses of 12677.6, 12839.7, and 12938.2 Da, each of which contains seven disulfide bridges. Only one mass (12839.7 Da) fits to the calculated mass for the protein encoded by Ds3 gene. Further analysis of the CNBr fragments of the enzyme showed the heterogeneity of large 5.5 kDa peptide at positions 64 (threonine or arginine) and 67 (histidine or arginine) in the wild-type amino acid sequence. One CNBr peptide, with Arg and His at positions 64 and 67, respectively, correlates in the molecular mass with the protein encoded by Ds3. In addition, we have found a new acid form of destabilase-lysozyme, P-Ac, which differs from all known destabilase-lysozyme structures by its N-terminal amino acid sequence.
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Keywords: Destabilase-lysozyme; Enzyme isoforms; Mass spectrometry

Destabilase-lysozyme is a component of the medicinal leech (*Hirudo medicinalis*) salivary gland secretion [1]. Its capacity to dissolve *Micrococcus lysodeikticus* is higher than that of hen egg white lysozyme [1]. Three relative genes Ds1, Ds2, and Ds3 encoding three enzyme forms were determined [2,3]. Based on the nucleotide sequences of these genes, complete primary structures of the encoded proteins Ds2-P and Ds3-P and nearly complete primary structure of Ds1 were deduced [3]. As much as 115 or 116 amino acids of these enzymes comprise 14 highly conserved Cys-residues [2]. We found that amino acid sequence of destabilase shares high homology with amino acid sequences of members of a new protein family, invertebrate lysozymes [1]. This was confirmed in subsequent studies in other laboratories [4–6]. A baculovirus system in *Spodoptera frugiperda* cell was used to express Ds2 and Ds3 mature enzymes and their lysozyme activity was determined [1].

Amino acid sequence of destabilase-lysozyme previously known as destabilase was determined by amino acid sequencing of its CNBr-fragments [2]. Molecular mass heterogeneity of one of the four CNBr peptides was revealed.

In this work three new forms of destabilase-lysozyme were identified and their molecular masses were calculated using ESI-TOF mass spectrometry. It was found that the heterogeneity of a CNBr-fragment (molecular mass about 5.5 kDa) is dependent on variations at positions 64 and 67.

Materials and methods

Chemicals. CNBr, Tris, α-cyano-4-hydroxycinnamic acid, and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO).

☆ *Abbreviations:* Ds1, Ds2, and Ds3, genes encoding destabilase-lysozymes; Ds1-P, Ds2-P, and Ds3-P, proteins encoded by genes Ds1, Ds2, and Ds3, respectively; P1, P2, and P3, wild forms of destabilase-lysozyme proteins, detected by mass-spectrometry; P-TR, P-TH, and P-RH, wild forms of destabilase-lysozyme proteins which are characterised by the amino acid variations in 64 (T or R) and 67 (H or R) positions; P-Ac, isoform of destabilase-lysozyme of calculated acid p*I* value.

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Endoproteinase Glu-C was obtained from Boehringer–Mannheim (Germany). Other chemicals were of analytical grade. All solutions were prepared using Millipore water.

Leeches. Medicinal leeches *Hirudo medicinalis* of 1.5–2.0 g were purchased from Hirud I.N. (Balakovo, Russia). They were starving at least for four months.

Preparation of destabilase-lysozyme. One kilogram of leeches frozen at −70 °C was homogenised and the total volume of resultant homogenate was adjusted to 5 L with 20 mM Tris–HCl buffer, pH 7.4. After extraction at 4 °C for 10 h the homogenate was centrifuged for 30 min at 12,000 rpm using J2-21 Beckman centrifuge (rotor JA-14) and 10% Polimin P (final concentration 0.35%) was added to the supernatant. The sediment formed was separated by centrifugation at 12,000 rpm for 15 min. For the subsequent purification two methods have been employed.

- *Method I*. Resultant supernatant was sequentially fractionated by ultrafiltration on Minitan (Millipore) using cut-off membranes of 100, 30, and 10 kDa. The fraction retained on the cut-off membrane of 10 kDa was further separated by size-exclusion HPCL using Superose 12 HR 10/30 column (Pharmacia) in 20 mM Tris–HCl buffer, pH 7.4. Fractions possessing lysozyme activity were pooled and purified by reversed-phase HPLC on Vydac C4 column (4.6 × 150 mm) using acetonitrile gradient 0–60% in the presence of 0.1% TFA during 120 min.
- *Method II*. Resultant supernatant was applied on DEAE-Toyopearl column. Protein fraction non-bounded with the resin and contained 85–90% of starting lysozyme activity was used for the isolation of P-forms of lysozyme-destabilase by a method described earlier [2].

Isolation of P-Ac form of destabilase-lysozyme. Proteins bounded with DEAE-Toyopearl column were eluted with 0.2 M NaCl in 20 mM Tris–HCl buffer, pH 7.4, where about 10% of starting lysozyme activity was detected. After the dialyses against 20 mM Tris–HCl buffer, pH 7.4, it was applied onto Mono-Q HR 10/10 column and eluted using NaCl gradient (0–0.2 M) in 20 mM Tris–HCl buffer, pH 7.4. The fractions possessing lysozyme activity were concentrated and purified by reversed-phase HPLC using Vydac C4 column as described [2]. By this way it was isolated P-Ac form of destabilase-lysozyme as more acetic protein than destabilase-lysozyme P-forms which were bounded with DEAE-Toypearl in the same conditions.

Lysozyme activity. This was determined as described previously by lysis of *Micrococcus lysodeikticus* cell wall [1].

Reduction and alkylation of destabilase-lysozyme. This was carried out as described in [7] with minor modifications. Briefly, 3 nmol of the enzyme dissolved in 80 μl of 10 mM Tris–HCl buffer, pH 7.8, containing 6 M guanidine chloride and 5% mercaptoethanol was incubated at 37 °C for 12 h. After addition of 1.5-fold excess of 4-vinylpyridine (with respect to mercaptoethanol) the mixture was incubated at 22 °C for 10 min. The alkylation reaction was terminated by injection of the reaction mixture onto the Vydac C4 column (4.6 × 150 mm). Protein eluted by the acetonitrile gradient 0–60% in the presence of 0.1% TFA during 120 min. The samples were dried on a Speedvack concentrator.

Cleavage of destabilase-lysozyme by CNBr. This was carried out in 70% TFA at 25 °C for 12 h in the darkness [8]. The obtained fragments were separated on a Vydac C18 column (4.6 × 250 mm) using the acetonitrile gradient 0–60% in the presence of 0.1% TFA during 120 min.

Hydrolysis of destabilase-lysozyme fragment 26–70 by proteinase Glu-C. This was carried in 50 mM ammonium acetate buffer, pH 4.0, as described [9]. The mixture of peptides was separated on the Vydac C18 column (4.6 × 250 mm) as described for CNBr peptides (see above).

Protein and peptide sequenation. These were carried on the Protein Sequencer model 477 (Applied Biosystems).

Mass-spectrometry. Electrospray ionisation mass spectra of protein samples were acquired using a single–quadrupole mass spectrometer (model 201, Vestec). Protein samples dissolved in 1% acetic acid and 50% methanol (5–25 pmol/μl) were infused by a syringe pump (Orioin Research) at a flow rate of 0.3 μl/min. Full-scan mass spectra were acquired in the positive-ion mode. The instrument was calibrated with multiple charged ions of β-lactoglobulin. MALDI mass spectra of peptides were obtained on time-of-flight mass spectrometer Vision 2000 (Thermo Finnigan) using α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid as matrixes. For subsequent data evaluation the software package GPMWA 4.0 (LightHouse Data, Odense, Denmark) was used.

Results

CNBr fragments of destabilase-lysozyme

Mass spectrometric analysis of two samples of destabilase-lysozyme purified by method II revealed the presence of three forms with molecular masses of 12677.6 (P1), 12839.7 (P2), and 12938.2 Da (P3). The molecular masses of intact forms P1–P3 and after reduction and alkylation with vinylpyridine differed by 1491, 1489, and 1498.7 Da, respectively. These data demonstrate the presence of 14 cysteine residues in each form. As molecular masses of these proteins after alkylation without prior reduction stayed unchanged the existence of seven disulfide bridges in the DL molecule was concluded.

CNBr cleavage of destabilase-lysozyme isolated by method I followed by subsequent fractionation of the resultant mixture on C18 column yielded 11 peptide fractions and their molecular masses (m/z) and N-terminal sequences were determined (Fig. 1). Based on the primary structure of destabilase-lysozyme [2] the positions of amino acid residues in each of the resultant peptides were determined and their molecular masses (m/z) were calculated (Table 1). The differences between determined and calculated peptide molecular masses comprise 167.5–550 Da, that depended on the presence of modified with vinylpyridine Cys residues and Met residues transformed into homoserine or in homoserinlacton.

Based on the positions of amino acid residues in the polypeptide chain of destabilase-lysozyme, all peptides were subdivided into four groups: group 1–25 (fractions 4,6), group 26–70 (fractions 7–11), group 71–94 (fraction 1), and group (95–115) (fractions 2,3). These groups corresponded to four CNBr-fragments of destabilase-lysozyme. Microheterogeneity of molecular masses of isolated peptides in groups 1–25, 26–70, and 95–115 suggested a possibility of existence of several protein forms characterised by amino acid substitutions at the same positions. So we investigated into more details of the most heterogeneous CNBr-fragment, group 26–70 (fractions 7–11).

Analysis of heterogeneity of peptide fractions corresponding to the group 26–70

Peptides of fractions 7–11 were further digested by proteinase Glu-C, fractionated on C18 column, and

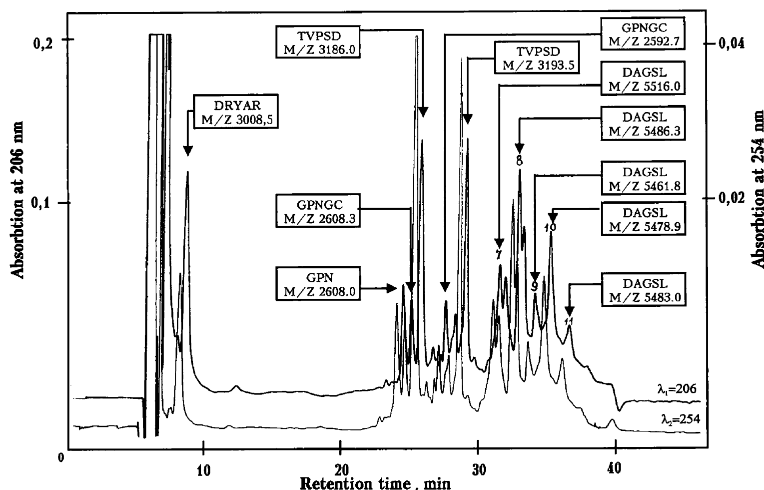

Fig. 1. Separation of CNBr-fragments of destabilase-lysozyme by RP-HPLC on a Vidac C18 column.

Table 1
Characteristics of fractionated CNBr-cleaved fragments of destabilase-lysozyme preparation

Fraction no.	N-terminal sequence	Position of residues	$[M+H]^+$ value		
			Determined	Calculated	Mass difference
1	DRYAR-	71–94	3008.5	2841	167.5
2	GPNGC-	95–115	2608.0	2378	230.0
3	GPN-	95–115	2608.3	2378	230.3
4	TVPSD-	1–25	3186.0	2701	485
5	GPNGC-	95–115	2592.7	2378	214.7
6	TVPSD-	1–25	3193.5	2701	492.5
7	DAGSL-	26–70	5516.0	4933	583
8	DAGSL-	26–70	5486.3	4933	553.3
9	DAGSL-	26–70	5461.8	4933	528.8
10	DAGSL-	26–70	5478.9	4933	545.9
11	DAGSL-	26–70	5483.0	4933	550

sequenced. This resulted in identification of the hexapeptide corresponding to amino acid positions of 64–69. There were several variants of this hexapeptide characterised by different amino acid residues at 64 and 67 positions (Table 2). The data of this table show that T-64 was identified in fractions 7 and 10 whereas other fractions (8, 9, and 11) contained R-64. There were some variations in the 67 amino acid residue. Fractions 8–11 contained H-67 whereas in fraction 7 there was equal probability for R-67 and H-67. This suggests the existence of the following hexapeptides corresponding to 64–69 region of wild type destabilase-lysozyme: 64-TCVHAY, 64-RCVHAY, and 64-TCVRAY. These peptides represent fragments of three forms of destabilase-lysozyme with calculated molecular masses of 12784.4 (P-TH), 12839.8 (P-RH), and 12803.4 Da (P-TR) (Table 3). Calculated p*I* values of recombinant and wild-type destabilase-lysozyme show similarity between wild-type and Ds3-P destabilase-lysozyme and difference between Ds1-P and Ds2-P forms.

Table 2
Analysis of heterogeneity of hexapeptide 64–69 of destabilase-lysozyme

Fraction no.	Amino acid at position 64	Amino acid at position 67
7	T	H or R
8	R	H
9	R	H
10	T	H
11	R	H

P-Ac form of destabilase-lysozyme

We have isolated a new form of destabilase-lysozyme which differs from other forms of the wild-type enzyme (P). This form was prepared in more alkali conditions (see "Materials and methods") and therefore it was marked as more acetic protein, P-Ac form. Figs. 2A and B introduced the differences in time retention of P (46 min) and P-Ac (48 min) forms of destabilase-lysozyme during the fractionations by RP-HPLC on a Vidac

Table 3
Calculated characteristics of various forms of destabilase-lysozyme

Form of destabilase-lysozyme	Amino acid residue of positions 64 and 67 (64/67)	Calculated molecular mass (Da)	Calculated p*I* value
Ds1-P	T/H	12724.1	6.13
Ds2-P	T/R	12749.7	10.44
Ds3-P	R/H	12839.5	9.62
P-TH	T/H	12784.4	9.36
P-TR	T/R	12803.4	9.62
P-RH	R/H	12839.8	9.62

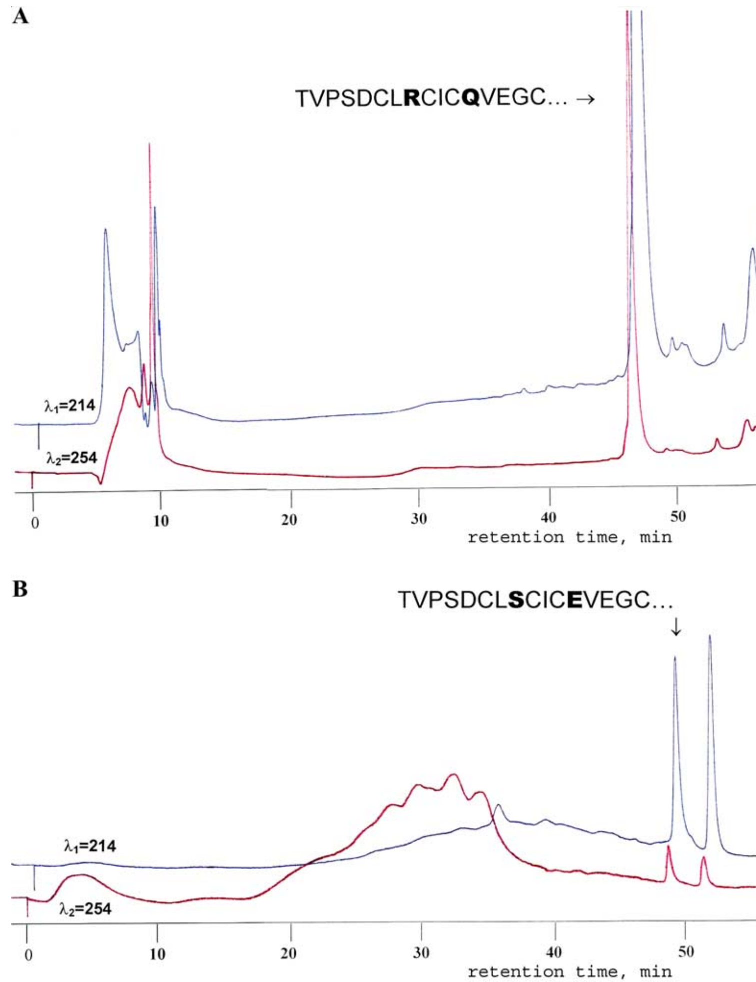

Fig. 2. Separation of destabilase-lysozyme preparations by RP-HPLC on a Vidac C4 column: (A) destabilase-lysozyme and (B) new form of destabilase-lysozyme, P-Ac.

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        1        10          20          30          40          50        60
P       TVPSDCLRCICQVEGCNNEIGRCGMDAGSLSCGPYQIKEPYWIDCGRPGGGYQQCTKEKA
Ds3-P   ------------------------------------------------------------
Ds1-P   -------S---E----DK------D---------------------S--A---E------
Ds2-P   QFTDS------K----DSQ--K----V------------K-------K-----ES---N--

                    70        80          90         100       110
P       CSETCVHAYMDRYARRCTGGRQPTCQDYAKIHNMGPNGCQSSNNH·YWDNVRRCLG
Ds3-P   ---R-----------------------------------------·----------
Ds1-P   -------------F----R--E-------------------RRTS-T·--NKANA--N
Ds2-P   ------R---K--G-F-----T-------R---G--R--K--ATVG--NK-QK--R
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Fig. 3. Amino acid sequences deduced from destabilase-lysozyme genes *Ds1*, *Ds2*, and *Ds3* and natural enzyme (P) [3].

C4 column. N-terminal sequencing of P-Ac revealed two substitutions compared with P: R-8 to S-8 and E-12 to Q-12. The sequence of 16 N-terminal amino acid residues of P-Ac was identical to that of Ds1-P [3], which is actually characterised by lower calculated p*I* value (Table 3).

Discussion

In 1996 we found a family of genes composed of at least three related cDNAs (Ds1, Ds2, and Ds3) encoding destabilase proteins, Ds1-P, Ds2-P, and Ds3-P [2,3]. Later, the repeated sequencing of these cDNAs allowed us to clarify the positions of amino acid residues in Ds1-P: H-67, A-68, F-74, R-81, and E-82 (Fradkov A.F. et al., unpublished data), which were previously designated [3] as "X." Fig. 3 shows deduced amino acid sequences of Ds1-P, Ds2-P, and Ds3-P, and primary structure of destabilase-lysozyme (P) obtained on the basis of sequencing of its CNBr-fragments. Ds1-P shares high homology with Ds3-P, which reproduces one variant of wild-type natural destabilase-lysozyme. Ds2-P significantly differs from other forms (Fig. 3, Table 3). This suggests that genes Ds1 and Ds3 were derived from one common predecessor gene, which was separated from Ds2 on early stages of evolution.

Studying heterogeneity of CNBr-fragment of highly purified enzyme which included amino acid residues 26–70 we revealed the existence of natural variants of destabilase-lysozyme. These variants are characterised by amino acid substitutions in the positions 64 and 67. Wild-type destabilase-lysozyme corresponds to proteins Ds1-P, Ds2-P, and Ds3-P by these positions. Table 3 also shows that Ds1-P, Ds2-P, and Ds3-P correspond to the variants T-64/H-67, T-64/R-67, and R-64/H-67, respectively. The latter suggests that such heterogeneity is a characteristic feature of this protein family.

Comparison of experimental and calculated molecular masses revealed that P2 form of destabilase-lysozyme is identical to P-RH and Ds3-P forms. Data of Table 3 suggest that Ds1-P is the only form of destabilase-lysozyme with calculated acidic p*I* and therefore conditions of its isolation from the natural source should differ from those employed for isolation of other forms. In fact we were unable to isolate this form using standard methods used for purification of destabilase-lysozyme. However, the employment of two sequential steps of anion exchange chromatography (instead of anion and cation exchange chromatography) at the last stages of the enzyme purification resulted in isolation of the fifth more acid form of wild-type destabilase-lysozyme (P-Ac). Its N-terminal sequencing of 16 residues revealed two substitutions (S-8/E-12) compared with P-forms (R-8/Q-12) and complete identity with Ds1-P, which also has S-8 and E-12. It is possible that such variants of amino acid residues in these positions are characteristic features of destabilase protein family. All members of invertebrate lysozyme family, including destabilase-lysozyme, are cysteine-rich proteins (14 residues per molecule) [4]. This is consistent with our data of mass spectrometric analysis. Thus, we identified the following forms of destabilase-lysozyme which differ by molecular masses and positions of some amino acid residues:

1. **P1** (12677.6 Da),
2. **P3** (12938.2 Da),
3. **P2** = **P-RH** = Ds3-P (12839.7 ± 0.2 Da),
4. **P-TR** (12803.4 Da),
5. **P-TH** (12784.4 Da),
6. Ds1-P (12724.1 Da),
7. Ds2-P (12749.7 Da),
8. **P-Ac**, is similar to Ds1-P by N-terminal sequence.

Fig. 3 shows, that in spite of the detected distinctions, the positions of cysteine residues and also Y-41 W-42 (responsible for binding of glycoside substrates) and E14 (constituting catalytic site) are strictly conservative [4,5].

Thus, results of the present study confirm the existence of several forms and/or isoforms of proteins of destabilase-lysozyme family. One of the possible reasons for such diversity of the enzymatic forms may be heterogeneity of source of destabilase-lysozyme, which included homogenates of several variants of the medicinal leech *Hirudo medicinalis*, such as *Hirudo medicinalis medicinalis*, *Hirudo medicinalis officinalis*, and *Hirudo medicinalis orientalis* [10].

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