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Effect of oxidation of the non-catalytic β-propeller domain on the substrate specificity of prolyl oligopeptidase from *Pleurotus eryngii*

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ABSTRACT

Enzymes belonging to the S9 family of prolyl oligopeptidases are of interest because of their pharmacological importance and have a non-catalytic β-propeller domain. In this study, we found that the oxidation of Met203, which lies on surface of the β-propeller domain, leads to change in the substrate specificity of eryngase, an enzyme from *Pleurotus eryngii* and a member of the S9 family of prolyl oligopeptidases. The activity of eryngase for L-Phe-*p*-nitroanilide was maintained following hydrogen peroxide treatment but was dramatically reduced for other *p*-nitroanilide substrates. MALDI-TOF MS analysis using tryptic peptides of eryngase indicated that the change in substrate specificity was triggered by oxidizing Met203 to methionine sulfoxide. In addition, mutations of Met203 to smaller residues provided specificities similar to those observed following oxidation of the wild-type enzyme. Substitution of Met203 with Phe significantly decreased activity, indicating that Met203 may be involved in substrate gating.

1. Introduction

Enzymes belonging to the prolyl oligopeptidase (POP) family (clan SC, family S9 in the MEROPS peptidase database [1], http://merops.sanger.ac.uk/) are widely distributed in nature, and have the classic Ser/His/Asp catalytic triad [2]. Based on their primary structures and biochemical characteristics, the enzymes of this family are further classified into four distinct subfamilies: S9a, S9b, S9c, and S9d. Subfamily S9a POP in humans is involved in the degradation of neuropeptides responsible for the modulation of memory processes and thus POP is implicated in Alzheimer's disease [3]. In contrast, oligopeptidase B (OpdB) of subfamily S9a is believed to be associated with the pathogenesis of *Trypanosoma brucei* and *T. evansi*, which cause trypanosome infection [4,5].

A common feature of the S9 POP family of enzymes is that they cleave peptides shorter than 30 residues [6–8]. Most enzymes of this family consist of a characteristic β-propeller domain and an α/β hydrolase catalytic domain [8–11]. The former domain is believed to block the entry of large protein substrates into the α/β hydrolase catalytic domain [9]. Therefore, enzymes of this family with a β-propeller domain are unable to hydrolyze large proteins. The mechanisms underlying substrate recognition and substrate entry to the active site are of interest to many researchers because of the pharmacological importance of family S9 POPs, but these mechanisms remain unclear.

The enzyme activities of several family S9 POPs are affected by thiol-reacting regents and salt. Morty et al. identified three cysteine residues in OpdB from *T. brucei* as key residues for the thiol-reactivation of the enzyme [12]. Their report suggested the possibility that the activity-enhancing effect of reducing reagents is correlated to a conformational change of the enzyme. Usuki et al. examined a cysteine-free mutant of OpdB from *Streptomyces griseus* and confirmed that the mutant was markedly activated by general thiol-reactive reagents [13]. They suggested that a conformational change and activity-enhancing effect induced by thiol-reacting reagents can originate in a thiol-independent manner. Salt also affects these enzymes, and Polgar reported that the activity of porcine POP was enhanced by high concentrations of NaCl [14].

Abbreviations: pNA, *para*-nitroanilide; POP, prolyl oligopeptidase; DTT, dithiothreitol.

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Polgar further reported that the rate-determining step for POP catalysis is its conformational change, which might depend on several factors such as pH and salt concentration [14–16]. In addition, an enzyme kinetics study of porcine OpdB revealed a change in the substrate specificity in the presence of high concentrations of NaCl [17]. Details of structural changes associated with the change in substrate specificity are currently unavailable and therefore the identification of other factors that induce changes in substrate specificity would provide new insights into the substrate recognition mechanisms of family S9 POPs, thereby enhancing their emerging therapeutic utility.

We previously identified an enzyme from *Pleurotus eringii* that belongs to the family S9 POPs and named this enzyme eryngase [18,19]. A preliminary study demonstrated that the substrate specificity of eryngase is altered by oxidative treatment with hydrogen peroxide. However, the mechanism by which the substrate specificity of the enzyme changes following oxidation remains unknown. Eryngase belongs to subfamily S9c (based on its primary structure), yet the overall structure of all S9 POPs, including eryngase, are extremely similar to each other. We therefore focused on changes in the substrate specificity of eryngase following treatment with hydrogen peroxide. In this study, we attempted to identify the key residue responsible for the change in substrate specificity following oxidation and identified the 203th residue (Met203), which lies on the surface of the β-propeller domain. We mutated Met203 and assessed the substrate specificity of the mutants to elucidate the role of this residue in substrate recognition.

2. Materials and methods

2.1. Materials, bacterial strains, and plasmids

Aminoacyl-*p*-nitroanilides (pNAs) were purchased from Bachem AG, Aldrich Chemical Co. Inc., Sigma Chemical Co., Novabiochem Co., or Wako Pure Chemical Industries Ltd. Plasmid pET22b/Ery, constructed in an earlier study, was used for expression and as a template for mutation. *Escherichia coli* (*E. coli*) JM109 was used as a host for plasmid construction and *E. coli* Rosetta (DE3) was used as a host for expression.

2.2. Enzyme assay

Enzyme activity was determined using a continuous spectrophotometric assay with aminoacyl-pNAs. Aminoacyl-pNA (20 μL, 2 mM) was added to 80 μL of a mixture containing 75 μL of 200 mM Tris−HCl (pH 8.0) and 5 μL of a diluted enzyme solution in a 96-well microtiter plate at room temperature. The increase in absorbance at 405 nm due to the release of pNA was monitored each minute using a microtiter plate reader (Bio-Rad Laboratories Inc.). The initial activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$) was determined from the linear portion of the optical density profile.

2.3. Site-directed mutagenesis

Mutant enzymes were constructed by site-directed mutagenesis using pET-22b/Ery, inverse PCR, and a pair of primers containing a point mutation (Table S1). The PCR program comprised 18 cycles of 1 min at 98 °C and 1.5 min at 68 °C. The PCR products were treated with Dpn I at 37 °C for 6 h, then the products were transfected into competent *E. coli* JM109 cells according to the manufacturer's protocol. The plasmid was extracted and correct cloning was confirmed by sequencing.

2.4. Expression and purification of recombinant eryngase and its variants

E. coli Rosetta (DE3) harboring pET-22b/Ery or the expression vector for mutant eryngase production was cultivated at 15 °C for 72 h in 20 mL Overnight Expression Instant TB medium (Novagen Inc.) containing 50 μg mL^{-1} ampicillin. The harvested cells were suspended in 20 mM Tris-HCl (pH 8.0) containing 1 mM DTT (buffer A) and disrupted by ultrasonication on ice. After removal of the cell debris, the supernatant was brought to 50% saturation with ammonium sulfate, then the resulting supernatant was brought to 70% saturation with ammonium sulfate. The resulting precipitate was dissolved in buffer A containing 10 mM $CaCl_2$, then the solution was heated at 55 °C for 30 min. The precipitate was removed by centrifugation and the supernatant was brought to 40% saturation using ammonium sulfate. The solution was loaded onto a butyl Toyopearl (Tosoh Corp.) column equilibrated with buffer A containing 40% ammonium sulfate. After washing the column with buffer A containing 30% ammonium sulfate, the enzyme was eluted with buffer A containing 20% ammonium sulfate. Fractions exhibiting high activity were pooled and then dialyzed against buffer A. The dialysate was loaded onto a Vivapure-Q spin column (Sartorius AG) equilibrated with buffer A. After washing with buffer A containing 0.25 M NaCl, the bound protein was eluted with buffer A containing 0.4 M NaCl. Eluate with high activity was used as the purified enzyme solution.

2.5. Identification of the oxidation site

Eryngase was treated with 1% hydrogen peroxide for 0, 1, and 8 h, heated at 95 °C for 10 min, then digested using approximately 0.05 mg mL^{-1} trypsin for 2 h at room temperature. The tryptic eryngase peptides were desalted using a Zip Tip (Merck Millipore Co.), then analyzed using an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics Inc.) using sinapinic acid as the MALDI matrix.

2.6. Time dependence of oxidation on the activity of eryngase

Solutions of wild-type or mutant eryngase (0.02 mg mL^{-1}) were treated with 1% hydrogen peroxide for between 20 min and 8 h at 30 °C. The oxidation reaction was terminated by adding a solution of catalase to the eryngase solution to a final concentration of 0.2 mg mL^{-1}. This mixture was used to characterize oxidized eryngase.

2.7. Enzyme kinetics

The K_m and k_{cat} values of wild-type, oxidized, and mutant eryngase for the catalysis of L-Phe-pNA and L-Leu-pNA were measured by conducting the reactions at different concentrations of the respective substrates (0.003125–0.4 mM) under the conditions described in subsection "2.2 *Enzyme assay*" and constructing Lineweaver-Burk plots. The molecular weight of erymgase used for these calculations was 72,840 g mol^{-1}.

3. Results

3.1. Change in the substrate specificity of eryngase following treatment with hydrogen peroxide

As shown in Fig. 1A, the pNA-release activity of wild-type eryngase for L-Phe-pNA increased after 20 min treatment, then gradually decreased as the treatment time increased, whereas its activity for L-Leu-pNA dramatically decreased within 60 min.

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