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Synthesis and properties of tachyplesin I, a lipopolysaccharide-binding peptide, from *Tachypleus tridentatus*

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Tachyplesin I, a cationic heptadecapeptide amide containing two disulfide bonds isolated from hemocytes of *Tachypleus tridentatus*, which binds to lipopolysaccharide, was synthesized by a solid-phase procedure. Reduction and oxidation of the deprotected peptide amide yielded an identical peptide to native tachyplesin I in terms of chemical and biological properties. Tachyplesin I was found to bind to alkylphosphorylcholine accompanied by perturbation of its tyrosyl and tryptophyl residues but bound only weakly to *N*-acetyl-D-glucosamine tetramer.

Tachyplesin; Lipopolysaccharide; Solid-phase synthesis; Alkylphosphorylcholine; Difference spectroscopy; (*Tachypleus tridentatus*)

1. INTRODUCTION

Tachyplesin I, a cationic heptadecapeptide amide (fig.1), isolated from the hemocyte membrane of Japanese horseshoe crab (*Tachypleus tridentatus*), is able to bind to lipopolysaccharide (LPS) and to neutralize the *Limulus* factor C-activating activity of LPS [1]. Thus tachyplesin I prevents initiation of an activation cascade leading to coagulation of the *Limulus* lysate [2–4]. In addition, the peptide has been found to exhibit strong antimicrobial activity against both Gram-positive and -negative bacteria [1]. The total synthesis of this peptide and its variants appears to be very promising for elucidation of the structure-function relationship of this functionally interesting peptide. Here, we describe the synthesis of tachyplesin I and compare data on its affinity for binding to alkylphosphorylcholine and *N*-acetyl-D-glucosamine tetramer [$(GlcNAc)_4$], both of which contain components involved in LPS.

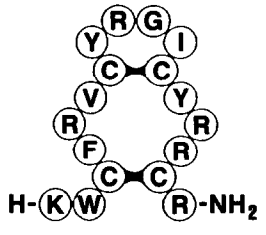

Fig.1. Structure of tachyplesin I.

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Abbreviations: Boc, *t*-butoxycarbonyl; Pam, phenylacetamidomethyl; DMF, dimethylformamide; LPS, lipopolysaccharide; LPC, laurylphosphorylcholine; $(GlcNAc)_4$, *N*-acetyl-D-glucosamine tetramer; HPLC, high-performance liquid chromatography; all amino acids are of the L-configuration

2. EXPERIMENTAL

2.1. *Materials*

Aminomethylated (~0.7 mmol/g) copoly(styrene-divinylbenzene) [1%]) resin (200–400 meshes) was obtained from Pierce. Boc-N^g-tosylarginyl-4-(oxymethyl)phenylacetic acid was prepared according to Tam et al. [5]. Boc-amino acids were supplied by the Peptide Institute (Osaka). Laurylphosphoryl-

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choline (LPC) was synthesized as described by Hirt and Berchtold [6] and purified as in [7]. *N*-Acetyl-D-glucosamine tetramer was a gift from Dr N. Yamasaki (Faculty of Agriculture, Kyushu University). Other reagents used were of the highest grade available.

2.2. *Protected heptadecapeptidyl-4-(oxymethyl)phenyl-acetamidomethyl resin*

Boc-Arg(Tos)-4-(oxymethyl)phenylacetamidomethyl (Pam) resin (0.284 mmol Arg/g) was prepared from the amino-methylated resin and Boc-Arg(Tos)-4-(oxymethyl)phenylacetic acid according to Mitchell et al. [8], followed by acetylation of the remaining amino group with acetic anhydride-pyridine (1:4). Successive additions of Boc-amino acids to Boc-Arg(Tos)-4-(oxymethyl)-Pam resin (1.0 g) along the sequence of tachyplesin I were conducted on a Beckman 990E peptide synthesizer according to the method of Wong and Merrifield [9] with minor modifications. The side-chain-protected amino acids employed were N^g-tosylarginine, N^ϵ-2-chlorobenzyloxy-carbonyllysine, *S*-4-methylbenzylcysteine, *O*-benzyltyrosine and N^{in}-formyltryptophan. The final yield of the protected heptadecapeptidyl-4-(oxymethyl)-Pam resin amounted to 1.78 g (79% of theoretical value).

2.3. *Heptadecapeptide amide·7HCl*

Protected heptadecapeptidyl-4-(oxymethyl)-Pam resin (1.0 g) was suspended in a mixture of anhydrous dimethylformamide (DMF) (12 ml) and methanol (10 ml), dried NH_3 gas being bubbled through the mixture at 0°C until saturation. The vessel was tightly stoppered and allowed to stand for 3 days at room temperature. The filtrate from the resin was evaporated and the residue solidified by addition of water (392 mg). Reprecipitation with a DMF-methanol system gave a white solid (295 mg). The protected peptide amide (150 mg) was then treated with HF (8 ml) in the presence of anisole (1.2 ml) and 1,2-ethanedithiol (0.8 ml) at 0°C for 3 h. After removal of HF and extraction with ethyl acetate (three times), the peptide was dissolved in 5% acetic acid containing 0.1 M HCl (10 ml) and, after filtration, the solution was lyophilized. The peptide was chromatographed on a Sephadex G-10 column (2.0 × 25 cm) with 0.05 M HCl, the fraction containing the peptide being subsequently lyophilized (97 mg).

2.4. *Reduction and oxidation*

The peptide (40 mg) obtained above was dissolved in 0.05 M Tris-HCl (pH 8.5) (8 ml) containing 220 μl 2-mercaptoethanol and the solution allowed to stand overnight at room temperature. The solution was acidified (pH 2) using 5.7 M HCl and passed through a Sephadex G-10 column (2.0 × 25 cm) with 0.05 M HCl. The fraction (12 ml) containing the peptide was diluted to 200 ml with water and the pH adjusted to 8.5 with 0.5 M NaOH. The solution was left to stand uncovered in a 500 ml beaker at room temperature for 3 days with occasional gentle stirring. The solution was then concentrated to 10 ml by evaporation in vacuo at 35°C and gel-filtered on a Sephadex G-10 column (2.0 × 25 cm) with 0.05 M HCl. The peptide-containing fraction was lyophilized (15.7 mg), followed by fractionation of the peptide by HPLC on a TSK gel ODS-120T column (0.78 × 30 cm), using a solvent system comprising 0.1% trifluoroacetic acid (A)-acetonitrile containing 20% A (B). The peptide was eluted with a linear concentration gradient of B.

2.5. *Tachyplesin assay*

Tachyplesin was assayed with respect to inhibitory activity towards LPS-mediated activation of *Limulus* factor C as in [1].

2.6. *Spectroscopic titrations of peptides with LPC and (GlcNAc)$_4$*

Difference spectra were recorded on a Hitachi 100-60 spectrophotometer by titrating synthetic peptide and native tachyplesin I ($8.9–9.0 \times 10^{-5}$ M) with increasing concentrations of LPC (0.09–2.71 mM) or $(GlcNAc)_4$ (0.15–1.82 mM) at pH 8.0 (0.02 M Tris-HCl containing 0.1 M NaCl).

3. RESULTS AND DISCUSSION

Tachyplesin I has an amide group at the C-terminus (fig.1). The absence of an ester-type side-chain-protecting group in the protected peptide that is linked to the supporting resin via the ester bond enabled us to cleave the peptide via ammonolysis, leaving an amide group at the C-terminus. As described in section 2, the protected heptadecapeptide amide was successfully cleaved from the resin. All protecting groups were then removed with HF.

Fig.2 shows HPLC profiles of the crude synthetic peptide, HPLC-purified peptide, and native tachyplesin I. The purified synthetic peptide coeluted with native tachyplesin I as a single sharp peak and gave a single peak on HPLC using a hydroxyapatite column (TSK gel HA-1000) (not shown). The yield after reduction and oxidation was 12%. The amino acid composition of a hydrolysate (3 M mercaptoethanesulfonic acid) of the synthetic peptide was in accord with that of native tachyplesin I (theoretical values in parentheses): Gly 1.1 (1), cystine 3.0 (4), Val 1.0 (1), Ile 1.0 (1), Tyr 1.8 (2), Phe 0.9 (1), Lys 0.9 (1), Trp 0.8 (1), Arg 5.1 (5).

The ultraviolet absorption spectrum of the synthetic peptide with a maximum at 276 nm and shoulders at 281 and 289 nm was superimposed with that of native tachyplesin I (not shown). The ID_{50} values for neutralizing 2.5 pg LPS were 1.25 and 2.95 μg for synthetic and native tachyplesin I, respectively. The somewhat higher activity of the synthetic peptide vs the native form of tachyplesin I is probably due to partial adsorption of LPS-like material from the environment onto native tachyplesin.

The interactions of the synthetic peptide and native tachyplesin I with LPC, an analog of phosphatidylcholine, were investigated by means

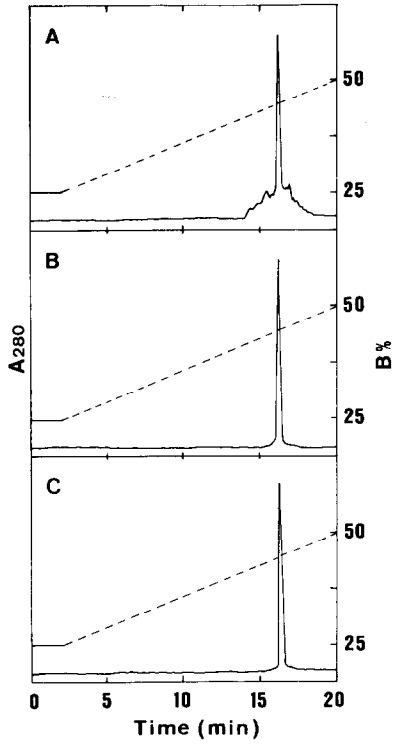

Fig.2. HPLC profiles of crude synthetic (A), purified synthetic (B) and native (C) tachyplesin I. TSK gel ODS 120T column (0.46 × 25 cm). Solvent system: 0.1% trifluoroacetic acid (A)-acetonitrile containing 20% A (B). Elution conducted using a linear concentration gradient of B, 25% for 2 min and 25–50% for 20 min. Flow rate, 1.0 ml/min.

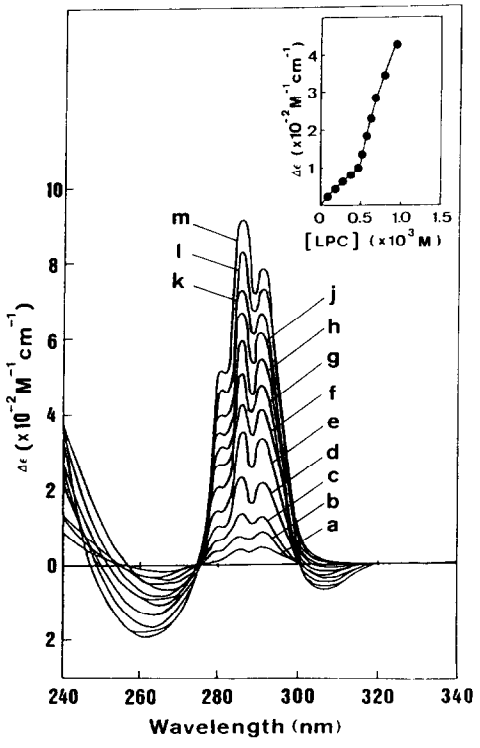

Fig.3. Ultraviolet difference spectra of synthetic tachyplesin I when titrated with increasing concentration of LPC at pH 8.0 (0.02 M Tris-HCl containing 0.1 M NaCl). Synthetic tachyplesin I, 8.9×10^{-5} M: LPC concentrations (in mM) (a) 0.17, (b) 0.34, (c) 0.51, (d) 0.67, (e) 0.84, (f) 0.99, (g) 1.15, (h) 1.30, (j) 1.45, (k) 1.59, (l) 1.88, (m) 2.15 mM. Magnitudes of difference spectra were expressed by the difference molar absorption coefficient ($\Delta\epsilon$).

of difference spectroscopy at pH 8.0. When both materials were titrated with LPC, identical difference spectra were obtained (fig.3 for the synthetic peptide). The inset to fig.3 shows a plot of the difference molar absorption coefficients at 285 nm vs LPC concentrations. The biphasic titration curve could be ascribed to a critical micellar concentration (0.6–0.7 mM) for LPC [7]. The interactions differ below and above the critical micellar concentration. The dissociation constants for binding to LPC in the monomeric dispersed state were computed to be $4.4–4.6 \times 10^{-4}$ M for the synthetic and native peptides, indicating the ability of tachyplesin I to bind to phospholipids. Maxima at 285 and 292 nm in the difference spectra could be ascribed to perturbation of both tyrosyl and tryptophanyl residues, respectively. Tachyplesin I contains two tyrosyl residues at the positions 8 and 13, one or both of which could be responsible for producing the characteristic spectrum. On the other hand, we observed that only the tyrosyl residue(s) showed a minor degree of perturbation when tachyplesin I was titrated with $(GlcNAc)_4$ (not shown). The results obtained suggest that tachyplesin I binds to LPS in the lipid moiety more strongly than in the saccharide moiety.

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