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# Comparative Study of the Action of Bovine Duodenal Proteinases (Duodenases) on Polypeptide Substrates

**E. A. Sokolova[1]*, O. A. Mirgorodskaya[2], P. Roepstorff[3], N. V. Savelyeva[2], and T. S. Zamolodchikova[1]**

*[1]Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, Moscow, 117871 Russia; fax: (095) 310-7007; E-mail: elena@enzyme.siobc.ras.ru*
*[2]Institute of Cytology, Russian Academy of Sciences, Tikhoretskaya nab. 4, St. Petersburg, 194064 Russia; fax: (812) 247-0341*
*[3]Department of Molecular Biology, Odense University, DK-5230, Odense M, Denmark*

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**Abstract**—A comparative study of substrate specificity of bovine duodenal proteinases—chymotrypsin-like duodenase (ChlD) and dual-specificity duodenase (dsD)—was carried out using oligopeptide substrates (human proinsulin, glucagon, melittin, angiotensinogen fragment 1-14). ChlD displayed mainly chymotrypsin-like properties towards these substrates, hydrolyzing peptide bonds carboxy-terminally to bulky aliphatic or aromatic residues. In melittin, ChlD additionally cleaved peptide bonds after Thr and Ser residues. Dual-specificity duodenase (dsD) significantly restricted its specificity to only trypsin-like or only chymotrypsin-like or displayed full activity, combining both specificities, depending on substrate. Both ChlD and dsD efficiently hydrolyzed a single peptide bond (Phe8–His9) in angiotensinogen fragment 1-14. The kinetic parameters of angiotensinogen fragment 1-14 cleavage by ChlD and dsD were determined ($k_{cat}/K_m$ = 80,500 $M^{-1}\cdot sec^{-1}$ for ChlD and 103,000 $M^{-1}\cdot sec^{-1}$ for dsD).

*Key words*: duodenases, serine proteases, substrate specificity

Two bovine duodenal proteinases (duodenases) were recently identified and described [1, 2]. Duodenases belong to the serine protease superfamily and structurally resemble hemopoietic cell serine proteinases including mast cell chymases, T-cell granzymes, and leukocyte cathepsin G [2, 3]. The study of substrate specificity of duodenases has revealed remarkable differences between the enzymes. One of them, earlier reported as duodenase [1, 3, 4], possessed dual specificity. It combined both trypsin-like and chymotrypsin-like activities; in this paper it is referred to as dual-specificity duodenase (dsD). Another bovine duodenase was described as a chymotrypsin-like enzyme [2], and it was named chymotrypsin-like duodenase (ChlD). Both duodenases, dsD (26.5 kD) and ChlD (25.0 kD), are highly basic (p$I$ > 10.0) single-chain monomeric proteins [2].

It was shown [4] that dsD is synthesized in epitheliocytes of Brunner's (duodenal) glands and enters the duodenal lumen. The enzyme presumably activates the zymogen of enteropeptidase [5], which is known to initiate the cascade of activation of digestive proteases. The complete amino acid sequencing of dsD revealed that the substrate-binding pocket of the enzyme has alternative (compared with trypsin) location of negative charge (Asp226 according to the numbering of chymotrypsinogen A), this being crucial for specificity. This is believed to determine the unusual dual trypsin-like and chymotrypsin-like specificity of dsD [3]. The catalytic properties of dsD were well characterized using synthetic and natural polypeptide substrates [4]. The optimal pH range of dsD catalytic activity is 7.9-8.2 [1].

We have determined the N-terminal sequence (23 amino acids) of ChlD [2]. The substrate specificity of ChlD was studied using peptide 4-nitroanilide substrates. The best ChlD substrates appeared to be the same as the best hydrophobic substrates of dsD [2]. ChlD displays its maximum catalytic activity within the pH range 8.0-10.0 [2].

Inhibitor analysis of ChlD in comparison to dsD showed that both duodenases are sensitive to protein serine proteinase inhibitors from various beans and chymotrypsin inhibitor II from potato [2].

Here we report the results of comparative study of the ChlD and dsD specificities towards polypeptide sub-

*Abbreviations*: ChlD) chymotrypsin-like duodenase; dsD) dual-specificity duodenase; pNA) 4-nitroanilide.

* To whom correspondence should be addressed.

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strates of known amino acid sequence. Since the enzymes show a remarkable degree of selectivity in the bonds that are ultimately hydrolyzed, this study will be useful to understand the unique specificities of the duodenases and their physiological function.

## MATERIALS AND METHODS

**Reagents.** Glucagon and angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) were purchased from Serva (Germany). Melittin and dithiothreitol were from Sigma (USA). Recombinant human proinsulin was prepared at the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (Moscow, Russia).

Proteinases dsD and ChlD were purified as described previously [1, 2].

**Proteolytic degradation of melittin and glucagon by ChlD.** Enzymatic digestion of melittin (140 μM) by ChlD was performed in 0.05 M Tris-HCl, pH 8.0, at 37°C for 1 h at enzyme/substrate molar ratio 1 : 100. The reaction was stopped by adding 100% trifluoroacetic acid to obtain pH below 3.0. Hydrolysis products were separated by HPLC on a Lichrosorb RP-4 (Supelco Inc., USA) macroporous column (4.6 × 50 mm) in a 0-50% linear gradient of acetonitrile concentration in 0.07% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min. The individual HPLC fractions were analyzed by mass spectrometry (MS). Additionally, hydrolysis of melittin was performed at pH 7.8 (25 mM $NH_4HCO_3$ buffer) at 25°C for 1 h and the samples were analyzed by MS. Hydrolysis of glucagon (60 μM) by ChlD was performed in 0.05 M Tris-HCl, pH 8.0, at 37°C for 30 min at enzyme/substrate molar ratio 1 : 300. After stopping the reaction with trifluoroacetic acid, the sample was subjected to HPLC separation followed by MS analysis as described for melittin. To determine the primary sites of glucagon hydrolysis, glucagon (0.1 mg/ml) was incubated with ChlD (1 μg/ml) for 5 min, 1 h, and 6 h in 25 mM $NH_4HCO_3$ buffer, pH 7.8, at 25°C. After adding trifluoroacetic acid, the samples were analyzed by MS.

**Hydrolysis of human proinsulin.** Proinsulin (1 mg/ml) was digested with ChlD in 25 mM $NH_4HCO_3$ buffer, pH 7.8, at 25°C for 3 min and 19 h. The enzyme/substrate molar ratio was 1 : 50. The reaction mixture was incubated with dithiothreitol (130 mM) for 10 min at 37°C before MS analysis.

**Mass-spectrometric analysis.** Matrix assisted laser desorption ionization (MALDI)-time-of-flight-MS analysis of samples was performed in a Voyager-Elite Biospectrometry Workstation (Perseptiv Biosystem Inc., Framingham, MA, USA) with a delayed extraction ion source in the reflector mode. 2,5-Dihydroxybenzoic acid (25 g/liter) was used as a matrix solution in a mixture with acetonitrile containing 0.1% trifluoroacetic acid at volume ratio 70 : 30. The samples were prepared by the dried-drop method. Trifluoroacetic acid (2%, 0.3 μl) was mixed with 0.3 μl of the sample (0.5-2 pmol on the target) and 0.3 μl of the matrix solution.

**Kinetic study of angiotensinogen fragment 1-14 cleavage by dsD and ChlD.** The kinetic parameters of hydrolysis of angiotensinogen fragment 1-14 with dsD and ChlD were determined at pH 8.9 (0.05 M $(NH_4)_2CO_3$ buffer containing 1% methanol, 37°C). The substrate concentration was varied from 5 to 25 μM; the enzyme concentration was 7.4 nM. Under these conditions, the product formation was linear with respect to time over the duration of incubation. The reaction was terminated by adding 10% acetic acid. Angiotensinogen hydrolysis products were separated by HPLC on a Nucleosil $5C_{18}$ column (Macerey Nagel, Germany). Elution was performed with a linear gradient of acetonitrile concentration from 10 to 50% (v/v) in 0.05% trifluoroacetic acid. The concentrations of proteolysis products were estimated from the peak areas using the molar extinction coefficient calculated by the additive scheme [6]. Kinetic constants were determined according to Eisenthal and Cornish-Bowden [7]. Additionally, kinetic data for the cleavage of angiotensinogen fragment 1-14 by dsD were obtained at pH 8.0, when the substrate (7 μM) and the enzyme (120 nM) were incubated in 0.05 M Tris-HCl buffer at 37°C. At certain times, samples were withdrawn and the reaction stopped by adding trifluoroacetic acid. The cleavage products were separated by reverse-phase HPLC on a $5C_{18}$ column (4 × 150 mm) (NPO Diagnostikum, Russia) in a linear gradient of acetonitrile concentration from 25 to 60% in 0.06% trifluoroacetic acid. The data were interpreted by Walker–Schmidt analysis [8]. The operative concentrations of the enzymes were determined as described in our previous reports (dsD [4] and ChlD [2]).

## RESULTS

**Peptide bond specificity of ChlD.** The peptide bond specificity of ChlD was investigated using human proinsulin, glucagon, and melittin as substrates. The sites of cleavage of native proinsulin by ChlD (3 min incubation) are presented in Table 1. It was found that the primary specificity of ChlD was similar to that of chymotrypsin. ChlD preferentially cleaved peptide bonds with carbonyl groups of Leu, Phe, Ile, and Gln residues. After prolonged incubation with the enzyme (19 h), three additional cleavage sites containing Leu and Ile at the $P_1$ position (nomenclature of Schechter and Berger [9]) appeared (Table 1). No evidence for cleavage after charged or small aliphatic residues was found. The major sites of cleavage of proinsulin by ChlD appeared to be between aromatic and/or hydrophobic residues (Leu-Tyr, Tyr-Leu, Phe-Phe). Three of thirteen bonds

**Table 1.** Peptide bonds in human proinsulin hydrolyzed by ChlD

| Residue at position | | | | | | Cleaved bond location |
|---|---|---|---|---|---|---|
| $P_3$ | $P_2$ | $P_1$ | $P_1'$ | $P_2'$ | $P_3'$ | |
| Gln | His | **Leu** | 1/2**Cys** | Gly | Ser | 6-7* |
| Glu | Ala | **Leu** | **Tyr** | Leu | Val | 15-16 |
| Ala | Leu | **Tyr** | **Leu** | Val | 1/2Cys | 16-17 |
| Leu | Tyr | **Leu** | **Val** | 1/2Cys | Gly | 17-18 |
| Arg | Gly | **Phe** | **Phe** | Tyr | Thr | 24-25 |
| Asp | Leu | **Gln** | **Val** | Gly | Gln | 38-39 |
| Val | Gly | **Gln** | **Val** | Glu | Leu | 41-42 |
| Val | Glu | **Leu** | **Gly** | Gly | Gly | 44-45 |
| Gln | Pro | **Leu** | **Ala** | Leu | Glu | 56-57 |
| Arg | Gly | **Ile** | **Val** | Glu | Gln | 67-68* |
| Val | Glu | **Gln** | 1/2**Cys** | 1/2Cys | Thr | 70-71 |
| Thr | Ser | **Ile** | 1/2**Cys** | Ser | Leu | 75-76 |
| 1/2Cys | Ser | **Leu** | **Tyr** | Gln | Leu | 78-79* |

Note: Proinsulin was incubated with ChlD for 3 min. Cleavage sites marked with asterisks were observed under prolonged incubation (19 h). Amino acid residues forming the cleaved bond are marked with bold letters.

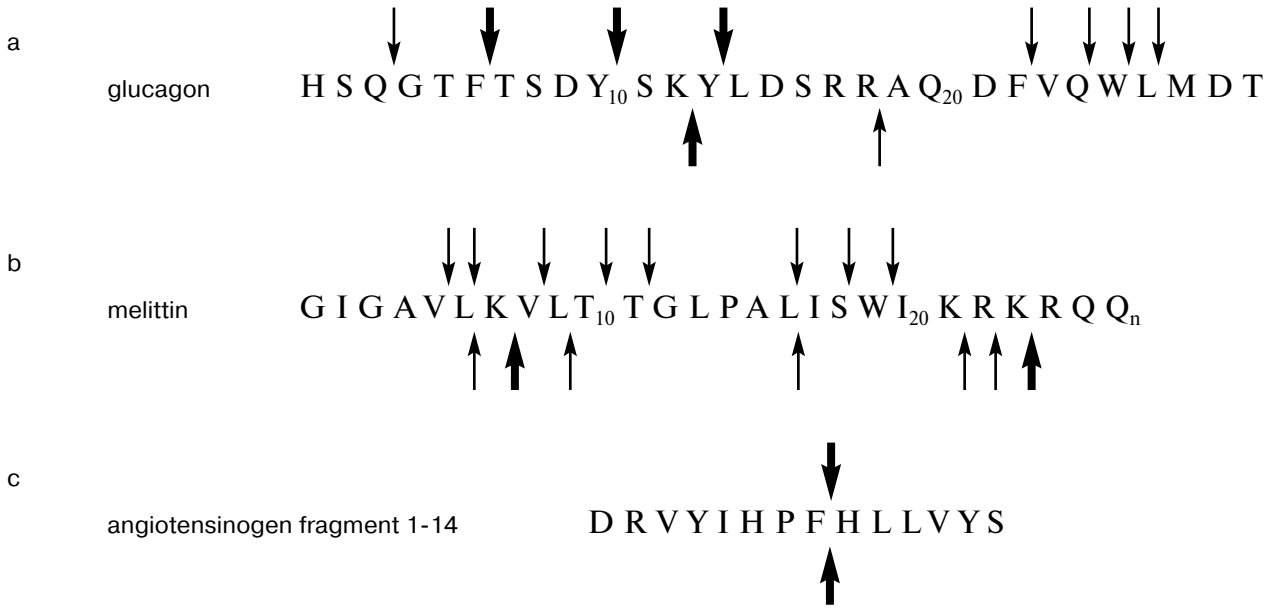
cleaved by ChlD in proinsulin have a half-cystine residue at the $P_1'$ position. No clear preference of any certain residues at the $P_2$ and $P_3$ position was observed. For comparison, the earlier discovered dual-specificity duodenase (dsD) cleaved only two peptide bonds in the proinsulin molecule (Lys29-Thr30 and Lys64-Arg65) [10].

Similar results were obtained when glucagon was hydrolyzed with ChlD (figure, panel (a)). Generally, the same set of $P_1$ residues characteristic of ChlD hydrolysis of proinsulin was observed with glucagon. Three of eight hydrolyzed bonds were cleaved by ChlD during 5 min incubation (Phe6-Tyr7, Tyr10-Ser11, and Tyr13-Leu14). With extended incubation time, secondary cleavages after Gln3, Phe22, Gln24, Trp25, and Leu26 were also noted. The cleavage sites determined for dsD under similar conditions are also presented in the figure (panel (a)). Again, in glucagon dsD cleaved two bonds containing positive charged residues at the $P_1$ position (Lys12-Tyr13, Arg18-Ala19), thus displaying trypsin-like specificity.

The sites of cleavage of melittin with ChlD in comparison to those observed for the dsD are presented in the figure (panel (b)). In addition to the $P_1$ residues found for proinsulin and glucagon hydrolysis, ChlD cleaved peptide bonds carboxy terminal to Thr and Ser in melittin. For comparison, the primary cleavage sites determined for dsD-catalyzed hydrolysis of melittin [11] were Lys7 and Lys23. The secondary cleavages were after Leu6, Leu9, Lys21, and Arg22.

**Hydrolysis of angiotensinogen fragment 1-14 by ChlD and dsD.** When ChlD and dsD were incubated with a tetradecapeptide substrate of renin (angiotensinogen fragment 1-14), the proteinases yielded the same peptide products. The rates of substrate hydrolysis were high for both enzymes. Mass-spectrometric analysis of individual chromatographic fractions allowed identification of the hydrolysis products and determination of the cleaved peptide bond. The single cleavage site of angiotensinogen fragment 1-14 hydrolysis observed for both proteinases was Phe8-His9 (figure, panel (c)). This site contained Phe and Pro residues at the $P_1$ and $P_2$ positions, respectively. Therefore, both enzymes produced angiotensin II directly from the tetradecapeptide substrate. Cleavage at Leu10, which yields angiotensin I, was not observed with the enzymes. Kinetic study of angiotensinogen fragment 1-14 hydrolysis by ChlD and dsD showed very similar kinetic constant values for this process by both enzymes (Table 2). The catalytic efficiencies ($k_{cat}/K_m$) of the renin substrate hydrolysis at pH 8.9 by ChlD and dsD were 80,500 $M^{-1}\cdot sec^{-1}$ and 50,000 $M^{-1}\cdot sec^{-1}$, respectively. The dual-specificity duodenase hydrolyzed angiotensinogen fragment 1-14 with twofold greater $k_{cat}/K_m$ value (103,000 $M^{-1}\cdot sec^{-1}$) at pH 8.0, the pH value which is optimal for the enzyme activity (Table 2).

For comparison, human chymase (an enzyme that is structurally related to duodenases and believed to be involved in angiotensin II production [12]) cleaved the same peptide bond in the substrate with about 2-4-times




Action of duodenases (ChlD and dsD) on oligopeptide substrates. Hydrolysis of glucagon (a), melittin (b), and angiotensinogen fragment 1-14 (c). Designations: ↓, cleavage by ChlD; ↑, cleavage by dsD. Primary cleavage sites are marked with bold arrows.

higher catalytic efficiency ($k_{cat}/K_m$ value) than the duodenases (Table 2). The determined catalytic parameters for hydrolysis of angiotensinogen fragment 1-14 by ChlD and dsD show that this substrate is the most reactive among the substrates studied for the two investigated duodenases.

## DISCUSSION

The present investigation was intended to compare substrate specificities of two related duodenal proteinases (duodenases) using oligopeptides with known amino acid sequence. In our earlier report [2], the enzymatic proper-

**Table 2.** Kinetic parameters of hydrolysis of angiotensinogen fragment 1-14 and the best 4-nitroanilide substrates by ChlD and dsD

| Substrate | Enzyme | $k_{cat}$, $sec^{-1}$ | $K_m$, mM | $k_{cat}/K_m$, $M^{-1}\cdot sec^{-1}$ |
|---|---|---|---|---|
| Angiotensinogen fragment 1-14 | ChlD* | 2.1 | 0.027 | 80,500 |
| | dsD* | 2.1 | 0.042 | 50,000 |
| | dsD** | 3.1 | 0.030 | 103,000 |
| | human chymase*** | 6.3 | 0.025 | 253,200 |
| Suc-Val-Pro-Phe-pNA | ChlD** | 2.8 | 1.2 | 2,300 |
| | dsD** | 0.55 | 0.64 | 850 |
| Tos-Gly-Pro-Lys-pNA | ChlD** | 0 | 0 | |
| | dsD** | 13 | 0.37 | 35,000 |

Note: The data for 4-nitroanilide substrates are from [2]. The data for hydrolysis by human chymase are from [12].
* 0.05 M $(NH_4)_2CO_3$ buffer, pH 8.9, 37°C.
** 0.05 M Tris-HCl buffer, pH 8.0, 37°C.
*** 0.02 M Tris-HCl buffer, pH 8.0, containing 0.5 M KCl, 0.01% Triton X-100, 37°C [12].

ties of the duodenases were compared using synthetic substrates (peptide 4-nitroanilides) and inhibitor analysis. It was demonstrated that the best substrates for ChlD were among the best hydrophobic substrates for dsD. Besides, most inhibitors good for ChlD were also efficient with dsD. Thus, it might be assumed that the catalytic properties of the duodenases are quite similar. But we believed that restriction of the investigated substrates to small synthetic compounds could not provide detailed characterization of the substrate preferences of the studied enzymes. Since proteinases hydrolyze various polypeptides as natural substrates *in vivo*, it was of interest to compare the action of ChlD and dsD on oligopeptide substrates of known amino acid sequence.

The results of the present study clearly demonstrate that the hydrolytic actions of ChlD or dsD on the investigated substrates can be either quite different or similar. This depends on nature of the substrate. Thirteen sites of cleavage by ChlD were observed in native human proinsulin (Table 1), and each of them contained bulky hydrophobic (Leu, Phe, Ile) or polar (Tyr, Gln) amino acid residues at the $P_1$ position. This is in agreement with the earlier determined chymotrypsin-like specificity of ChlD using synthetic substrates. When dsD was examined with human proinsulin, it was found that the enzyme behaved as a Lys-specific proteinase, cleaving only the two Lys-containing bonds present in the proinsulin molecule. Similar results were obtained with glucagon. Again, ChlD cleaved eight peptide bonds containing the same $P_1$ residues (plus Trp) as were found with proinsulin (figure, panel (a)), whereas dsD hydrolyzed the single Lys-containing bond and, additionally, one Arg-containing bond. Thus, dsD did not display its second, chymotrypsin-like specificity with proinsulin and glucagon.

The situation changed when we investigated melittin hydrolysis by the duodenases. Melittin is a useful model substrate because it contains a number of potential cleavage sites both for trypsin-like and chymotrypsin-like proteinases [11]. We found that ChlD cleaved melittin carboxy terminally to Ser and Thr as well as to the hydrophobic residues (figure, panel (b)). Hence, the substrate specificity of ChlD appeared to be broader than we had earlier assumed based on our previous results [2] and than the described specificities of chymotrypsin and various chymases [13-17]. We found that most peptide bonds cleaved by ChlD in the analyzed oligopeptide substrates were positioned between bulky aliphatic and/or aromatic residues. Analogous peculiarities of specificity were observed for dog and rat mast cell chymases, which are structurally related to ChlD [14, 17].

The dual-specificity duodenase demonstrated both types of its specificity with melittin. In addition to four cleavage sites carboxy terminal to Lys and Arg residues (trypsin-like specificity), dsD hydrolyzed three peptide bonds formed by carboxyl groups of Leu residues (figure, panel (b)).

Unexpected results were obtained when the angiotensinogen fragment 1-14, which contains several potential sites for hydrolysis by duodenases, was hydrolyzed by ChlD and dsD. With this substrate, both duodenases behaved similarly, hydrolyzing the single Phe8-His9 peptide bond and producing the octapeptide hormone angiotensin II (figure, panel (c)). The generated angiotensin II was not further degraded by either ChlD or dsD. The hydrolytic action of the duodenases on the renin substrate was similar to that observed for human mast cell chymase, which cleaved the same Phe8-His9 peptide bond in angiotensinogen fragment 1-14 and was reported as a highly efficient angiotensin II-producing enzyme [12] (Table 2).

Kinetic study of angiotensinogen 1-14 peptide hydrolysis revealed that it was the best peptide substrate for both duodenases (Table 2). The value of $k_{cat}/K_m$ found for angiotensinogen fragment 1-14 hydrolysis by ChlD was about 35 times greater than the $k_{cat}/K_m$ value earlier determined for hydrolysis of the most effective peptide 4-nitroanilide substrate (Suc-Val-Pro-Phe-pNA) [2]. In the case of dsD, the $k_{cat}/K_m$ value for angiotensinogen fragment 1-14 hydrolysis at pH 8.0 was about 3-fold higher than that for its best charged substrate (Tos-Gly-Pro-Lys-pNA). The best hydrophobic substrate of dsD (Suc-Val-Pro-Phe-pNA) was about 120-fold less effective ($k_{cat}/K_m$ value) than the renin substrate (Table 2).

Based on the results of this investigation, we suggest that the bovine mucosal duodenases have many more differences in their substrate preferences than might be concluded from the comparison of their hydrolytic action on 4-nitroanilide peptide derivatives [2]. Generally, the earlier observed chymotrypsin-like specificity of ChlD was confirmed with the oligopeptide substrates. However, besides the $P_1$ residues known as favorable for chymotrypsin and chymotrypsin-like chymases (Phe, Tyr, Leu, Gln, Trp) [15-17], $P_1$ residues such as Ser and Thr were found to be appropriate for recognition by ChlD. The proteolytic action of ChlD on the studied substrates is generally similar. Dual-specificity duodenase (dsD) may significantly restrict its specificity to trypsin-like or chymotrypsin-like or display its full activity (combining both types of its specificities) depending on the nature of the examined substrate.

Based on the results obtained with ChlD—substrate specificity (present work), N-terminal sequence and susceptibility to inhibitors [2], and the localization of the enzyme in tissues (unpublished)—we propose ChlD to be the first member of an uncharacterized family of bovine mast cell chymases.

Finding of changeable activity of dsD can broaden our conception about the role of the enzyme. It is quite possible that the function of dsD is not restricted to proenteropeptidase activation when the enzyme displays its Lys-specific activity. In the future, an alternative physiological hydrophobic substrate for dsD might be discovered.

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