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Site-directed mutagenesis of myofibril-bound serine proteinase from *Crucian carp*: possible role of Pro95, A127 and I130 on thermal stability

Meng-Si Li[a,b], Ting Li[a,b], Xue Lu[a,b], Le-Chang Sun[a,b], Yu-Lei Chen[a,b], Hong Liu[a,b], Min-Jie Cao[a,b,*], Guang-Ming Liu[a,b,*]

[a] College of Food and Biological Engineering, Xiamen Key Laboratory of Marine Functional Food, Fujian Provincial Engineering Technology Research Center of Marine Functional Food, Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Jimei University, 43 Yindou Road, Xiamen, 361021, Fujian, PR China
[b] Engineering Research Center for Aquatic Products Processing of Fujian Province, Xiamen, 361021, PR China

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ABSTRACT

The myofibril-bound serine proteinase (MBSP) from the skeletal muscle of fish belongs to a class of trypsin-like serine proteinases with higher thermal stability than trypsin. To investigate the mechanism responsible for the thermal stability of MBSP from *Crucian carp*, mutagenesis of key residues were identified by amino acid sequence alignments and Protein Mutant Stability Prediction online prediction software. Meanwhile, five mutants (MutP95T, MutR125N, MutA127S, MutI130D, and MutA127S/I130D) were obtained using site-directed mutagenesis. The mutants and wild-type enzymes were expressed in *Pichia pastoris* (SMD1168), the secondary structure of the mutants of MBSP was approximately similar to that of wild-type MBSP by circular dichroism spectroscopy analysis. The thermal Inactivation parameters showed that the half-life for thermal inactivation ($t_{1/2}$) of MutP95T was reduced by approximately 10 min, whereas MutA127S, MutI130D and MutA127S/I130D were more stable than the wild-type and increased by approximately 18 min, 15 min and 24 min, respectively. The unfolding free energy difference ($\Delta\Delta G$) between the variants and wild-type of MutA127S, MutI130D and MutA127S/I130D was enhanced when compared with the wild-type. As a result, the mutants were efficiently produced with altered thermal stability, and MBSP could potentially be used as novel tool enzyme for mass spectrometric identification.

1. Introduction

The myofibril-bound serine proteinase (MBSP) belongs to a class of trypsin-type serine proteinases, which can specifically recognize the carboxyl terminal of arginine or lysine residues during protein degradation and has higher thermal stability than trypsin [1]. Currently, trypsin is used in protein mass spectrometric identification to obtain peptides for mass fingerprinting analysis [2]. As the cleavage specificity of MBSP is similar to trypsin and has better thermal stability, MBSP could potentially be used as a novel enzyme for mass spectrometric identification. However, the content of native MBSP is extremely low in muscle and purification is not practical for commercial applications. When 300 g of crucian carp muscle was purified, only 1.2 mg of purified MBSP was obtained [3], and it is a time-consuming and expensive purification procedure.

To achieve high-yield production, it is necessary to use genetic engineering technology to produce recombinant MBSP. In previous studies, the cDNA sequence of MBSP from crucian carp, carp and silver carp was cloned [3–5], which laid the groundwork for expression. The cloning results showed that the mature protein of MBSP (excluding the signal peptide) from the three types of freshwater fish all contained 222 amino acid residues, and their sequence identity was greater than 88%, which indicated that the gene sequence was relatively conservative in freshwater fish. The active site center of MBSP consists of His, Asp and Ser residues (the catalytic triad) responsible for the acyl transfer mechanism of catalysis [3], and both His and Asp residues are located in the N-terminal domain while Ser residues are found in the C-catalytic domain.

* Corresponding authors at: College of Food and Biological Engineering, Xiamen Key Laboratory of Marine Functional Food, Fujian Provincial Engineering Technology Research Center of Marine Functional Food, Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Jimei University, 43 Yindou Road, Xiamen, 361021, Fujian, PR China.
E-mail addresses: mjcao@jmu.edu.cn (M.-J. Cao), gmliu@jmu.edu.cn, liugmo@sina.com (G.-M. Liu).

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Recently, our group successfully expressed MBSP from *Crucian carp* in *Escherichia coli* [6] and *Pichia pastoris* [1]. Unfortunately, the recombinant MBSP was mainly expressed in the form of inclusion bodies and revealed no biological activity after renaturation in *Escherichia coli*, and a glycosylation MBSP obtained with amino acid residue Ala35 mutated to Val utilized the GS115-pPIC9 K expression system. The glycosylation MBSP was confirmed by phenol-sulfuric acid method and PAS reaction.

MBSP and trypsin have the same catalytic triad and the similar substrate binding site, which suggests that they have a similar catalytic mechanism. However, mechanism for high stability of MBSP has not been elucidated. To determine the relationship between the thermal stability mechanism and protein structure, a suitable and efficient method must be chosen, for example, the site-directed mutation technique and homology modeling method have been used [7–10]. To date, the homology modeling method has been widely used to construct the tertiary structure of proteins quickly and with high accuracy [11,12].

Many successful protein engineering examples for thermostability studies have been reported. For instance, the thermal stability of the lipase mutant was improved by the formation of hydrophobic interactions and salt bridges, which enhanced the rigidity of the protein surface [13]. Proline improved the thermal stability by enhancing the rigidity of the luciferase flexible region [14]. Similarly, the increased thermal stability of the double mutant (Q7K/G234P) of tyrosinase was due to the formation of hydrogen bonds and salt bridges which made its conformation more stable; while the substitution of glycine enhanced its rigidity [15]. To improve one property such as the thermostability of an enzyme, widely used strategies include hydrogen bonding, the formation of disulfide bridges, hydrophobic or aromatic interactions, and ion pairing [16]. The thermostability of MBSP from *Crucian carp* by genetic engineering has not been reported, and is an interesting research area.

Therefore, in this study, in order to obtain bioactive unglycosylated recombinant MBSP, we adopted the *Pichia pastoris* expression system SMD1168-pPICZαA. Special emphasis was placed on investigating the association between enzyme thermostability and significant residues. We performed site-directed mutagenesis at various positions and observed the influence of amino acid mutation on the thermostability of MBSP. The results indicated that the mutants MutP95T, MutA127S, MutI130D and MutA127S/I130D influenced thermal stability.

2. Materials and methods

2.1. Materials

E. coli DH5α, *Pichia pastoris* SMD1168 strain and pPICZαA plasmid were conserved by our laboratory. The recombinant plasmid pGEM-T-*MBSP* was constructed by our laboratory [1]. Restriction enzyme *Eco*RI, *Not*I, *Sac*I, DNA Marker and Protein Marker were purchased from TaKaRa (Dalian, China). TIANprep Mini Plasmid Kit and the Universal DNA Purification Kit were purchased from TIANGen (Beijing, China). The fluorogenic peptide substrate Boc-Gln-Arg-Arg-MCA was purchased from the Peptide Institute (Osaka, Japan). Zeocin was purchased from Invitrogen (Beijing, China). All other chemicals used were of the highest reagent grade from commercial sources.

2.2. Sequence alignment and site-directed mutagenesis

To investigate thermostability of the MBSP, multiple sequence alignment and mutation prediction analysis using the protein sequence were performed online with the Espript 3.0 software (https://espript.ibcp.fr/Espript/) and PoPMuSic (https://soft.dezyme.com/query/create/hot). Compared with MBSP and trypsin, the weak spots of MBSP were selected, as the critical point in studying the thermal stability mechanism of an enzyme is to pinpoint the weak spots [8,17]. When the weak spots were identified, the critical points regarding enzyme thermal stability were identified by amino acid substitution.

Table 1
Oligonucleotide primers used in the overlap extension PCR.

Target sites	Oligonucleotide Sequence[a]
F-P95T R-P95T	CAAGCTGAAACAAACTGCCACCCTC
	TATCTGTTGAGGGTGGCAGTTTG
F-125N	GCTTGGTTTCTGGATGGGGTAATACTG
R-125N	AAGCTATGCCGTCCGCTGTATTGCC
F-A127S	GGATGGGGCAGAACTTCAGACG
R-A127S	TGCCGTCTGAAGTTCTGCCCCAT
F-I130D	TGCAGACGGCGATGCTTC(T)ACCCT
R-I130D	AGGGTGGAAGCATCGCCGTCTG
F-A127S/I130D	CAGAACTTCAGACGGCGATGCTT
R-A127S/I130D	AAGTATCGCCGTCTGAAGTTCTGCCCCAT

[a] The nucleotide changes are underlined.

Site-directed mutagenesis was carried out using splicing by overlap extension PCR [18] to create mutants of MBSP. The plasmid of pGEM-T-*MBSP* served as the template for mutagenesis which involved the application of primers as described in Table 1. The PCR products were purified using the universal DNA purification kit. The recombinant gene and mutant genes with restriction sites *Eco*RI and *Not*I were amplified using a forward primer: 5′-TAGAATTC (*Eco*RI) ATCATTGGTGGTTACGAGTGTAGG-3′ and a reverse primer: 5′-TAGCGGCCGC (*Not*I) TTAGTTACTAGCTATGGTGG-3′, then they were sub-cloned into pPICZαA and verified by sequencing.

2.3. Recombinant MBSP expression in Pichia pastoris

The recombinant expression vectors were linearized by digestion with *Sac*land transformed into the *Pichia pastoris* SMD1168 strain by the electroporation method [19]. Transformants were screened on YPD plates (1% yeast extract, 2% peptone, 2% glucose) containing different concentrations of Zeocin (100 μg/mL, 500 μg/mL, 800 μg/mL, 1 mg/mL) to obtain multi-copy recombinant *Pichia pastoris* strains, and the phenotype was identified by 3′AOX and 5′AOX primers. The multi-copy transformants were inoculated into 50 mL BMGY medium (1% yeast extract; 2% peptone; 1.34% YNB without amino acids; 100 mM phosphate buffer, pH 6.0; 4×10^{-5}% biotin; 1.0% glycerol) and induced in 80 mL BMMY medium (1% yeast extract; 2% peptone; 1.34% YNB without amino acids; 100 mM phosphate buffer, pH 6.0; 4×10^{-5}% biotin; 1.0% methanol) at 28 °C for 96 h at 220 g. 100% methanol was added at a final concentration of 1.0% methanol every 24 h to maintain induction. The supernatant containing the enzyme was collected by centrifugation at 10,000*g* for 10 min at 4 °C. Ammonium sulfate between 30 and 60% saturation was added to fractionate the recombinant enzyme. After the precipitate was dissolved and dialyzed in 25 mM Tris-HCl (pH 8.0), the secreted protein was detected by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The preparation of anti-MBSP peptide polyclonal antibody was carried out using the recombinant MBSP expressed in *E. coli*.

Purification of MBSP was performed using a Q-Sepharose column and the active fraction was collected and desalted.

2.4. Enzyme activity assays

The proteolytic activity of the enzyme was routinely determined using Boc-Gln-Arg-Arg-MCA as the substrate. The ability of the