Catalytic residues and substrate specificity of scytalidoglutamic peptidase, the first member of the eqolisin in family (G1) of peptidases

Yukiko Kataoka[a], Katsumi Takada[b], Hiroshi Oyama[a], Masahiko Tsunemi[b], Michael N.G. James[c,1], Kohei Oda[a,*]

[a] *Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan*
[b] *Peptide Institute, Inc., Minoh, Osaka 562-8686, Japan*
[c] *CIHR Group in Protein Structure and Function, Department of Biochemistry, University of A1berta, Edmonton, Alta., Canada T6G 2H7*

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Abstract **Scytalidoglutamic peptidase (SGP) is the first-discovered member of the eqolisin family of peptidases with a unique structure and a presumed novel catalytic dyad (E136 and Q53) [Fujinaga et al., PNAS 101 (2004) 3364–3369]. Mutants of SGP, E136A, Q53A, and Q53E lost both the autoprocessing and enzymatic activities of the wild-type enzyme. Coupled with the results from the structural analysis of SGP, Glu136 and Gln53 were identified as the catalytic residues. The substrate specificity of SGP is unique, particularly, in the preference at the P_3 (basic amino acid), P_1' (small a.a.), and P_3' (basic a.a.) positions. Superior substrates and inhibitors have been synthesized for kinetic studies based on the results reported here. k_{cat}, K_m, and k_{cat}/K_m of SGP for D-Dap(MeNHBz)-GFKFF*ALRK(Dnp)-D-R-D-R were 34.8 s^{-1}, 0.065 μM, and 535 $\mu M^{-1} s^{-1}$, respectively. K_i of Ac-FKF-(3S,4S)-phenylstatinyl-LR-NH_2 for SGP was 1.2×10^{-10} M. Taken together, we can conclude that SGP has not only structural and catalytic novelties but also a unique subsite structure.**

Keywords: Fluorescence resonance energy transfer; Glutamic peptidase; Catalytic dyad; Pepstatin-insensitive; Inhibitor; Scytalidopepsin

1. Introduction

In 1972, scytalidoglutamic peptidase (SGP) (formerly called scytalidopepsin B) was discovered as a pepstatin-insensitive carboxyl proteinase in the culture filtrate of the wood-destroying fungus, *Scytalidium lignicolum* [1–3]. The enzyme is active at acidic pH but it is insensitive to pepstatin. Since 1972, several other pepstatin-insensitive carboxyl proteinases have been found in bacteria and in fungi [4–6]. Among them, the tertiary structure of *Pseudomonas* sp. carboxyl proteinase, sedolisin, a typical bacterial enzyme, was determined in 2001 [7]. The enzyme has the subtilisin fold but with a unique catalytic triad, Ser-Glu-Asp. Sedolisin and its family have now been classified into the S53 peptidase family of serine peptidases in the MEROPS database [7,8].

In 2003, we solved the three-dimensional structure of SGP and the results were published in 2004 [9]. The SGP molecule is a β-sandwich structure composed of two 7-stranded antiparallel ß-sheets. This structure was the first to be reported among the known peptidases. In addition, the catalytic mechanism is unique. SGP was presumed to have a catalytic dyad of Glu136 (E) and Gln53 (Q). The most likely hydrolytic mechanism involves nucleophilic attack of a general base (Glu136)-activated water (OH^-) on the *si*-face of the scissile peptide carbonyl-carbon atom to form a tetrahedral intermediate. Electrophilic assistance and oxyanion stabilization is provided by the side-chain amide of Gln53. Protonation of the leaving-group nitrogen is accomplished by the general acid function of the protonated carboxy group of Glu136. Based on these unique features, SGP has now been established as the sixth family of peptidases, the glutamic peptidase (G1) family in the MEROPS database [8,9]. This family has been named the eqolisins.

In order to clarify the structure–function relationships of SGP, mutational analysis of the Glu136 and Gln53 residues, which have been presumed to be the catalytic residues of SGP, has been carried out. In addition, the substrate specificity of SGP was investigated by using a novel type of FRET substrates [10]. Based on the results obtained here, a highly sensitive substrate for kinetic studies has been designed and synthesized. Furthermore, a potent subnanomolar inhibitor, useful for structural analysis, has also been synthesized.

2. Materials and methods

2.1. Materials

A fragment corresponding to the SGP precursor gene was cloned into expression vector pET15b (Novagen) using a PCR-based method [11]. The resultant plasmid, pET15b/SLB was a generous gift from Prof. Tsuru (Sojo University, Kumamoto, Japan). Enzymes used for construction of the expression and mutation plasmids were purchased from TOYOBO Co. (Osaka, Japan). Other reagents were purchased from Wako Pure Chemicals (Osaka, Japan). FRET substrates and the inhibitors were synthesized in the Peptide Institute, Inc. (Osaka Japan).

*Corresponding author. Fax: +81 75 724 7760.
E-mail address: bika@ipc.kit.ac.jp (K. Oda).

[1] Canada Research Chair.

Abbreviations: FRETS, fluorescence resonance energy transfer substrate; MCA, peptidyl-4-methyl-coumaryl-7-amides; AMC, 7-amino-4-methylcoumarin; Bz, benzoyl; MeNHBz, 2-(*N*-methylamino)-benzoyl; Dap, D-2,3-diamino propionic acid; Dnp, 2,4-dinitrophenyl; LC–MS, liquid chromatography mass spectrometer

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2.2. Construction of expression plasmids

A 0.7-kbp fragment of pET15b/SLB was amplified for the recombinant SGP (wild-type) using a sense primer containing an *Eco*RI site 5′-CAT GAA TTC ATG AAG TTC ACT ACC GCT GCC-3′ (S-1) and an antisense primer containing a *Hin*dIII site 5′-TAA AAG CTT TTA AAC GTA AGA GCA GGA GAC-3′ (AS-1). The expression plasmids for the SGP mutants were prepared by PCR-based mutagenesis. The oligonucleotide primers (forward and reverse) used for mutagenesis have the following sequences; D43A: 5′-GTT GGC ATT GCC GGT GAT AC-3′ and 5′-ATC ACC GGC AAT GCC AAC CC-3′; Q53A, 5′-CCA TCT TGG CAA CTG GTT TC-3′ and 5′-GAA ACC AGT TGC CAA GAT GG-3′; Q53E, 5′-CCA TCT TGG AAA CTG GTT TC-3′ and 5′-GAA ACC AGT TTC CAA GAT GG-3′; E136A, 5′-TTC ATC ATC GCG GAC TTC GA-3′ and 5′-GAA GTC CGC GAT GAT GAA CT-3′. The first PCR was carried out using pET15b/SLB as a template and two sets of primers for each mutant; one is a set of a primer S-1 and the reverse primer, and another is a set of a primer AS-1 and the forward primer. The second PCR was carried out using both of the amplified products as templates and a set of primers S-1 and AS-1. Each amplified fragment was digested with *Eco*RI and *Hin*dIII, and it was inserted into the *Eco*RI-*Hin*dIII site of pKK223-3. Nucleotide sequences of all the expression plasmids (pWT, pD43A, pQ53A, pQ53E, and pE136A) were determined and verified correct.

2.3. Expression, refolding, and acid activation

Escherichia coli JM109 cells harboring the expression plasmids were cultured at 30 °C in 2 l of M9 medium containing 50 μg/ml of ampicillin until absorbance at 660 nm = 0.6. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM and the growth was continued for an additional 2 h. After disruption of the cells by sonication, hydrochloric acid (1 N) was added to the suspension until pH 5. The resultant precipitant was dissolved in 80 ml of a denaturation buffer (8 M urea, 0.1 M ß-mercaptoethanol, 1 mM EDTA, 50 mM NaCl, 50 mM Tris–HCl, pH 8.0). After incubation for 1 h at room temperature, ethanol was then added to a final concentration of 30%. After centrifugation at 48 000 × *g* for 30 min, the recombinant protein was purified by DEAE-Sepharose CL-6B equilibrated with a denaturation buffer. The eluate (0.2–0.3 M NaCl) was dialyzed against each 20 times volume of 6, 4, or 2 M urea in PIPES buffer (50 mM NaCl, 1 mM EDTA, 0.1 M PIPES-NaOH buffer, pH 6.5) for 2 h at 4 °C. In addition, the sample was dialyzed against PIPES buffer overnight at 4 °C. In order to activate the expressed SGP, the resultant solution was dialyzed against 100 times volume of 10 mM acetate buffer, pH 4.0 at 4 °C for 12 h. Detection of the expressed proteins was performed by Western hybridization using a mouse anti-SGP antibody.

2.4. Assay of peptidase activity and protein concentration

Enzyme activity was assayed at 37 °C in 0.1 M acetate buffer, pH 4.0 containing 10 mM $CaCl_2$ and 0.005% Triton X-100 using D-Dap(MeNHBz)-GFKFF*ALRK(Dnp)-D-R-D-R (*, cleavage site) as a substrate. Upon the cleavage of any peptide bond between D-Dap(MeNHBz) and Lys(Dnp) in the substrate, the fluorescence at λ_{ex} = 340 nm and λ_{em} = 440 nm increases in proportion to the release of Nma fluorophore from the internal Dnp quencher. Initial velocity for hydrolysis was measured with 1 μM of the substrate. Protein concentration was estimated from the amino acid analysis of each sample.

2.5. Substrate specificity

SGP used in this study was purified according to the method of Oda et al. [1]. The FRETS-25Xaa libraries (D-Dap(MeNHBz)-G-Zaa-Yaa-Xaa-A-F-P-K(Dnp)-D-R-D-R) used to study amino acid preference at the P_1 position, have been described previously [10]. Xaa represents the fixed position where each of the 19 natural amino acids excluding Cys was incorporated. A mixture of five amino acids (P, Y, K, I, and D) was incorporated at the Yaa position along with a mixture of five amino acids (F, A, V, E, and R) at the Zaa position for each fixed Xaa. This provides a peptide mixture of 25 combination of each Xaa series resulting in a combinatorial library with a total of 475 peptide substrates in 19 separate pools.

FRETS-25F (D-Dap(MeNHBz)-G-Zaa-Yaa-F-A-F-P-K(Dnp)-D-R-D-R) was used for analyzing the P_2 and P_3 preferences. Cleavage of the substrate was monitored with increase of the fluorescence intensity (λ_{ex} = 340 nm and λ_{em} = 440 nm). Molecular weight of the cleaved product was determined by LC–MS according to the previous report [10]. Based on its molecular weight, the primary structure of the cleaved product and cleavage site were identified.

For analyzing the primed-side preferences of eqolisin, three combinatorial libraries (D-Dap(MeNHBz)-GFKFF*Xaa-Yaa-Zaa-K(Dnp)-D-R-D-R) were synthesized. Xaa, Yaa, and Zaa represent the fixed position where each of the 19 natural amino acids excluding Cys was incorporated (FRETS-19FP, Yaa and Zaa were fixed by Phe and Pro; FRETS-A19P, Xaa and Zaa were fixed by Ala and Pro; FRETS-AF19, Xaa and Yaa were fixed by Ala and Phe).

2.6. Kinetics assays

D-Dap(MeNHBz)-GFKFF*ALRK(Dnp)-D-R-D-R (sub-1) and D-Dap(MeNHBz)-GFKFF*AFPK(Dnp)-D-R-D-R (sub-2) were synthesized for kinetics study. The initial velocities were measured at several different concentrations of substrate at 37 °C (100 mM acetate buffer pH 4.0 containing 10 mM $CaCl_2$ and 0.005% Triton X-100). K_m and V_{max} were derived from the direct fitting to the Michaelis–Menten equation using a non-linear regression analysis program, with at least five values of the initial substrate concentration used.

2.7. Inhibition study

SGP (0.28 nM) was mixed with varying amounts of inhibitor (0–1.63 nM) and incubated for 10 min at 37 °C and pH 4.0. Because the K_i value of the inhibitor was of the same order as the enzyme concentration, we analyzed the inhibition according to the procedure described by Tashiro et al. [12], and by Laskowski and Kato [13], which have been widely used in the analysis of tightly-binding inhibitors. Residual enzyme activity was estimated from the initial linear part of fluorescence intensity increase. The slopes were converted into free enzyme concentrations together with the initial concentrations of the inhibitor to calculate the association equilibrium constants, K_a (=1/K_i), using personal software devised by Tashiro. Each titration curve was fitted to the theoretical equation of Bieth and Frechin [14] using a non-linear least-squares analysis program. The best-fit line through the data points yielded K_a.

3. Results and discussion

3.1. Identification of catalytic dyad, Glu136 and Gln53

SGP is synthesized as a precursor protein consisting of a prepro region (54 residues) and a mature region (206 residues) [15]. The mature enzyme has significant sequence similarity to the other fungal carboxyl peptidases, *Aspergillus niger* carboxyl peptidase (ANCP), *Sclerotinia sclerotiorum* carboxyl peptidase, *Cryphonectria parasitica* peptidases B and C, and *Talaromyces emersonii* carboxyl peptidase [9].

We selected the residues Glu136, Gln53, and Asp43 for mutational analysis. These residues are conserved in all of the enzymes described above. Glu136 and Gln53 were identified as the presumed catalytic residues by structural analysis [9]. Asp43 which corresponds to the Asp53 in ANCP, had been suggested as one of the catalytic residues by site-directed mutagenesis of ANCP [16].

Wild-type SGP was expressed as a precursor form in *E. coli*. After refolding, the precursor was autocatalytically converted to the mature form under acidic conditions (Fig. 1). The mature enzyme showed normal peptidase activity. E136A, Q53A, and Q53E mutants were expressed as precursor forms in the same manner as the wild-type enzyme (Fig. 1). However, none of the refolded precursors were converted to a mature form under acidic conditions (Fig. 1). In addition, they did not show any peptidase activity. These results strongly support the contention that residues Glu136 and Gln53 are involved in the catalytic function as predicted by James [9].