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Asteropsins B–D, sponge-derived knottins with potential utility as a novel scaffold for oral peptide drugs

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ABSTRACT

Background: Known linear knottins are unsuitable as scaffolds for oral peptide drug due to their gastrointestinal instability. Herein, a new subclass of knottin peptides from Porifera is structurally described and characterized regarding their potential for oral peptide drug development.
Methods: Asteropsins B–D (ASPB, ASPC, and ASPD) were isolated from the marine sponge *Asteropus* sp. The tertiary structures of ASPB and ASPC were determined by solution NMR spectroscopy and that of ASPD by homology modeling.
Results: The isolated asteropsins B–D, together with the previously reported asteropsin A (ASPA), compose a new subclass of knottins that share a highly conserved structural framework and remarkable stability against the enzymes in gastrointestinal tract (chymotrypsin, elastase, pepsin, and trypsin) and human plasma.
Conclusion: Asteropsins can be considered as promising peptide scaffolds for oral bioavailability.
General significance: The structural details of asteropsins provide essential information for the engineering of orally bioavailable peptides.

1. Introduction

Peptides are attractive drug candidates because of their potent biological activities as well as high target specificities [1]. However, the short in vivo half-lives of peptide therapeutics remain a major issue. In particular, oral delivery, which provides improved patient compliance, remains a primary target of peptide drug development. Recently, head-to-tail cyclized knottins (cyclotides) have been placed in the spotlight as novel scaffolds for oral peptide drug administration because of their extraordinary proteolytic stability and relatively straightforward chemical and recombinant syntheses [2–5].

Knottin peptides share a rigid molecular disulfide arrangement (III–VI through I–IV, II–V), also called a 'knot', and a triple-stranded antiparallel β-sheet fold. In late 2004, the knottin, ω-conotoxin MVIIA (or ziconotide) from the cone snail *Conus magus* became the first fully approved drug obtained directly from the marine environment for the treatment of chronic pain [6]. Several other knottins are currently undergoing preclinical or phase I/II clinical trials for the treatment of pain related diseases [7]. In addition to their potential as drug candidates, the use of knottins, especially cyclotides, has been extended to peptide engineering for the development of protease-resistant ligand scaffolds [3,4,8–12]. The cyclotide kalata B1, which exhibits stability towards pepsin, trypsin, and chymotrypsin, is a promising scaffold for the development of orally effective peptide drugs; however, the oral bioavailability of kalata B1 was found to be dramatically reduced when its head-to-tail cyclization was not performed [3,4]. The linear knottins from squash (SE-EM and SE-EP), human agouti related protein (SE-AGAZ), and even hybrid recombinant knottins (SE-ET-TP-020 and SE-MC-TR-020) were found to be extensively degraded by chymotrypsin or trypsin [13–15]. The approved drug ziconotide, which is a linear knottin, is also unstable to trypsin, and thus, must be administered intrathecally [16,17]. In a recent study by Clark et al., it was found that the cyclic product obtained by cyclizing a 16-residue conotoxin with a hydrophobic linker consisting of six aliphatic amino acids was orally effective, whereas the linear native conotoxin remained orally ineffective [18]. Linear knottins reported so far are not suitable as scaffolds for oral delivery, although their syntheses are less complicated because they do not require an additional step for head-to-tail cyclization.

Knottins have been reported in many organisms, though most have been discovered in spider or cone snail venoms. Relatively few marine-derived knottins have been discovered in sources other than cone snails. In 2006, Fusetani et al. first identified asteropine A (APA; a bacterial sialidase inhibitor) in the marine sponge *Asteropus simplex* [19]; and

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later, we isolated asteropsin A (ASPA) from the same sponge genus and validated Porifera as a source of unusual knottin-like peptides [20]. The *N*-terminal modification, absence of basic residues, the *cis* conformation of prolines of ASPA and its unique bioactivity distinguish it from other reported knottins.

Herein we report three unusual knottin-like peptides, asteropsins B–D (ASPB, ASPC and ASPD) from the same sponge *Asteropus* sp. In addition to their moderate sequence homologies, NMR derived solution structures revealed that these sponge-derived peptides have highly conserved tertiary structures. Asteropsins A–D share several conserved residues at special locations that are likely to maintain structural stability. In addition, *N*-terminal blocking and the absence of basic residues make them inherently stable to aminopeptidases and trypsin. In our evaluation of their proteolytic resistances against major gastrointestinal proteases (chymotrypsin, elastase, pepsin, and trypsin) and human plasma, all four asteropsins exhibited notable resistance, which suggests that they can be utilized as novel linear knottin scaffolds for orally delivered peptide drugs.

2. Materials and methods

2.1. Animal material and peptide purification

The marine sponge *Asteropus* sp. (2.4 kg, wet weight) was collected by hand at a depth of 20 m in 2006 off the coast of Geoje Island, Korea, and stored at −20 °C until used. The frozen sponge (2.4 kg, wet weight) was extracted with MeOH at room temperature, and the extract (166 g) was then partitioned between water and CH_2Cl_2 (1:1, v/v). The aqueous layer was further partitioned with BuOH and water (1:1, v/v), and the organic layer (5.1 g) obtained was subjected to step-gradient MPLC (ODS-A, 120 Å, S-30/50 mesh) using 20–100% methanol as eluant. Asteropsins B–D (7.5, 68, and 2.7 mg, respectively) were purified by reversed-phase HPLC equipped with an RI detector (YMC ODS-H80 column 250 mm × 10 mm, i.d. 4 μm, 80 Å) using 60% MeOH + 0.2% HCOOH at a flow rate of 1 mL/min.

2.2. Reduction and alkylation of peptides

A portion of each peptide (100 μg) was dissolved in 100 μL of denaturation buffer (7 M guanidine hydrochloride in 0.4 M Tris-acetate-EDTA buffer, pH 8.3) and 10 μL of 45 mM dithiothreitol (DTT) was then added. The mixture was incubated at 60 °C for 90 min and then 20 μL of 100 mM iodoacetamide was added and incubated at room temperature for 45 min. The reaction mixture was purified by reversed-phase HPLC equipped with a UV detector (Waters ODS-2 column 250 mm × 4.6 mm, i.d. 5 μm; wavelength: 220 nm) using a linear gradient (30–80% solvent B; solvent A: H_2O + 0.1% TFA, solvent B: 90% ACN + 0.1% TFA) to afford hexacarboxamidomethyl derivatives (*m/z* 4267.7 $[M + Na]^+$, 4135.7 $[M + Na]^+$, and 3774.3 $[M + H]^+$ for ASPB, ASPC, and ASPD, respectively).

2.3. MALDI-TOF MS

Each peptide (0.1 mg) was dissolved in DMSO and then diluted tenfold with α-cyano-4-hydroxycinnamic acid matrix solution (7 mg/mL in 50% ACN, 0.1% TFA). Then, 1 μL of each sample/matrix solution was spotted onto a MALDI plate and inserted into a MALDI-TOF unit (Applied Biosystems 4700 proteomics analyzer, Framingham, MA).

2.4. N-terminal deblocking and sequence analysis

The hexacarboxamidomethyl derivatives (<30 μg) of asteropsins B–D were digested with 2 mU of pyroglutamate aminopeptidase (Takara Bio Inc., Shiga, Japan) dissolved in 100 μL of supplied buffer (50 mM sodium phosphate buffer containing 10 mM DTT and 1 mM EDTA; pH 7.0) containing 5% glycerol at 50 °C for 10 h. Digests were desalted with a sample preparation cartridge (Prosorb, Applied Biosystems, Foster City, CA), and subjected to automatic Edman degradation in a protein sequencing system (Procise 491; Applied Biosystems). Sequence analysis was performed at KBSI (Korea Basic Science Institute, Seoul). Sequence alignment was performed using the program ClustalX (ver. 2.0, http://www.clustal.org).

2.5. NMR spectroscopy

ASPB and ASPC were dissolved in DMSO-d_6 and CD_3OH (Sigma Aldrich Chemical Co., St. Louis, MO, USA), respectively, degassed, and topped with argon to minimize moisture absorption. Residual hydroxyl signal suppression was achieved using a standard presaturation method. Standard experimental parameters were used for the acquisition of HSQC (Adiabatic Decoupling), z-TOCSY (DIPSI, mixing time = 80 ms), DQF-COSY, and NOESY (mixing time = 100 and 300 ms) using Varian 900 and 600 MHz (^{13}C = 150 MHz) INOVA at 298 K with reference to the internal lock signal. Processing was completed using NMRpipe (ver. 5.5, NIH) and chemical shift analysis using SPARKY (ver. 3.114, UCSF) [21], with spectra image rendering using MestReNova (ver. 6.2.0, MestreLab Research S.L.).

2.6. Structure calculations

Interproton distance constraints were obtained from the 100 ms and 300 ms mixing time NOESY spectra recorded in DMSO-d_6 or CD_3OH for ASPB and ASPC, respectively. NOESY spectra were analyzed with the program SPARKY [21]. Cross peaks were categorized into four classes by peak intensity (2.5, 3.0, 4.0, and 5.0 Å, which corresponded to strong, medium, weak, and very weak correlations). Pseudo-atoms were applied for methyl, non-stereospecifically assigned methylene, and aromatic protons according to a standard method [22]. Dihedral angle constraints were generated from $^3J_{NH\text{-}H\alpha}$ values > 8.0 Hz in DQF-COSY. Hydrogen bond restraints were collected by long-range NOE correlations together with hydrogen–deuterium exchange experiments using CD_3OH and CD_3OD as solvents. In addition, chemical shift indices (CSI) [23,24] were used to determine secondary structures.

Solution structural calculations were initially performed by CYANA 2.1 [25] using distance, dihedral angle, and hydrogen bond constraints, and further refined by simulated annealing within CNS 1.3 [26]. A final set of 200 structures was calculated within the CNS program, and the 20 structures with lowest energies and no residual restraint violations were used to represent the solution structures of the peptides. The calculated three-dimensional structures were analyzed with the program PYMOL [27] and MOLMOL [28]. Structure qualities were validated by the PSVS (protein structure validation software suite) server (http://psvs-1_4-dev.nesg.org/) [29]. The solution structures of ASPB and ASPC have been deposited in the RCSB Protein Data Bank (PDB ID: 2LZX and 2LZY, respectively).

2.7. Homology modeling of ASPD

Prediction including loop refinement of ASPD was performed in Prime (Schrödinger, LLC) using the solution structure of ASPC (PDB ID: 2LZY), its closest sequence homolog, as a template. The ASPD model obtained was further refined using ModRefiner [30], and energy minimization was performed by CHARMm force field. The resulting model was analyzed using PYMOL [27] and PSVS server (http://psvs-1_4-dev.nesg.org/) [29].

2.8. Gastrointestinal enzymatic stability of asteropsins A–D

Enzymatic degradations by chymotrypsin (Sigma C3142, 54 BTEE units/mg), elastase (Sigma E7885, 4 *N*-succinyl-L-Ala-Ala-Ala-*p*-nitroanilide units/mg), and trypsin (Sigma T4799, 1753 BAEE units/mg) were performed at protease concentrations typically found in human