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Two variants of the major serine protease inhibitor from the sea anemone *Stichodactyla helianthus*, expressed in *Pichia pastoris*

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ABSTRACT

The major protease inhibitor from the sea anemone *Stichodactyla helianthus* (ShPI-1) is a non-specific inhibitor that binds trypsin and other trypsin-like enzymes, as well as chymotrypsin, and human neutrophil elastase. We performed site-directed mutagenesis of ShPI-1 to produce two variants (*r*ShPI-1/K13L and *r*ShPI/Y15S) that were expressed in *Pichia pastoris*, purified, and characterized. After a single purification step, 65 mg and 15 mg of protein per liter of culture supernatant were obtained for *r*ShPI-1/K13L and *r*ShPI/Y15S, respectively. Functional studies demonstrated a 100-fold decreased trypsin inhibitory activity as result of the K13L substitution at the reactive (P1) site. This protein variant has a novel tight-binding inhibitor activity of pancreatic elastase and increased activity toward neutrophil elastase in comparison to *r*ShPI-1A. In contrast, the substitution Y15S at P2′ site did not affect the K_i value against trypsin, but did reduce activity 10-fold against chymotrypsin and neutrophil elastase. Our results provide two new ShPI-1 variants with modified inhibitory activities, one of them with increased biomedical potential. This study also offers new insight into the functional impact of the P1 and P2′ sites on ShPI-1 specificity.

1. Introduction

Kunitz-type protease inhibitors belonging to the Bovine Pancreatic Trypsin Inhibitor (BPTI) family (PFAM: PF00014) are among the most extensively studied protein-protein binding systems. Site-directed mutagenesis studies of this family have mainly focused on mammalian proteins, such as BPTI [1–4]. Furthermore, most studies have focused on the reactive residue, the P1 site according to the Schechter and Berger notation, which is the main contributor to the binding association energy and specificity [5,6]. Typical trypsin inhibitors have Arg/Lys at this P1 site whereas chymotrypsin inhibitors have aromatic residues, Leu or Met, and elastase inhibitors usually contain aliphatic reactive residues at this position [1,7]. The functional impact of other positions has been also studied through site-directed mutagenesis but mainly on the Pn side (P5–-P1 sites) of the binding loop [8].

Abbreviations: 3D, three-dimensional; BAPA (Bz-Arg-pNA), N-benzoyl-arginine-*p*-nitroanilide; BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; K_i, inhibition constant; K_M, Michaelis constant; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; MW, molecular weight; PDB, protein data bank; PI, protease inhibitor; RT, room temperature; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ShPI-1, *Stichodactyla helianthus* protease inhibitor 1.

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We have previously reported the purification, characterization and recombinant expression of a BPTI-Kunitz type inhibitor, isolated from the sea anemone *Stichodactyla helianthus* (ShPI-1, UNIPROT ID: P31713) [9]. Wild-type ShPI-1 bears a basic residue at the P1 site, which explains its activity mainly toward trypsin-like enzymes, such as trypsin, kallikrein and plasmin. Like the Lys (P1)-containing inhibitor BPTI, ShPI-1 is able to bind chymotrypsin and human neutrophil elastase (HNE) [10,11]. The presence of Tyr15 at P2′ position of ShPI-1 is suggested to support these activities [9], taking into account the hydrophobic nature of their S2′ pockets. Nevertheless, the impact of this hydrophobic residue on the activity of ShPI-1 has been not tested by site-directed mutagenesis. Other remarkable activities of ShPI-1 include its unusual ability to interact with cysteine and aspartic proteases and the strong inhibition of other serine proteases, such as kallikrein, plasmin and HNE [9]. Together with its novel functional features, ShPI-1 has potential applications in biotechnology [12,13] and in biomedicine, the latter as a result of its antiparasitic effect against trypanosomatids [14,15]. These facts emphasize the importance of understanding the functional determinants of ShPI-1 and of characterizing new variants with different specificities or higher selectivity toward enzymes of biomedical interest.

Here we report the site-directed mutagenesis of ShPI-1 at the P1 and P2′ sites resulting in two variants named *r*ShPI-1/K13L and *r*ShPI-1/Y15S. Both recombinant proteins were expressed in *Pichia pastoris* and purified. Kinetic studies demonstrated their modified specificity and inhibitory strength, in comparison with the pseudo wild-type inhibitor *r*ShPI-1A that is produced in the same host [12]. This is the first report describing mutagenesis at different positions of ShPI-1 and its functional effect on the activity against nine serine proteases. Modification at the P1 site reduced binding to trypsin-like enzymes and significantly increased the inhibitor selectivity toward elastases. The functional effect of mutation at P2′ site, initially designed to decrease inhibition of chymotrypsin, was lower than expected. Thus, the hydrophobic residue Tyr15 may not has a great influence into chymotrypsin binding, which suggests that other positions should be studied.

2. Materials and methods

2.1. Strains and molecular biology reagents

Escherichia coli TOP10 and *Pichia pastoris* KM71H strains, both from Invitrogen (USA), were used as cloning and expression hosts systems, respectively. Restriction enzymes, DNA ligase, *Taq* (*Thermus aquaticus*) and *Phusion*™ (*Pyrococcus* sp.) polymerases and their buffers, were obtained from Fermentas (Germany) or NEB (USA).

2.2. Cloning and site-directed mutagenesis

The gene of wild-type ShPI-1 was amplified by PCR using as a template the plasmid pBM301 that contains the gene of the pseudo wild-type inhibitor ShPI-1A [12]. A first PCR was performed with site specific primers to eliminate the additional residues on the *r*ShPI-1A gene [12]. The sense primer (5′-TCTCTCGAGAAAA-GA**TCCATCTGCAGCGAACCG**-3′) included the 5′ end of the ShPI-1 gene that encodes for amino acids 1 to 6 (bold letters), an *Xho*I restriction site, and the recognition site of the Kex2 enzyme. The antisense primer (5′-TGTTCTAGATTACTA**CGCGCGGCAGATAGC**-3′) hybridized at the 3′ end of the ShPI-1 gene that encodes amino acids 51–55 (bold letters) and additionally includes two stop codons and an *Xba*I restriction site. The PCR mixture (50 μL) contained 100 μM dNTPs, 0.5 μM each primer, 40 ng of template and 0.5 U of *Taq* DNA polymerase. The program [1 × (2 min, 95 °C); 20 × (30 s, 95 °C; 45 s, 72 °C); 1 × (5 min, 72 °C); 1 × 20 °C] was performed in a *Mastercycler*® (Eppendorf, Germany). The purified product was cloned into the *Xho*I/*Xba*I-digested pPICZαA vector (Invitrogen, USA). The resultant vector (pPICSh) was used as template for the site-directed mutagenesis following the standard Quick Change method (Stratagene, USA) using the primers shown in Table 1. Mutagenesis was performed in 50 μl of the supplied GC buffer, 5–10 ng template, 0.2 μM each primer, 200 μM dNTPs and 1U of Phusion DNA polymerase. After the initial denaturation step at 98 °C for 30 s, the mutagenesis reaction was conducted for 30 cycles with denaturation at 98 °C for 10 s, primer annealing at Tm+3 °C for 30 s and DNA synthesis at 72 °C for 2 min, with a final extension at 72 °C during 5 min. The final product was digested with 10U of *Dpn*I for 90 min at 37 °C before transformation of *E. coli* TOP10 cells by plasmid electroporation. Resulting vectors were sequenced at the University Medical Center Hamburg (Germany).

2.3. Production of rShPI-1/K13L and rShPI-1/Y15S

Transformation of *Pichia pastoris* and further analysis were performed according to the Easy Select *Pichia* Expression Kit (Invitrogen, USA). *P. pastoris* strains for production of *r*ShPI-1/K13L and *r*ShPI-1/Y15S were isolated by increasing concentrations of zeocine in the culture media up to 100 μg/mL. The pseudo wild-type inhibitor *r*ShPI-1A and the *r*ShPI-1/K13L variant were produced in a 1.5 L fermenter as previously reported [12]. The variant *r*ShPI-1/Y15S was produced using a similar protocol and after 48 h of culture at 28 °C and 200 rpm, the cells were harvested by centrifugation, inoculated in 150 mL minimal medium [9] and further incubated for 72 h at 28 °C with the addition of 1% (v/v) methanol every 24 h. Protein secretion to the medium was evaluated by SDS-PAGE [14] and Western-blotting. The inhibitory activities against pancreatic trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were tested using the substrates Bz-Arg-pNA [15] or Suc-Ala-Ala-Pro-Phe-pNA [16], respectively (Table 2).

Protein purification from culture supernatants was performed in a Streamline Direct HST-1 multi-modal resin, as previously reported [12]. Protein concentrations were estimated by measuring the absorbance at 280 nm, using the specific extinction coefficient of ShPI-1 (E_{280} 1% = 5.2) [9]. The integrity and identity of the proteins was verified by molecular mass determination and peptide mapping, as further described.

2.4. Immunodetection of the recombinant proteins

Immunodetection was performed using a polyclonal antibody against the pseudo wild-type inhibitor *r*ShPI-1A generated in a BalB/C mouse (unpublished). The serum was diluted 1:5000 in 0.1% (m/v) bovine serum albumin and dissolved in Tris-buffered saline (TBS). An alkaline phosphatase conjugated anti-mouse IgG was used as secondary antibody and the reaction was visualized using Nitro Blue Tetrazolium (0.1%) and 5-bromo-4-chloro-3-indolyl phosphate (0.5%) in Tris-HCl, pH 9.5.

2.5. Peptide mapping and molecular mass determination

Proteins were treated with 40 mM dithiothreitol (DTT) for 1 h at 37 °C and the reduced Cys residues were modified with iodoacetamide or acrylamide (100 mM) by incubation in the dark for 30 min at 37 °C. Subsequently, the variant *r*ShPI-1/K13L was digested with trypsin and *r*ShPI-1/Y15S with endoproteinase Glu-C (*Staphylococcus. aureus* V8) or a Glu-C/lysil endopeptidase (LEP) mixture (1:1), for 2 h at 37 °C. Mass spectroscopy was done on a MALDI-TOF-TOF Axima Performance mass spectrometer (Shimadzu, Japan), equipped with a 337 nm pulsed nitrogen laser.