Published in final edited form as:

Biochem Biophys Res Commun. 2019 May 07; 512(3): 623–628. doi:10.1016/j.bbrc.2019.03.064.

Insight into subtilisin E-S7 cleavage pattern based on crystal structure and hydrolysates peptide analysis

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Abstract

The X-ray crystallographic structure of the mature form of subtilisin E-S7 (SES7) at 1.90 Å resolution is reported here. Structural comparisons between the previously reported propeptide-subtilisin E complex (1SCJ) and our mature form subtilisin E-S7 (6O44) provide insight into active site adjustments involved in catalysis and specificity. To further investigate the protease substrate selectivity mechanism, we used SES7 to hydrolyze skim milk and analyzed the hydrolysates by LC-MS for peptide identification. The cleavage pattern suggests a high preference for proline at substrate P2 position. The results based on the peptide analysis are consistent with our structural observations, which is instrumental in future protein engineering by rational design. Furthermore, the ACE-inhibitor and NLN-inhibitor activity of the hydrolysates were determined to assess the utility of SES7 for further industrial applications; IC_{50} -ACE = $67 \pm 0.92 \mu\text{g/mL}$ and IC_{50} -NLN = $263 \pm 13 \mu\text{g/mL}$.

Keywords

Subtilisin E-S7; crystal structure; structural comparisons; ACE-inhibitor; NLN-inhibitor

1. Introduction

Subtilisins have wide applications in the detergent, leather, and food industry, due to their relatively broad substrate specificity and neutral to alkaline pH preference[1]. Especially in the dairy industry, their ability has a great potential in making bioactive hydrolysates. Several proteases from the human body, like trypsin[2] or pepsin[3], have been reported to release bioactive peptides from the milk. Those proteases could be used to pre-digest milk protein. But the only protease with different selectivity could release different bioactive

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peptide and work as compensation for gastrointestinal digestion. Here we report a serine proteases subtilisin E-S7 (SES7) with preferable solubility and catalytic preference that could be used in skim milk digestion.

About 25% of the adult population worldwide was diagnosed with hypertension in 2000 and the figure is predicted to increase to 29% (1.56 billion) in 2050[4]. Food components containing bioactive peptides, especially those with properties to relieve high blood pressure, are gaining extra attention. Angiotensin-converting enzyme (ACE, EC3.4.15.1) could indirectly increase blood pressure by hydrolyzing the dipeptide of angiotensin-I and converting it to the active form (angiotensin-II)[5]. Neurolysin (NLN, EC3.4.24.16) is another antihypertensive target which helps control the levels of the neurotensin[6]. It is also implicated in schizophrenia[7], cardiovascular disorders[8,9] Parkinson's diseases[10]. Thus, ACE-inhibitor and NLN-inhibitor peptides have great potentials to show antihypertensive effects and other bioactivities, and identifying those in milk hydrolysates is of high priority in research.

Although many earlier reports describe the hydrolysates with potential antihypertensive activity and other bioactive peptides derived from milk proteins, the mechanism of how the protease release these bioactive peptides were not discussed. Selectivity or specificity is not just controlled by one or two residues but is affected by intricate hydrogen bond networks and motions of the flexible loops surrounding the whole catalytic pocket [11-13]. It has been difficult to understand the mechanisms underlying the broad substrate selectivity of subtilisins, with relatively few structures available in the PDB and more hydrolysates peptides reported without an obvious sequence pattern.

In this study, we investigated the proteolysis mechanism of SES7 by combining protease crystal structure analysis and hydrolysates peptide analysis. The mature form of SES7 crystal structure has been solved in this paper to help us understand the domain motion around the catalytic pocket. Structural comparison between a reported structure of propeptide-subtilisin E complex and our mature form subtilisin E-S7 identifies changes of key residues and the catalytic caves. On the other hand, we examined the released peptide sequences in hydrolysates to comprehensively analyze the cleavage pattern of SES7. The results suggest that SES7 is really promising in dairy industry application.

2. Materials and methods

2.1. Strains and plasmids

Bacillus subtilis (JN-S7) was used as the source of SubtilisinE-S7 (SES7) gene. The forward (5'-CGGGATCCGCCGGAAGCAGTACAGAAAAG-3') and the reverse (5'-CCCTCGAGTTGTGCAGCTGCTTGTACGTTGAT-3') primers were used. *E. coli* 5α (NEB, US) was used as the host cells for gene cloning and DNA amplification, and BL21(DE3) (Lucigen, US) was used for protein expression. Expression vector pET-24a (+) was obtained from Novagen (US).

2.2. Protein expression and purification

The transformed *E. coli* cells were grown at 37 °C in LB medium supplemented by kanamycin (40 mg/L) till OD600 reached 1.0, after which the expression of SES7 was induced by addition of isopropyl β -D-1-thiogalactopyranoside into the medium to the final concentration of 50 μ M. The induced bacteria were further incubated overnight at 18°C, pelleted by centrifugation, resuspended in Buffer A (200 mM Tris-HCl (pH 7.4), 500 mM NaCl, 5 mM β -mercaptoethanol) and stored in a -20°C freezer. To purify SES7, the cells were disrupted by sonication on Branson Sonifier 450. Then the lysate was centrifuged at $48384 \times g$ (4 °C) for 1 hour (Beckman Avanti J-25 I, JA-25.50). Following centrifugation, the supernatant was applied to the HisPur™ Ni-NTA resin (Thermo) equilibrated by Buffer A. The resin was washed with the same buffer containing 5 mM imidazole, and subsequently His-tagged SES7 was eluted by further increasing the imidazole concentration to 300 mM. SES7 was then further purified *via* size exclusion chromatography on a HiLoad™ 26/600 Superdex™ 75 pg column (GE Healthcare) operating with 30 mM Tris-HCl (pH 7.4), 150 mM NaCl.

2.3. Crystallization

The purified SES7 was concentrated to about 30 mg/ml. Plate-shaped crystals grew in 0.15 M ammonium sulfate, 15% (w/v) PEG 4,000, 0.1 M Tris-HCl (pH 8.0).

All data sets used for model building and refinement were collected at the Advanced Photon Source at Argonne National Lab on beamlines 24-ID-C and 24-ID-E at 100 K and using the x-ray wavelength of 0.979 Å. The data were indexed, integrated, and scaled by the Rapid Automated Processing of Data beamline software (XDS[14] and CCP4[15]) or in HKL2000[16]. The PHENIX program suite was utilized for initial molecular replacement phasing, model building, and refinement. Manual model building and further refinement were performed in Coot[17] and PHENIX[18] Refine.

2.4. Preparation of skim milk hydrolysates

Skim Milk solutions (10% w/v) were sterilized by autoclaving at 110 °C for 15 min. To hydrolyze the Skim Milk proteins, the reaction mixture contained one part of the enzyme preparation and one hundred parts by volume of the Skim Milk solutions. The mixture was incubated at 37 °C for 48 h, and the reaction was stopped by heating at 80 °C for 15 min and adding PMSF to 1 mM. The hydrolysates were centrifuged (6000g for 15min), and the supernatants were collected. The hydrolysates were passed through ultrafiltration membranes (Millipore) with a cutoff of 3 kDa. The filtrates were collected for in vitro inhibitory assay and peptide analysis.

2.5. In vitro assay of ACE-inhibitory and neurolysin-inhibitory effect

The angiotensin-converting enzyme (ACE, peptidyl-dipeptidase A, EC3.4.15.1) was obtained from Sigma-Aldrich (St. Louis, MO). Rat neurolysin was recombinantly expressed in *E. coli* and purified. The working solution was added to the blank (B), control (C), or samples (S) as reported[5]. The reaction was started by adding the fluorogenic substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-p-Phe (NO₂)-Pro-OH) (0.45 mM, Bachem Feinchemikalien, Bubendorf, Switzerland). The reaction mixtures were

incubated at 37 °C in 96-well microplates. The fluorescence of the samples was measured in a Spark™ 10M multimode microplate reader (Tecan).

The activity of each sample was tested in triplicate. Inhibitory activity was expressed as the protein concentration required to inhibit 50% of the initial activity (IC₅₀). The percentage of ACE-inhibitory and Neurolysin-inhibitory activity was calculated as $100\% \times \text{Sample/Control}$. This parameter was plotted versus protein concentration, and curve fitting using nonlinear regression was performed to estimate the IC₅₀ values with the GraphPad Prism 7 software.

2.6. Peptide analysis by liquid chromatography-tandem mass spectrometry (LC- MS).

The analyses by LC-MS were performed on an Orbitrap mass spectrometer after sample clean-up. Data Analysis was used to process and transform the spectra. Peptide sequencing was performed by MASCOT (matrixscience.com)[19], using the bovine milk proteins. Error tolerance used were 0.1% for precursor masses and 0.5 Da for fragment masses. The matched MS spectra were interpreted using PEAKS 8.5 (PEAKS Studio)[20]. The Enzyme Predictor tool was used to help to analyze peptide sequences[21]. The QSAR platform AHT pin was used to predict antihypertensive Capacity in the variable length mode and amino acid composition model (SVM threshold was set to 0.9)[22].

3. Results and discussion

3.1. Cloning and sequence analysis of the subtilisin E-S7 (SES7) gene from *Bacillus subtilis* (JN-S7)

An 825-bp fragment was obtained as the major product of PCR amplification. The primers sequence is shown in Figure S1. SES7 have 3 amino acids different from subtilisin E (1SCJ); S85A, T130S, and T162S. It is worth noting that, Subtilisin E (1SCJ) has an insolubility issue during expression in *E. coli*. Thus, Subtilisin E is typically obtained in inclusion body and purified through a complicated renaturation process [23-25] and the highest yield was estimated to be 40-50 mg/liter of culture[26]. Recently, Gro Elin et al overcame this problem by fusing maltose-binding protein (MBP) or the small ubiquitin-related modifier (SUMO) to Subtilisins[27]. But we found that SES7 is soluble without such modifications (Figure S2). We hypothesize that the 3 amino acid differences mentioned above dramatically improve the solubility of SES7.

3.2. Expression of SES7 gene in *E. coli* and purification

As shown in Figure 1, the theoretical molecular mass of recombinant mature protease expressed in *E. coli* BL21 (DE3) is 28.8 kDa. The protease with propeptide is 37.4 kDa and the propeptide alone is 8.6 kDa. Other enzymatic properties were shown in Figure S3. To facilitate structural studies, we mutated the critical catalytic residues serine 221 of SES7 to a cysteine to avoid protease self-digestion. Subsequently, we successfully separated the zymogen from mature SES7 through size-exclusion chromatography (Figure 1). Both zymogen and the mature (cleaved) SES7 co-eluted with the propeptide. The cleaved propeptide is likely to be bound non-covalently with mature protease through hydrogen-bonds. In the end, we got the mature SES7 structure without propeptide suggesting that the

condition we used for crystallization allowed the mature SES7 to preferentially form crystals by itself. The reason for co-elution of zymogen with propeptide is unknown.

3.3. Molecular structure

X-ray diffraction data were collected at the beamline 24-ID-E. The summary of data collection and model refinement statistics is shown in Table 1. The asymmetric unit of the crystal structure consists of two molecules of SES7 in slightly different conformations. The all-atom RMSD is 0.312 Å (1958 atoms) after superposing these two molecules through PyMOL align function. Neither molecule is bound to the cleaved propeptide; thus our structure allows a detailed view of the mature protease without propeptide. The amino acid sequence difference between SES7 and Subtilisin E (1SCJ) are marked on the structure with spheres on Figure 2A. From the surface B-factor demonstration of the mature SES7, some loops are much more mobile and flexible (shown in yellow to red in Figure 2B) compare to other areas in the structure.

We superimposed each molecule of the SES7 structures onto the Subtilisin E-propeptide complex (1SCJ). As shown in Figure 2D&E, in both of the mature SES7 molecules, loops that surround the catalytic pocket and are within hydrogen-bonding distance from the propeptide show significantly higher B-factors than the rest of the molecule. Compared to the propeptide-bound structure (Figure 2C), the high relative B-factors of these loops in mature SES7 is also pronounced. This suggests that these three loops become more flexible once the cleaved propeptide is released from the mature protease and they may also play an important role in substrates selectivity and catalytic reaction.

To better understand how the bound propeptide affects the structure and dynamics of Subtilisin E, we docked the propeptide to our SES7 crystal structure. When we zoom into the catalytic pocket, it looks like a horseshoe-like gorge. The substrate must form a U-shape chain to fit in. The complex structure of Subtilisin E (1SCJ) is in the intermediate state with the cleaved propeptide still occupying the catalytic pocket. The Loop 100 lines a deep pocket (cave) that accommodates the P2 residue of the substrate (Figure 3A). In the propeptide-bound structure, the GLY100 position shifts by 0.9 Å when compared to that of SES7 molecule II to widen the cave. In addition, CYS 221 side chain is also turned away in the propeptide-bound structure to widen the adjacent cave for the P1 residue of the substrate. The open conformation of the catalytic pocket with the propeptide bound, and its transition to a more closed state in the mature peptidase structure may help release the cleaved product (Figure 3D). Both SES7 molecules I and II show the flexibility of GLY100. It shifts by 0.7 and 0.9 Å compared to that in 1SCJ, respectively. We can see the change in the size of the cave lined by Loop 100 clearly (Figure 3B&C). Smaller cave could give tighter binding to the substrate or higher selectivity when SES7 in the substrate-free state.

When looking at this horseshoe-like catalytic pocket, we could partially understand how SES7 substrate selectivity is achieved. Three loops form this special structure and GLY127 is in the middle, requiring the substrates to form a U-shape chain to fit in this pocket. Normally, if a proline is in the P2 of the substrate chain, it could help the chain bend into U-shape itself. We also can see from Figure 3, that SER62 and GLY100 form a P2 cave. The flexibility of GLY100 may allow longer side chain residues, like glutamine, leucine and

glutamic acid to fit in the pocket whereas SER62 could form hydrogen bonds either as a donor or acceptor which may also help stabilize the substrate chain in a U-shape conformation.

3.4. Angiotensin-Converting Enzyme-Inhibitory and Neurolysin-Inhibitory activity of the hydrolysates.

The skim milk hydrolysates (fractions containing peptides smaller than 3 kDa) were assayed for ACE-inhibitory and NLN-Inhibitory activity. The IC_{50} values are shown in Table 2. The SES7 hydrolysates exhibited considerable ACE-inhibitory activity and NLN-inhibitory activity.

During gastrointestinal digestion before absorption, the skim milk protein would go through and digested by pepsin (stomach), trypsin, α -chymotrypsin, elastase, carboxypeptidase A, carboxypeptidase B (small intestine), brush border peptidases (luminal), intracellular peptidases (enterocytes), serum peptidases (serosal)[28]. As mentioned in the introduction, we need to know how this compensatory digestion working before absorption. Here we use trypsin to make a comparison and partially simulated what might happen when the SES7 hydrolysates go through gastrointestinal digestion. After mixed with SES7 and trypsin, protein in skim milk could be digested to shorter peptide[29]. And the double-protease hydrolysates showed lower ACE-Inhibitory and higher NLN-Inhibitory activity than the hydrolysates generated by either peptidase alone. Many reports have verified that peptides with a strong ACE-inhibiting effect could inhibit neurolysin, too[6]. From our result, it seems that neurolysin can be inhibited by both shorter and longer peptides. On the other hand, the double-protease hydrolysates with a strong NLN-inhibiting effect could not inhibit Angiotensin-Converting Enzyme very well.

3.5. Cleavage patterns and hydrolysates peptide profile.

Totally 276 peptides in the hydrolysates were identified by LC-MS (Table S1). Figure 4 shows the amino acids frequency on the product peptide P3, P2, P1, and P1' positions. To determine the cleavage specificities of SES7 from the hydrolysates peptide products, we used the Enzyme Predictor tool to analyze the mass-spec results. Table 3 shows the most likely cleavage specificity obtained computationally. Strong specificity is observed for the position P2. Proline is most frequently seen at this position in the peptide products. It also shows a more even distribution than P3, P1, or P1'.

After comparing SES7 hydrolysates peptide profile with other previously reported bio-functional peptide sequences, we found that many peptides with Angiotensin-Converting Enzyme-Inhibitory activity were indeed generated (Table 4). Two high ACE--Inhibitory activity peptide were found in the peptide profile. YQKFPQY presented IC_{50} values of 20.08 μ M. HLPLPLL presented IC_{50} values of 34.4 μ M[30].

Some other hydrolysates sequences are very similar to the reported bio-functional peptide sequences. They have been analyzed by the QSAR platform AHT pin to predict antihypertensive Capacity. Table 5 shows their SVM score and prediction results from QSAR. It is easily found that highly ACE-inhibitory peptide and peptides with high SVM scores share similar sequence patterns. Their second or third amino acid from the C-

terminus (corresponding to P2 or P3 position) is proline. The C-terminal sequence of the released peptide is the main factor that affects the peptide activity [31]. Thus, the SES7 selectivity preference on P2 position for proline may account for the bioactivities of its milk hydrolysates peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Surajit Banerjee for his assistance during diffraction data collection at the Advanced Photon Source. This work was supported by the National Natural Science Foundation of China (31470160), Program for Changjiang Scholars and Innovative Research Team in University (No. IRT_15R26), and a grant from the US National Institutes of Health (R35 GM118047). And the China Scholarship Council fund for financial support.

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Figure. 1. SDS-PAGE analysis of protein purification through gel-filtration of SES7. Lane M: protein size markers; Zymogen is 37.4 kDa; Mature SES7 is 28.8 kDa; Propeptide is 8.6 kDa

Figure. 2.

(A) Overall structure of SES7 dimer in the crystal's asymmetric unit. Cartoon model shows that the two molecules of SES7 interact *via* the C-terminus of one molecule (red sphere) positioned close to the catalytic pocket (mesh) of another molecule. S85A, T130S, and T162S are shown with spheres. (B) Surface representation corresponding to the cartoon in A. The SES7 molecules are colored according to the individual atom B-factors (in a spectrum of blue to red for low to high B-factors). The high B-factors for the loops surrounding the catalytic pocket suggest high flexibility, which may be important in substrate binding. (C) The structure of 1SCJ. (D) SES7 Molecule I. (E) SES7 Molecule II.

Figure. 3.

The change of horseshoe-like catalytic pocket between intermediate state and substrate-free state. (A) Intermediate state (ISCJ) of Subtilisin E with the cleaved propeptide bound. The catalytic pocket gives two caves close to P1 and P2 of the substrate indicated by the red boxes. (B) Substrate-free state of SES7 Molecule I docked with propeptide. Two caves get smaller with the change of Loop 100 and Loop 130. (C) Substrate-free state of SES7 Molecule II docked with propeptide. Two caves are almost closed with the further change in Loop 100. (D) Side chain comparison between ISCJ (green) and SES7 Molecular II (red). The side chain of CYS221 changes its direction. The residue of GLY100 shifts by 0.9 Å.

Figure. 4.

Amino acids frequency on the product peptide P2, P1, and P1' positions

Table 1

Data collection and refinement statistics.

Data collection	
Resolution range (Å)	56.6 - 1.83 (1.90 - 1.83)
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell	
a,b,c (Å)	74.177 80.123 87.729
Total reflections	239262 (22450)
Unique reflections	46392 (4571)
Multiplicity	5.2 (4.9)
Completeness (%)	98.88 (99.13)
$I/\sigma(I)$	6.62 (1.13)
R-merge (%)	22.8 (157.4)
R-meas (%)	25.27 (176)
R-pim (%)	10.66 (76.92)
CC _{1/2}	0.963 (0.548)
Refinement	
Reflections in refinement	46326 (4567)
Reflections for R-free	2243 (227)
R-work	18.72 (30.89)
R-free	22.20 (32.40)
Number of non-hydrogen atoms	4393
macromolecules	3914
ligands	29
solvent	450
Protein residues	552
RMS(bonds)	0.003
RMS(angles)	0.63
Ramachandran favored (%)	95.62
Ramachandran allowed (%)	4.38
Ramachandran outliers (%)	0.00
Average B-factor	25.69
macromolecules	24.11
ligands	46.46
solvent	38.03
PDB entry	6044

Statistics for the highest-resolution shell are shown in parentheses.

Table 2

Angiotensin-Converting Enzyme-inhibitory and Neurolysin-inhibitory activity

	IC ₅₀ -ACE (μg/mL)	IC ₅₀ -NLN (μg/mL)
SES7	67 ± 0.92	263 ± 13
Trypsin	193 ± 23	262 ± 7
SES7&Trypsin	2101 ± 213	102 ± 13

Table 3

Cleavage patterns of SES7 determined by hydrolysates peptide profile

Cleavage specificity			
P3	P2	P1	P1'
Q,T,P,L,	P,V,Q	K,L,N,Q,F,	F,S,A

Table 4

Hydrolysates peptide sequences identified with their reported biological activity and origin

Fragment	Sequence	Reported biological activity	Reported enzyme or origin
α_{s2} -casein f(190–197)	MKPWIQPK	ACE-inhibitory IC ₅₀ =300 μ M	<i>Lactobacillus helveticus</i> [32]
α_{s2} -casein f(89–95)	YQKFPQY	ACE-inhibitory IC ₅₀ =20.08 μ M	pepsin[30]
β -casein f(134–140)	HLPLPLL	ACE-inhibitory IC ₅₀ =34.40 μ M	pepsin[30]
β -casein f(199–209)	GPVRGPFPIIV	Antihypertensive	pepsin[30]
β -casein f(200–209)	PVRGPFPIIV	Antihypertensive	pepsin[30]

Table 5

Hydrolysates peptide sequences analyzed by the QSAR

Sequence form SES7	SVM score	Prediction	Similar Reported Fragment	Similar Reported sequence	Enzyme or origin	Reported biological activity
TPVVVPFLQPE	2.18	AHT	β -casein f(81–89)	PVVVPFLQ	pepsin	Antihypertensive[30]
RGPFPIIV	1.50	AHT	β -casein f(203–209)	GPFPIIV	pepsin	Antihypertensive[30]
KAVPYPQR	0.83	AHT	β -casein f(177–183)	AVPYPQR	Trypsin	Cytomodulatory; ACE-inhibitory IC ₅₀ =15 μ M[33]
KTMTPLW	0.22	AHT	α s1-casein f(194–199)	TTMTPLW	Trypsin	Immunomodulatory[34]
QSKVLPVPQ	1.41	AHT	β -casein f(169–174)	KVLPVPQ	<i>Lactobacillus helveticus</i> CP790 protease and synthetic	ACE-inhibitory IC ₅₀ =16 μ M[35]
SKVLPVPQK	1.74	AHT	α -casein residues f(169–176)	KVLPVPQK	synthesized peptide	antioxidant activity[36]
VYPFPGIPN	2.32	AHT	β -casein (f 60–70)	YFPFPGIPN	Gouda cheese	ACE-inhibitory IC ₅₀ =10 μ M[37]
QDKIHPF	1.61	AHT	β -casein f(47–51)	DKIHP	synthesized peptide	Antihypertensive[38]