Synthesis and structure-function study about tenecin 1, an antibacterial protein from larvae of *Tenebrio molitor*

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Abstract Tenecin 1, an inducible antibacterial protein secreted in the larvae of *Tenebrio molitor*, has a long N-terminal loop and common structural feature of insect defensin family corresponding to cysteine stabilized α/β motif. To study the function of the N-terminal loop and disulfide bridges, N-terminal loop deleted tenecin 1, reduced tenecin 1 and tenecin 1 were chemically synthesized and their activities were measured. N-terminal loop deleted tenecin and reduced tenecin 1 did not show antibacterial activity. Circular dichroism (CD) spectroscopy data revealed that the α-helical content of tenecin 1 and the other proteins increased in the presence of 50% (v/v) trifluoroethanol (TFE) and the α-helical content of tenecin 1 was much higher than that of the other proteins in buffer with or without 50% (v/v) TFE. These results suggest that disulfide bridges are necessary for the activity structure and the N-terminal loop plays an important role in the increase of α-helix in the membrane mimetic environment and the activity.

Key words: Tenecin 1; Antibacterial peptide; Disulfide bridge; Circular dichroism; N-terminal loop

1. Introduction

The insect defensin family, which had molecular weights of 3–4 kDa and three disulfide bridges, showed potent bactericidal activity against various Gram-positive bacteria and lesser activity against Gram-negative bacteria [1–3]. The study of the mechanism of sapecin and insect defensin A indicated that these defensin proteins acted on the cytoplasmic membrane by pore formation [4,5]. By NMR studies [6–8] the three-dimensional structure of the proteins in this family was obtained in aqueous solution. As shown in Fig. 1A, the insect defensin family shares a common structural feature of a short amphipathic α-helix followed by a C-terminal antiparallel β-sheet structure stabilized by two disulfide bridges (named cysteine stabilized α/β motif). Interestingly, the same structural motif was found in the structure of scorpion toxins [9,10]. As this structural motif was commonly used to maintain the different function of insect defensin and scorpion toxin, this motif has received attention as a scaffold for protein engineering [11]. As shown in Fig. 1B, aligned primary amino acid sequence of the insect defensin family showed that the three disulfide bridges were conserved; three amino acids are between the two cysteines in the helix region and one amino acid between the two cysteines in the β-sheet region and the general consensus sequence is C...CXXXC...C...CXC (X is any amino acid). However, many insect defensin proteins have a variable size and amino acids in the region of the N-terminal loop especially. The number of residues is from 6 to 17 between the two cysteines in the N-terminal loop, suggesting that this region may not be essential for antibacterial activity but specificity. For example, both *Aeshna* defensin and *Phormian* defensin A were active against Gram-positive bacteria, however, *Aeshna* defensin with a short N-terminal loop showed more activity against some of the Gram-positive bacteria strains than *Phormian* defensin A with a long N-terminal loop [12]. However, there is no study about the function of the N-terminal loop of the insect defensin family for structure and activity.

In the present study, we chose tenecin 1 as a model protein among the insect defensin family to investigate the function of the N-terminal loop and disulfide bridges. Tenecin 1, induced in the hemolymph of larvae of coleopteran *Tenebrio molitor*, had a very long N-terminal loop, the common structural feature named cysteine stabilized α/β motif and a potent bactericidal activity against Gram-positive bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA) [13]. We chemically synthesized reduced tenecin 1, tenecin 1 and N-terminal loop deleted tenecin 1 and measured their activities against Gram-positive bacteria. Tenecin 1 showed potent antibacterial activity while reduced tenecin 1 and N-terminal loop deleted tenecin 1 did not, which indicates that three disulfide bridges and the N-terminal loop are necessary for activity. By CD spectroscopy study, three disulfide bridges maintained the ordered structure in buffer and helped to induce the structure with high α-helical content in membrane mimetic environment. Also, the N-terminal loop played an important role in the increase of α-helix in membrane mimetic environment, which related to the activity.

2. Materials and methods

2.1. Peptide synthesis

Peptide was prepared by stepwise solid-phase synthesis on an Applied Biosystems model 431A automatic peptide synthesizer. The peptide chain was assembled on the hydroxymethylphenoxy polystyrene resin (HMP-resin) (Novabiochem, USA). The C-terminal amino acid Fmoc-Arg(Pmc)-OH was manually coupled to the HMP resin. All Fmoc-amino acid derivatives were purchased from Novabiochem. The fluorenylmethoxycarbonyl (Fmoc) group was employed for the protection of the α-amino group and the side chain protections were triphenylmethyl (Trt) for Cys, Gln, Asn and His, *tert*-butyl (tBu) for Thr, Glu, Ser and Asp, *tert*-butyloxycarbonyl (Boc) for Lys and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg. Cleavage of the peptide from the resin was achieved by treatment with trifluoroacetic acid (TFA)/thioanisole/ethanedithiol/H_2O in the ratio of 80:5:2.5:5 (v/v) at room temperature for 12 h. After filtration and washing of resin by TFA, a gentle stream of nitrogen removed excess TFA. Crude peptide was triturated with diethyl ether chilled at −20°C and then centrifuged at 3000 rpm for 10 min at −10°C. The synthetic peptide was purified by prep HPLC using a Vydac C-18 column. Amino acid analysis and electrospray ionization (ESI) mass spectrom-

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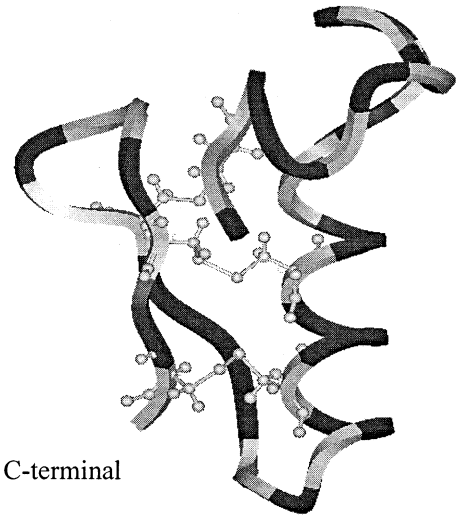
(A)



(B)

Tenecin 1	VTCDI	LSVEA	KGVKL	NDAAC	AAHCL	FRGRS	GGYCN	GKRVC	VCR	
Insect defensin A	ATCDL	LS	GTGI	NHSAC	AAHCL	LRGNR	GGYCN	GKGVC	VCRN	
Peptide B	VTCDL	LGFEI	AGTKL	NSAAC	GAHCL	ALGRR	GGYCN	SKSVC	VCR	
Peptide C	FTCDV	LGFEI	AGTKL	NSAAC	GAHCL	ALGRT	GGYCN	SKSVC	VCR	
Royalicin	VTCDL	LSF	KGQV	NDSAC	AANCL	SLGKA	GGHCE	KGVC	ICRKTS	
Sapecin	ATCDL	LS	GTGI	NHSAC	AAHCL	LRGNR	GGYCN	GKAVC	VCRN	
Sapecin C	ATCDL	LS	GIGV	QHSAC	ALHCV	FRGNR	GGYCT	GKGIC	VCRN	
Sapecin B	LTC		EI	DRSLC	LLHCR	LKGYL	RAYCS	QQKVC	RCVQ	

Fig. 1. A: Molecular model of tenecin 1 built from the three dimensional structure of insect defensin A [24]. B: Primary structures of tenecin 1 [13] and related insect defensin family proteins [15,25–27]. Dashed lines indicated disulfide bridges and gaps were introduced to align the maximal sequence homology.

etry on a Platform II (Micromass, UK) was used to characterize the purified peptide.

2.2. Folding and peptide mapping

The purified synthetic peptides were reduced by 50 mM of DTT (Aldrich, USA) in 10 mM phosphate buffer (pH 7.4) at 50°C for 4 h and purified by HPLC. For determination of the redox state of the cysteine residue, the purified peptide was characterized by Ellman assay and HPLC-mass spectrometer. Oxidation of linear tenecin 1 was carried out by the glutathione system (reviewed in [14]) and disulfide pairs of oxidized tenecin 1 were determined by the same method as described for the characterization of sapecin B [15]. The oxidized tenecin 1 (100 μg) was digested with Lys-C (2 μg) in a buffer (50 μl) containing 25 mM Tris-HCl, 1 mM EDTA, pH 6.8, at 35°C for 15 h and purified by HPLC. The peptide was further digested with trypsin (3 μg) in a buffer containing 0.2 M NH_4HCO_3 for 6 h at 30°C. The fragment was separated by HPLC and digested with thermolysin (2 μg) in a buffer containing 0.1 M Tris-HCl buffer, pH 8, for 24 h at 37°C. Each purified peptide in this process was characterized by ESI mass spectrometer to determine the connectivity of synthesized tenecin 1.

2.3. Antimicrobial assay

In vitro antimicrobial assay was done by a modified microdilution technique [16,17] using 96-well microplates (Nunc, Denmark). Antibiotic medium 3 (M3; pH 7.0 at 25°C, Difco) was used as antibacterial assay medium. Freshly grown cells on antibiotic medium 3 agar plate were suspended in physiological saline to 10^4 cells per 1 ml of 2× concentrated medium and used as the inoculum. Just before the assay, solid samples were dissolved, added to the wells (100 μl per well) and the wells were serially diluted twofold. After inoculation (100 μl per well, 5×10^3 cells per ml), plates were incubated at 37°C for 24 h, and the absorbance at 620 nm was measured by an ELISA reader (Spectra, Austria) to assess cell growth. Antifungal assay was done in Sabraud-2% dextrose broth (SB; pH 5.6 at 25°C, Merck) and the plates were incubated at 30°C for 24 h. The minimum inhibitory concentration (MIC) was defined as the concentration at which 100% inhibition was observed. All MICs were determined from two independent experiments performed in duplicate.

2.4. CD measurements

CD spectra were recorded on a J-715 spectropolarimeter (Jasco, Japan) using a quartz cell path length of 1 mm at wavelengths ranging from 190 to 250 nm. Trifluoroethanol (TFE) was obtained from Aldrich (Milwaukee, USA). The concentration of peptides was 2×10^{-4} (g/ml) in 10 mM sodium phosphate buffer (pH 7.4) with or without 50% TFE (v/v). The CD spectrum was recorded at room temperature and was obtained with a 0.5-nm bandwidth and a scan speed of 10 nm/min. Two scans were averaged to improve the signal-to-noise ratio. The helicity of the peptide was determined from the mean residue

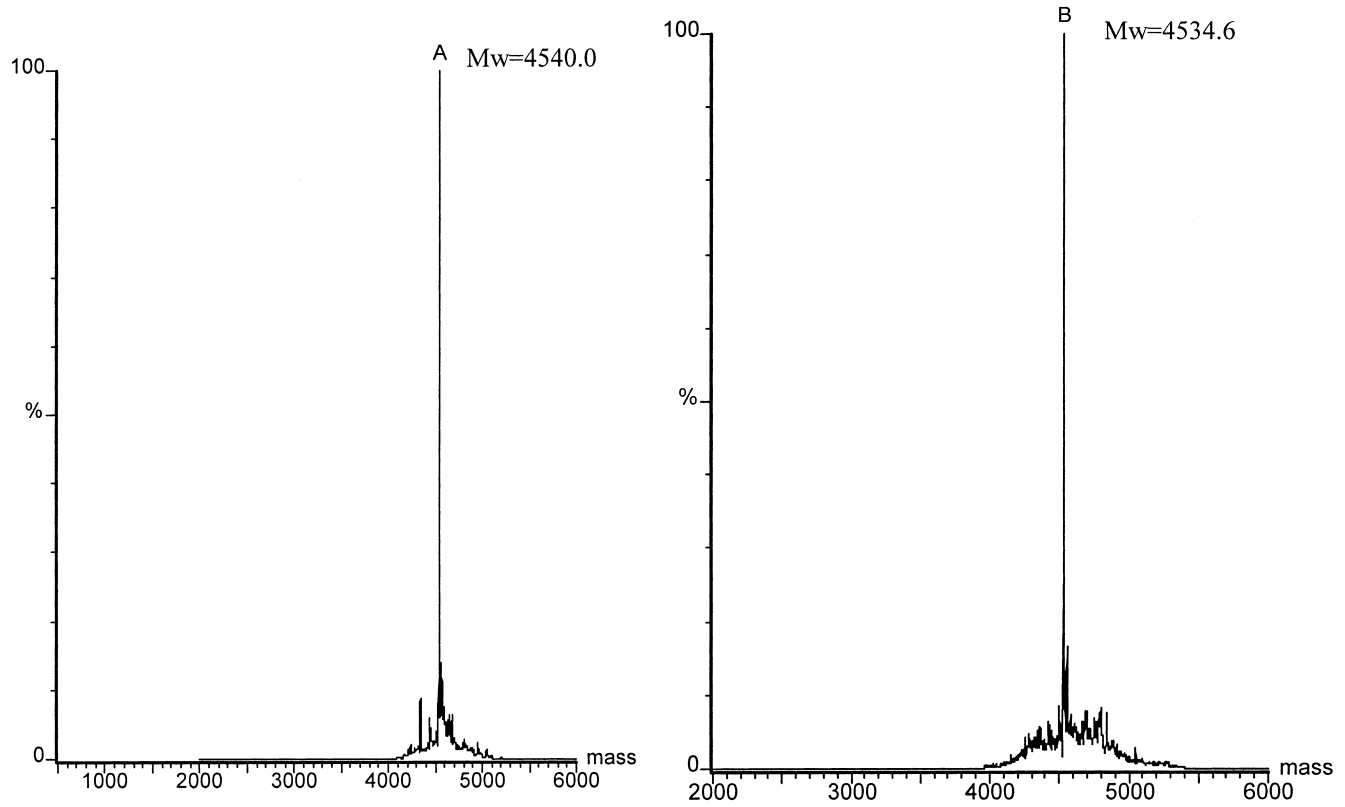


Fig. 2. Mass spectrum of reduced tenecin 1 (A) and tenecin 1 (B).

ellipticity ranging from 190 to 240 nm by the method of Greenfield and Fasman [18].

3. Results and discussion

3.1. Synthesis, folding and disulfide bond assignment

To investigate the function of the N-terminal loop and disulfide bridges, tenecin 1, N-terminal loop deleted tenecin 1, and reduced tenecin 1 were synthesized. Synthesized 43mer peptide was purified by prep HPLC and characterized using a mass spectrometer and amino acid analysis. The purity of the synthesized peptide corresponding to reduced tenecin 1 was above 95% as measured by analytical HPLC. Oxidation of this peptide performed by using the different ratios of oxidized (GSSG)/reduced (GSH) glutathione provided a single major peak of the product. The GSSG/GSH ratio corresponding to 10:1 provided almost a single product. Mass spectrum in Fig. 2 and Ellman assay confirmed the oxidation of the protein. As shown in Table 1, the mass of oxidized protein was in good agreement with the calculated mass of tenecin 1. To assign the connectivity of the disulfide bridge, the oxidized protein was digested by enzyme, separated by reverse-phase HPLC and characterized by mass spectrometer as described in Section 2. As shown in Table 1, the measured mass of digested fragments indicated that the oxidized protein was correctly folded. N-terminal loop deleted tenecin 1 was prepared from the deletion of the N-terminus in tenecin 1 by endopeptidase rather than the oxidization of N-terminal deleted linear protein for the following reasons. First, generally, the deletion of the N-terminal loop may have an effect on the folding of the protein [19,20]. Second, as the N-terminal truncated linear tenecin 1 has five cysteine residues, capping of the fourth cysteine may affect the cysteine stabilized α/β scaffold or folding. Digestion of tenecin 1 by aspartic protease provided two peptides. The peptide corresponding to N-terminal loop deleted tenecin 1 was purified by HPLC. Mass spectrum confirmed that only residues 4–16 were selectively deleted in this protein.

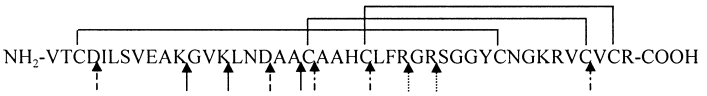
3.2. Antibacterial activity and conformation analysis

As shown in Table 2, chemically synthesized tenecin 1 showed the same antibacterial activity as shown in the literature [13]. However, reduced tenecin 1 and N-terminal loop deleted tenecin 1 did not show antibacterial and antifungal activity up to 100 μg/ml. Many structure-activity studies about antibacterial peptides indicate that an amphipathic α-helical or β-sheet structure and net positive charge are fundamental factors for the activity (reviewed in [21]). As N-terminal loop deletion or reduction of tenecin 1 maintains the net positive charge, we can find the structure-activity relationship of the protein. As membrane-active peptides act on the lipid membrane, the structure in lipid membrane is important for the interpretation of the activity (reviewed in [21]).

To get the conformation of each protein in buffer and lipid membrane, CD spectra of each protein were measured in the presence and absence of TFE, a well known membrane mimicking solvent [22,23]. TFE is known to induce the secondary structure in peptides and proteins and stabilize the helical structure in the native protein that has significant helical propensity [22]. As shown in Fig. 3, the CD spectrum of tenecin 1 measured in buffer exhibited double minimum bands at 208 and 227 nm, indicating the structure of the α-helix and β-

Table 1
Molecular mass of tenecin 1, its analog, and digested fragments

(A) Points cleaved by protease are shown by arrows (←—, endopeptidase Lys-C; ←···, trypsin; ←-·-, thermolysin, ←-- , endopeptidase Asp-N).



(B) Sequence and molecular mass of tenecin 1, its analog and digested fragments.

Peptides	Sequence	Calculated Mass	Measured Mass
Reduced Tenecin 1	NH_2-VTCDILSVEAKGVKLNDAACAAHCLFRGRSGGYCNGKRVCVCR-COOH	4542.2	4540.0±0.1
Tenecin 1	NH_2-VTCDILSVEAKGVKLNDAACAAHCLFRGRSGGYCNGKRVCVCR-COOH	4536.2	4534.6±1.4
N-terminal loop deleted tenecin 1	NH_2-VTC-COOH NH_2-DAACAAHCLFRGRSGGYCNGKRVCVCR-COOH	3187.4	3186.0±0.4
K1 fragment	NH_2-VTCDILSVEAK-COOH NH_2-SGGYCNGK-COOH	1598.8	1960.6±0.1
K2 fragment	NH_2-LNDAACAAHCLFR-COOH NH_2-RVCVCR-COOH	2133.9	2135.3±0.1
K3 fragment	NH_2-AAHC-COOH NH_2-VCR-COOH	774.3	773.7±0.3

sheet. 21% of the α-helix and 35% of the β-sheet were calculated from this CD spectrum using the method of Greenfield and Fasman [18]. This result is consistent with our previous molecular model of tenecin 1 built from the tertiary structure of inset defensin A [24]. Reductions of three disulfide bridges changed the major conformation to the random coil in buffer solution. N-terminal loop deleted tenecin 1 in buffer solution formed an ordered secondary structure that was not well defined. The deletion of the N-terminal loop had the same effect as deletion of the disulfide bridge between the first cysteine and the fourth cysteine. The two-disulfide bridges between the α-helix and β-sheet could not provide enough constraint for the conformation of tenecin 1 maintained by three disulfide bridges.

The CD spectrum of tenecin 1 in the presence of 50% TFE showed that the minimal band at 208 nm greatly increased in this condition, indicating that the α-helical conformation must be induced in the membrane environment. α-Helix and β-sheet content were calculated to be 39% and 34%, respectively. This result suggests that the random conformation of the N-terminal loop changes to an α-helix in the presence of TFE. The CD spectrum of N-terminal loop deleted tenecin 1 measured in the same condition indicates that even though this protein adopts the conformation with the α-helix and β-sheet, the α-helical content (10%) is even lower than that of tenecin 1 obtained in buffer and the β-sheet content is calculated to be 55%. In the N-terminal loop deleted tenecin 1, two disulfide bridges between the α-helix and β-sheet could provide some constraint for the ordered structure in buffer and helped to stabilize the structure with high β-sheet content in the presence of 50% (v/v) TFE. But this structure is not satisfactory for the structural requirement for activity because of the lack of α-helix. We suspect that the lack of α-helix in the presence of TFE must be due to the deletion of the N-terminal loop.

When we consider the structural change of tenecin 1 in

Table 2
Antimicrobial activity of tenecin 1 and its analogs

Strain	MIC (μg/ml)					
	Staphylococcus aureus ATCC 6538	MRSA (methicillin resistant *S. aureus*)	*Micrococcus luteus* ATCC 9341	*Escherichia coli* ATCC 2592	*Pseudomonas aeruginosa* ATCC 9027	*Candida albicans* ATCC 36232
Tenecin 1	6.25	25	3.12	>100	>100	>100
Reduced tenecin 1	>100	>100	>100	>100	>100	>100
N-terminal loop deleted tenecin 1	>100	>100	>100	>100	>100	>100

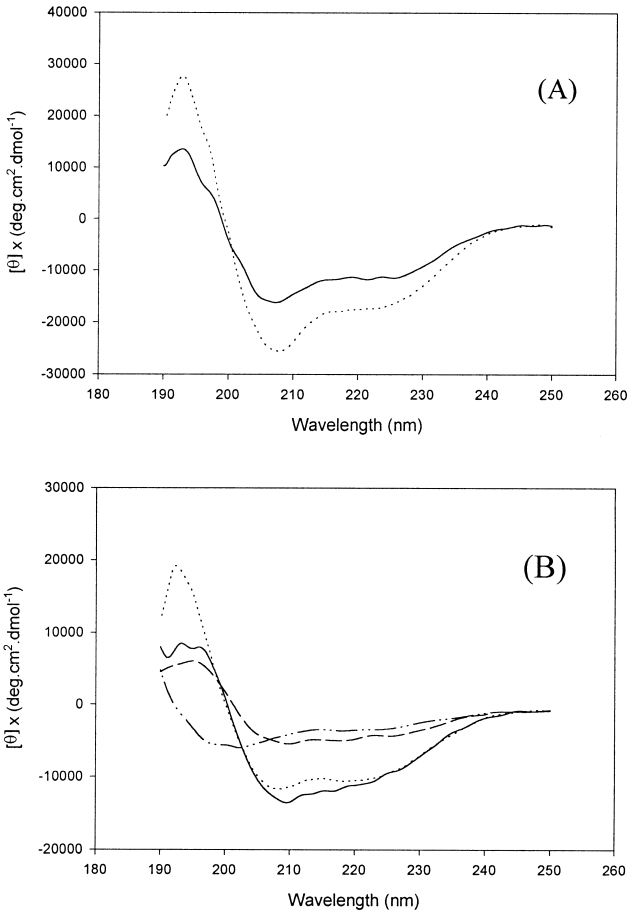


Fig. 3. A: CD spectra of tenecin 1 in buffer with or without 50% (v/v) TFE. ———, in buffer; – – –, in the presence of TFE. B: CD spectra of reduced tenecin 1 and N-terminal loop deleted tenecin 1 in buffer with or without 50% (v/v) TFE. – · · –, Reduced tenecin 1 in buffer; · · ·, reduced tenecin 1 in the presence of TFE; – – –, N-terminal loop deleted tenecin 1 in buffer; ———, N-terminal loop deleted tenecin 1 in the presence of TFE. Spectra of the proteins (200 μg/ml) in buffer with or without TFE 50% (v/v) were recorded on a Jasco J-715 spectropolarimeter in 1-mm cell at 22°C.

buffer and 50% TFE, this protein expected to have a large constraint by three disulfide bridges had some flexibility and adopted a more α-helical structure in the presence of 50% (v/v) TFE. In the presence of TFE, reduced tenecin 1, which has a random conformation in buffer, adopted an ordered structure with 20% α-helix and 22% β-sheet but was not active. Induced structure of reduced tenecin 1 may not be enough for the interaction with the lipid membrane to be biologically active because the α-helical content is much lower than that of tenecin 1 obtained in the presence of TFE. This result indicated that three disulfide bridges in tenecin 1 stabilized the ordered structure in buffer and introduced constraints for helping the induction of the structure with high α-helix in lipid membrane, which must be a critical function of the disulfide bridge for activity. Natori et al. [15] also showed that disulfide bridges of sapecin among the insect defensin family were necessary for antibacterial activity.

In conclusion, tenecin 1 adopted a higher α-helical structure in the presence of 50% (v/v) TFE and three disulfide bridges which were essential for the activity, helped to induce the higher α-helical structure in the membrane mimetic environment. The N-terminal loop also played an important role in the increase of α-helix in the membrane mimetic environment, which was necessary for the activity.

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References

[1] Hoffmann, J.A. and Hetru, C. (1992) Immunol. Today 13, 411–415.
[2] Matsuyama, K. and Natori, S. (1988) J. Biol. Chem. 263, 17112–17116.
[3] Lepage, P., Bitsch, F., Roecklin, D., Keppi, E., Dimarcq, J.L., Reichhart, J.M., Hoffmann, J.A., Roitsch, C. and Van Dorsselaer, A. (1991) Eur. J. Biochem. 196, 735–742.
[4] Cociancich, S., Ghazi, A., Hetru, C., Hoffmann, J.A. and Letellier, L. (1933) J. Biol. Chem. 268, 19239–19245.
[5] Matsuyama, K. and Natori, S. (1990) J. Biochem. (Tokyo) 108, 128–132.
[6] Bonmatin, J.M., Bonnat, J.L., Gallet, X., Vovelle, F., Ptak, M., Reichhart, J.M., Hoffmann, J.A., Keppi, E., Legrain, M. and Achstetter, T. (1992) J. Biomol. NMR 2, 235–256.
[7] Cornet, B., Bonamatin, J.M., Hetru, C., Hoffmann, J.A., Ptak, M. and Vovelle, F. (1995) Structure 3, 435–448.
[8] Hanzawa, H., Shimada, I., Kuzuhara, T., Komano, H., Kohda, D., Inagaki, F., Natori, S. and Arata, Y. (1990) FEBS Lett. 269, 413–420.
[9] Bontem, F., Roumestand, D., Gilquin, B., Menez, A. and Toma, F. (1991) Science 254, 1521–1523.
[10] Johnson, B.A. and Sugg, E.E. (1992) Biochemistry 31, 8151–8159.
[11] Vita, C., Roumestand, C., Toma, F. and Menez, A. (1994) Proc. Natl. Acad. Sci. USA 92, 6404–6408.
[12] Bulet, P. and Hoffman, J.A. (1992) Eur. J. Biochem. 209, 977–984.
[13] Moon, H.J., Lee, S.Y., Kurata, S., Natori, S. and Lee, B.L. (1994) J. Biochem. 116, 53–58.
[14] Fisher, B. and Goodenough, P. (1993) Biotechnol. Bioeng. 41, 3–13.
[15] Kusuhara, T., Nakajuma, Y., Natsuyama, K. and Natori, S. (1990) J. Biochem. 107, 514–518.
[16] Harwick, H.J., Kalmanson, G.M. and Guze, L.B. (1973) Antimicrob. Agents Chemother. 36, 1284–1289.
[17] Nakajima, R., Kitamura, A., Someya, K., Tanaka, M. and Sato, K. (1995) Antimicrob. Agents Chemother. 39, 1517–1521.
[18] Greenfield, N. and Fasman, G.D. (1969) Biochemistry 8, 4108–4116.
[19] Tsunemi, M., Kato, H., Nishiuchi, Y., Kumagaye, S. and Sakakibara, S. (1992) Biochem. Biophys. Res. Commun. 185, 967–973.
[20] Volkman, B.F. and Wemmer, D.E. (1997) Biopolymers 41, 451–460.
[21] Maloy, W.L. and Kari, P.U. (1995) Biopolymer 37, 105–122.
[22] Rajan, R. and Palaram, P. (1996) Int. J. Peptide Protein Res. 48, 328–336.
[23] Cammers-Goodwin, A., Allen, T.J., Oslick, S.L., McClure, K.F., Lee, J.H. and Kemp, D.S. (1996) J. Am. Chem. Soc. 118, 3082–3090.
[24] Lee, K.H., Hong, S.Y., Oh, J.E., Kwon, M., Yoon, J.H., Lee, J., Lee, B.L. and Moon, H.M. (1998) Biochem. J. 334, 99–105.
[25] Yamada, K. and Natori, S. (1993) Biochem. J. 291, 275–279.
[26] Bulet, P., Cociancich, S., Dimarq, J.L., Lambert, J., Reichhart, J.M., Hoffman, D., Hertu, C. and Hoffman, J. (1991) J. Biol. Chem. 266, 24520–24525.
[27] Fujiwara, S., Imai, J., Fumiwara, M., Yaeshima, T., Kawashima, T. and Kobayashi, K. (1990) J. Biol. Chem. 265, 11333–11337.