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Tachycitin, a Small Granular Component in Horseshoe Crab Hemocytes, Is an Antimicrobial Protein with Chitin-Binding Activity[1]

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Small granules of horseshoe crab hemocytes contain two known major antimicrobial substances, tachyplesin and big defensin (S5), and at least five protein components (S1 to S6), with unknown functions. In the present study, we examined the biological properties and primary structure of a small granular component S2, named tachycitin. This component was purified from the acid extract of hemocyte debris by two steps of chromatography. The purified tachycitin was a single chain protein with an apparent M_r=8,500 on Tricine-SDS-polyacrylamide gel electrophoresis. Ultracentrifugation analysis revealed tachycitin to be present in monomer form in solution. Tachycitin inhibited the growth of both Gram-negative and -positive bacteria, and fungi, with a bacterial agglutinating property. Moreover, tachycitin and big defensin acted synergistically in antimicrobial activities. The amino acid sequence and intrachain disulfide bonds of tachycitin were determined by amino acid and sequence analyses of peptides produced by enzymatic cleavages. The mature tachycitin consisted of 73 amino acid residues containing five disulfide bonds with no *N*-linked sugar. A cDNA coding for tachycitin was isolated from a hemocyte cDNA library. The open reading frame coded for an NH_2-terminal signal sequence followed by the mature peptide and an extension sequence of -Gly-Arg-Lys at the COOH-terminus, which is a putative amidating signal. The COOH-terminal threonine amide released after digestion of tachycitin with lysylendopeptidase was identified. The NH_2-terminal 28 residues of tachycitin shows sequence homology to a part of chitin-binding regions found in antifungal chitin-binding peptides, chitin-binding lectins, and chitinases, all of which have been isolated from plants. Tachycitin showed a specific binding to chitin but did not bind with the polysaccharides cellulose, mannan, xylan, and laminarin. Tachycitin may represent a new class of chitin-binding protein family in animals.

Key words: agglutinin, antimicrobial activity, chitin binding, horseshoe crab, host defense.

Horseshoe crabs have a unique host defense system differing from the vertebrate immune system (*1-6*). This system is carried by hemolymph, which contains granular cells comprising 99% of the total hemocytes (*7*) and play an important role in host defense of this animal. The cytosol is filled with two populations of secretory granules, one of which is larger with less electron-dense, named large granule, and the other is smaller with electron-dense, named small granule. The cells are highly sensitive to bacterial endotoxins, lipopolysaccharides (LPS), which are a major outer membrane component of Gram-negative bacteria, and respond to degranulate these granules after LPS stimulation. Biochemical and immunological studies of granular components demonstrated that the two granules selectively store granule-specific proteins participating in the host defense system. Large granules contain all the clotting factors essential for hemolymph coagulation (*1-6*), in addition to protease inhibitors including a Kunitz-type inhibitor (*8*), serpins (*9, 10*), a cystatin (*11*), anti-LPS factor (*12*), and lectins with LPS binding or bacterial agglutinating activity (*13, 14*). On the other hand, small granules contain tachyplesin (*15*), a strong antimicrobial substance, and also at least six cysteine-rich basic proteins, designated numerically S1 to S6, based on retention times on reversed-phase HPLC (*16*). Only two components of small granules, tachyplesin and big defensin (identical to

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Abbreviations: LPS, lipopolysaccharide; TPCK, tosyl phenylalanyl chloromethyl ketone; PCR, polymerase chain reaction; PTH, phenylthiohydantoin; *Ac*-AMP1, *Amaranthus caudatus* antimicrobial peptide 1; UDA, *Urtica dioica* lectin; WGA, wheat germ agglutinin.

S5) (*17*), have been functionally and structurally characterized. In the present study, S2, named tachycitin, was purified and biochemically characterized, and the amino acid and nucleotide sequences were also determined. Tachycitin proved to be a chitin-binding protein with a broad antimicrobial spectrum.

MATERIALS AND METHODS

Materials—Hemocyte debris from the Japanese horseshoe crab (*Tachypleus tridentatus*) was prepared as described (*18*). Zymogen factor C (*19*), tachyplesin (*15, 17*), and anti-LPS factor (*12*) were purified as described. Sources of materials used were as follows; Sephadex G-50 fine, Sephadex G-25, S-Sepharose FF, Sepharose CL-6B, and an electrophoresis calibration kit (cyanogen bromide peptides from horse-heart globin with molecular masses of 16.9, 14.4, 10.7, 8.2, 6.2, and 2.5 kDa) from Pharmacia LKB Biotechnology, Uppsala, chitin and chitooligo-agarose from Seikagaku, Tokyo, cellulose from Toyo Roshi Kaisha, Tokyo, xylan from Fluka Chemie AG, Buchs, endoproteinase Asp-*N* from Boehringer Mannheim Biochemica, Mannheim, lysyl endopeptidase from Wako Pure Chemical Industries, Osaka, asparaginyl endopeptidase from Takara Shuzo, Kyoto, TPCK-trypsin from Worthington Biochemical, Freehold, NJ, mannan, laminarin, and thermolysin from Sigma Chemical, St. Louis, MO, restriction endonucleases and DNA-modifying enzymes from Nippon Gene, Toyama, and Toyobo, Osaka, and [α-^{32}P]dCTP from Amersham Japan, Tokyo. Immobilized polysaccharides were prepared using AF-epoxy Toyopearl 650 (Tosoh, Tokyo) according to the manual provided by the manufacturer.

Purification of Tachycitin—Our foregoing studies indicated that tachycitin, separated from small granules by reversed-phase HPLC, is a 8-kDa protein identified on SDS-PAGE in a 15% gel and the NH_2-terminal sequence is YLAFRXGRYSPXLDXGPNV- (*16*). The hemocyte debris (30 g, wet weight) was extracted twice by homogenizing with 200 ml of 30% acetic acid and the supernatant obtained by centrifugation at 14,000×*g* for 15 min was lyophilized. The dried material was dissolved in 50 ml of 10% acetic acid and applied to a Sephadex G-50 column (3.6×110 cm), equilibrated with 10% acetic acid (data not shown). SDS-PAGE of every five tubes indicated the presence of the 8-kDa protein in fraction Nos. 60-70. The pooled fraction (Nos. 60-73) was lyophilized and then applied to an S-Sepharose FF column (2×32 cm), equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl. After washing with equilibration buffer, proteins were eluted with a linear NaCl gradient of 0.1 to 0.4 M in the same buffer. Two separated peaks both contained proteins with a 8-kDa band on SDS-PAGE in 15% gel. The partial NH_2-terminal sequences were YLAFR- for the protein in the first peak and NPLIP- for the second, respectively, indicating that the first peak contained tachycitin and the second one big defensin. The pooled fraction of the first peak was desalted by Sephadex G-25, equilibrated with 10% acetic acid, and then lyophilized. The purified tachycitin gave a single protein band with M_r=8,500 on Tricine-SDS-PAGE, under reducing conditions (data not shown). The extinction coefficient of tachycitin at 280 nm for 1% solution in water was calculated from data on amino acid analysis. The value of 28.5 was used for subsequent determination of tachycitin concentration. Through the procedures described above, tachycitin was purified reproducibly with the yield of 4-6 mg from 30 g of the hemocyte debris.

Antimicrobial, Bacterial Agglutinating, and Hemagglutinating Activities—Antimicrobial (*17*), bacterial agglutinating (*13, 14*), and hemagglutinating (*14*) activities were assayed, as described.

Isolation of Tachycitin-Derived Peptide, Peptide Sequencing, and Amino Acid Analysis—Tachycitin was reduced, *S*-alkylated with 4-vinyl pyridine, and then digested with lysylendopeptidase (E/S=1 : 40, w/w), endoproteinase Asp-*N* (E/S=1 : 100), or asparaginylendopeptidase (E/S=1 : 100) at 37°C for 16 h. The resulting peptides were separated by reversed-phase HPLC, using a Wakosil II 5C18 (4.6×250 mm, Wako Pure Chemical Industries) and TSK-GEL ODS-80 TM CTR columns (4.6×100 mm, Tosoh). Amino acid sequence analyses were performed using a gas-phase sequencer, model 477A (Applied Biosystems), with the chemicals and program provided by the manufacturer. For amino acid analysis, samples were hydrolyzed in 6 M HCl in evacuated and sealed tubes at 110°C for 24, 48, and 72 h. The hydrolyzates were analyzed using a Hitachi L-8500 amino acid analyzer and a PICO-TAG system (Waters, Millipore, Milford, MA) with the chemicals and program provided by the manufacturer.

Tachycitin-Specific DNA Probes and Screening of cDNA Library—The degenerate nucleotide sequences of the primers used for PCR were based on the amino acid sequences of DDGPNV (residue Nos. 14-20) and VNKECH (residue Nos. 64-69) of tachycitin. Sense and antisense nucleotides were synthesized with an *Eco*RI site at the 5′ end, using a Applied Biosystems DNA synthesizer model 380B with chemicals and program supplied by the manufacturer. Reactions for PCR contained the cDNA template (corresponding to 0.1 μg of poly(A)$^+$RNA) and 100 pmol each of the primer were carried out in a Perkin-Elmer Cetus thermal cycler. The PCR products were treated with *Eco*RI and purified with agarose gel electrophoresis. Fragments of interest were then ligated into plasmid Bluescript II SK$^+$ (Stratagene, La Jolla, CA) for sequence analysis, as described by Sambrook *et al.* (*20*). One clone with 0.2 kb contained the sequence of tachycitin and was used as a probe for screening the λZipLox cDNA library of the hemocytes prepared by Okino *et al.* (*14*). The PCR fragment, labeled with [α-^{32}P]dCTP, using a Ready-To-Go™ DNA-labeling Kit (Pharmacia LKB Biotechnology, Uppsala) served as a probe. After secondary screening, the plasmids containing the cDNA insert were prepared from the positive plaques, following *in vivo* excision protocols provided by the manufacturer and sequenced in both orientations by an Applied Biosystems 373A DNA sequencer, using sequencing primers.

Assignment of Disulfide Linkages in Tachycitin—The intact protein (30 nmol) was dissolved in 0.5 ml of 0.2 M Tris-HCl, pH 6.8, containing 2 M urea and digested with TPCK-trypsin (E/S=1 : 25, w/w) at 37°C for 15 h. The peptides generated were separated by reversed-phase HPLC using a Chemcosorb 5-ODS-H column (2.1×150 mm, Chemco Scientific, Osaka). Peptides were eluted from the column with a linear gradient of 0-80% acetonitrile in 0.06% trifluoroacetic acid at a flow rate of 0.2 ml/min.

Absorbance was monitored at 210 nm. Three peptides containing disulfide bonds were identified by amino acid analysis after performic acid oxidation and one of them, S2-T5, was further digested with thermolysin (10 μg) in 100 μl of 0.2 M sodium acetate, pH 6.5, containing 1 mM $CaCl_2$ at 65°C for 3 h. The digest was separated by reversed-phase HPLC on the same column, under the same conditions described above. The peptides obtained were used for amino acid and sequence analyses (*21*).

Computer-Assisted Analysis of Sequence Data—The amino acid sequence was compared with all entries in SWISS-PLOT protein sequence database [release, 1995, with GENE WORKS (IntelliGenetics, CA)].

SDS-PAGE—SDS-PAGE and Tricine-SDS-PAGE were performed according to Laemmli (*22*) and Schägger and von Jagow (*23*), respectively. The gels were stained with Coomassie Brilliant Blue R-250.

Analytical Ultracentrifugation—Tachycitin in 20 mM Tris-HCl, pH 7.5, containing 0.05 M NaCl was diluted using the same buffer. Sedimentation equilibrium run was performed for the solutions in a Beckman Optima XL-A Analytical Ultracentrifuge at 25,000 rpm at 20°C for 20 h. The reference cell was filled with the same buffer solution mentioned above. The partial specific volume was calculated to be 0.717 ml/g, based on the amino acid composition. The concentration gradients in the cells were determined spectrophotometrically at 280 nm.

RESULTS

Biological Activities of Tachycitin—The 50% inhibitory concentration (IC_{50}) of tachycitin for growth of various bacteria was determined using the microplate culture method (*17*). As summarized in Table I, tachycitin had an antimicrobial activity toward various bacteria. Tachycitin showed inhibitory effect on growth of both Gram-negative (*Escherichia* and *Salmonella*) and -positive bacteria (*Staphylococcus*) at IC_{50} values of 2.0 to 50 μg/ml. The growth of fungi, such as *Candida albicans*, was also inhibited by tachycitin. Furthermore, tachycitin was found to agglutinate both Gram-negative and -positive bacteria and the activity was more efficient for *Escherichia coli* strains than for Gram-positive bacteria (Table II). Tachycitin, however, showed no hemagglutinating activity against human A-, B-, and O-types of erythrocytes. These observations suggested that tachycitin recognizes the bacterial cell wall components. The LPS interaction of tachycitin was examined by the inhibitory activity of tachycitin against the LPS-mediated activation of the LPS-sensitive horseshoe crab protease zymogen, factor C (Fig. 1). Tachycitin neutralized LPS with the similar dose-response curve of big defensin. The 50% inhibitory concentration of 60 nM, however, is about 10-fold higher than for anti-LPS factor (4 nM) and tachyplesin (7 nM). Antifungal peptides isolated from

TABLE I. **Antimicrobial activity of tachycitin.**

	Antimicrobial activity [IC_{50} (μg/ml)]
Gram-negative bacteria	
Escherichia coli (clinical isolate)	33
Escherichia coli B	2.0
Salmonella typhimurium LT2 (S)	44
Salmonella minnesota R595 (Re)	41
Klebsiella pneumoniae	32
Gram-positive bacteria	
Staphylococcus aureus	56
Fungus	
Candida albicans	52

TABLE II. **Bacterial agglutinating activity of tachycitin.**

	Minimum agglutinating concentration (μg/ml)
Gram-negative bacteria	
Escherichia coli K12	53
Escherichia coli B	27
Gram-positive bacteria	
Staphylococcus saprophyticus	ND[a]
Micrococcus luteus	ND
Enterococcus hirae	53

[a]ND, not detectable at 107 μg/ml.

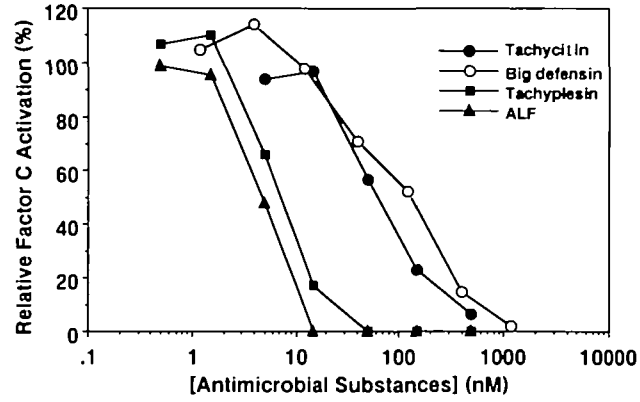

Fig. 1. **Inhibitory activity of tachycitin against the LPS-mediated activation of horseshoe crab factor C zymogen.** LPS (0.2 μg/ml) was preincubated with various concentrations (0.5 to 1,000 nM) of tachycitin (closed circle), big defensin (open circle), tachyplesin (closed square), or ALF, anti-LPS factor (closed triangle), in a total volume of 100 μl of 50 mM Tris-HCl buffer, pH 8.0, containing human serum albumin (0.5 mg/ml). Then 100 μl of zymogen factor C (0.3 μg/ml) was added and the factor C activation was assayed as described (*15*). Relative activation of zymogen factor C was expressed, taking the amidase activity of activated factor C generated in the absence of the inhibitors.

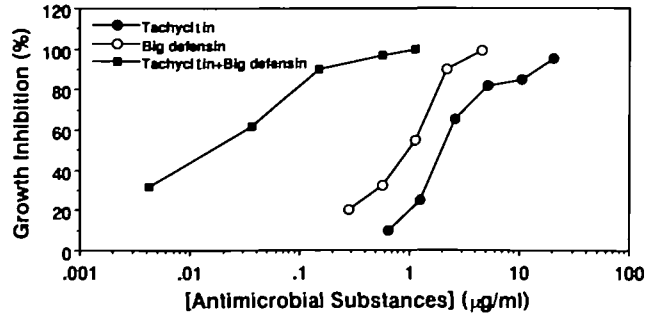

Fig. 2. **A synergistic effect between tachycitin and big defensin on antimicrobial activities.** Serial diluted samples of tachycitin, big defensin or the mixture of big defensin and a constant concentration of tachycitin (0.9 μg/ml) were incubated with *Escherichia coli B* in 10 mM sodium phosphate buffer, pH 7.0, and the antimicrobial activities were assayed as described (*17*). As a control experiment, the phosphate buffer was added to the bacterial suspension.

plant seeds have synergistic effects in combinations of the peptides (*24*). Figure 2 shows dose-response curves of antimicrobial activities of tachycitin, big defensin, and a mixture of big defensin and tachycitin against *E. coli B*, one of the most sensitive bacteria to tachycitin. IC_{50} values were 0.8 and 2 μg/ml for big defensin and tachycitin, respectively. Furthermore, in the presence of tachycitin at an almost noninhibitory concentration (0.9 μg/ml) the dose-response curve of big defensin dramatically shifted to lower concentrations with the IC_{50} of 0.015 μg/ml, suggesting synergistic enhancement of the antimicrobial activity of big defensin by tachycitin. Tachycitin appeared to be heat stable, since antimicrobial activity against *E. coli B* was not reduced by treatment of the protein (5 μg/ml) at 90°C for 1 h. On the other hand, *S*-alkylation of tachycitin with iodoacetamide after reduction led to a compete loss of the antimicrobial activity.

Peptide Sequencing, Isolation of a cDNA Clone, and Nucleotide Sequence of Tachycitin—The peptides derived from proteolytic digests of tachycitin were separated and sequenced, as described under "MATERIALS AND METHODS." The primary peptides used in the sequencing were produced by lysylendopeptidase cleavage (K1 to K6) and the secondary peptides required for overlaps were produced by endoproteinase Asp-*N* (AN1 to AN5) and asparaginylendopeptidase (NC1 to NC5). As a result of sequence analysis of these peptides (data not shown), the amino acid sequence containing 73 residues has been unambiguously determined (Fig. 3).

To obtain information on the precursor sequence of tachycitin, the cDNA clone was then screened. The tachycitin-specific probe of 0.2 kb was identified with oligonucleotides corresponding to the sequences of DDGPNV (residue Nos. 14-20) and VNKECH (residue Nos. 64-69), using PCR and DNA sequence analysis. When the probe was used to screen a hemocyte cDNA library, four positive clones were found and the longest clone with a 0.5 kb insert was subjected to sequence determination. The nucleotide and deduced amino acid sequences are shown in Fig. 3. The cDNA included 527 nucleotides and there were two candidates for an initiation Met at nucleotide positions 2 and 17, but both differed from the proposed optimal initiation sequence of ACCATGG for eukaryotic ribosomes (*25*). The stop codon at position 296 was followed by two polyadenylation signals, AATAAA, starting at position 416 and 491, and a poly (A) tail at position 510. Both open reading frames coded for a mature protein of 73 amino acid residues and an NH_2-terminal signal sequence with a typical hydrophobic core and the amino acid sequence of tachycitin determined by peptide sequencing corresponded exactly to the protein sequence deduced from the cDNA sequence (underlined in Fig. 3). Unexpectedly, the open reading frames also coded for a COOH-terminal extension peptide consisting of -Gly-Arg-Lys, an amidating signal for the carbonyl group of COOH-terminal residue. To determine if the COOH-terminus is amidated, intact tachycitin (4.2 nmol) was digested with lysylendopeptidase at 37°C for 12 h (S/E=100) and the resulting COOH-terminal threonine was recovered in the flow-through fraction of reversed-phase HPLC, the remaining six peptides were adsorbed to the column. The amino acid composition of the flow-through fraction was analyzed using the PICO-TAG system, without hydrolysis, and a single peak with the same retention time of authentic threonine amide (between Tyr and Val) was eluted with the yield of 530 pmol. The peak shifted to the retention time of Thr after hydrolysis. Thus, the COOH-terminal threonine of tachycitin is apparently amidated. The amino acid composition of tachycitin agreed well with that deduced from the cDNA and glucosamine and galactosamine were not detected (data not shown). The molecular weight calculated from the amino acid sequence was 8,363, a value in good agreement with that of the

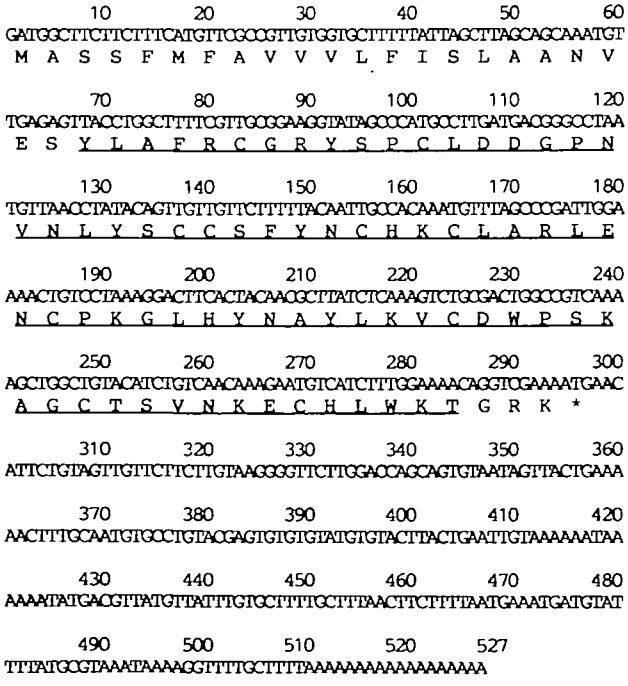

Fig. 3. **Nucleotide sequence and deduced amino acid sequence of tachycitin.** A single underline represents the amino acid sequence determined by peptide sequencing of the purified tachycitin. A star represents a stop signal.

TABLE III. **Amino acid compositions of Cys-containing peptides from tachycitin.**[a]

Amino acid	S2-T1	S2-T2	S2-T5	S2-T5-TL4	S2-T5-TL8
	(Residues/molecule)				
Cys	2.4(2)	2.1(2)	7.2(6)	3.5(4)	1.7(2)
Asp		2.0(2)	7.5(6)	1.1(1)	1.0(1)
Glu		1.0(1)	1.3(1)	1.2(1)	
Ser		0.8(1)	3.6(4)	1.1(1)	0.9(1)
Gly	1.0(1)		2.4(2)	1.0(1)	
His			1.5(2)	0.3(1)	
Arg	1.5(2)				
Thr			0.7(1)		
Ala	1.0(1)		0.8(1)	0.9(1)	
Pro		1.0(1)	2.0(2)		1.0(1)
Tyr			0.9(3)	0.3(1)	1.0(2)
Val		0.7(1)	2.1(2)	1.0(1)	
Leu	0.8(1)	0.7(1)	3.0(3)	0.8(1)	
Trp		ND(1)	ND(1)		
Lys		1.0(1)	2.0(3)	0.5(1)	
Position	6-8	52-58	9-32	22-25	9-12
	33-36	37-42	59-72	59-61	28-30
				64-69	

[a]Calculated from the 20-h hydrolysate after performic acid oxidation and values in parentheses are taken from the sequence data. ND, not determined.

purified protein estimated on Tricine-SDS-PAGE (M_r = 8,500).

Assignment of Disulfide Linkages in Tachycitin—The intact tachycitin was digested with a combination of TPCK-trypsin and thermolysin and four disulfide-containing peptides were isolated, as described under "MATERIALS AND METHODS." Amino acid analysis indicated that peptides S2-T1, S2-T2, and S2-T5-TL8 contained Cys^{6}-Cys^{33}, Cys^{40}-Cys^{53}, and Cys^{12}-Cys^{30}, respectively (Table III) and the disulfide bonds were confirmed by sequence analysis (Table IV). On the other hand, peptide S2-T5-TL4 contained 4 cysteines, composed of three peptide chains linked by two disulfides (Table III). Sequence analysis of S2-T5-TL4 revealed that PTH-cystine was recovered at the third and the fifth cycles of Edman degradation, clearly indicating that the two disulfide bonds were Cys^{24}-Cys^{61} and Cys^{25}-Cys^{68} (Table IV). The disulfide pattern of tachycitin is schematically shown in Fig. 4.

Analytical Ultracentrifugation—The concentration gradients obtained for tachycitin in the sedimentation equilibrium run could be well simulated on the assumption of the presence of a single species with the molecular weight of 7,848±47 (n=3). This indicates that the protein exists as a monomer in solution, under the conditions used.

Sequence Homology to Chitin-Binding Proteins and Chitin Binding Activity of Tachycitin—In overall length, tachycitin showed no significant sequence similarity to known proteins but the NH_2-terminal region had a partial sequence similarity to those of chitin-binding proteins, such as hevein (*26*), *Ac*-AMP1(*27*), stinging nettle (*Urtica dioica*) lectin (UDA) (*28*), wheat germ agglutinin (WGA) (*29*), and plant chitinases (*30, 31*) (Fig. 5A). Hevein (43 amino acid residues) and *Ac*-AMP1 (29 residues), antifungal peptides with chitin-binding properties and isolated from the rubber-tree (*Hevea brasiliensis*) latex and amaranth (*Amaranthus caudatus*) seeds, respectively. UDA and WGA are chitin-binding lectins, composed of repetitive hevein-like sequences arranged in tandem, two repeats in UDA and four repeats in WGA. On the other hand, chitinases from plants, such as poplar and bean, contain one hevein domain at their NH_2-terminal regions. To determine if tachycitin has chitin-binding ability, the purified protein was mixed with chitin equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and chitin was precipitated by centrifugation. After washing with buffer containing 1 M NaCl, the precipitated chitin was eluted with 10% acetic acid. Tachycitin was not recovered in the supernatant even in the presence of 1 M NaCl and could be eluted by the acid (Fig. 6A), therefore, tachycitin binds tightly to chitin, similar to the other chitin-binding proteins described above. Tachycitin could not be eluted with 1 M

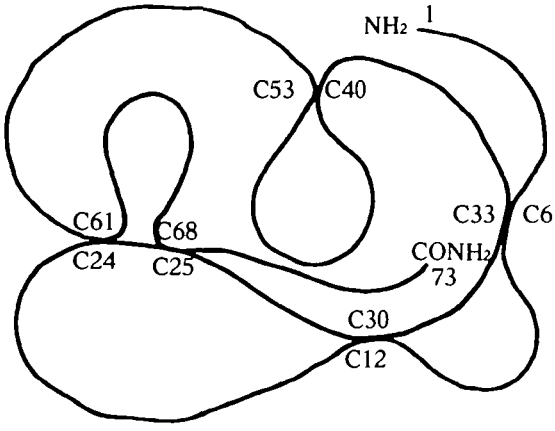

Fig. 4. **A structural model of tachycitin with five disulfide linkages.**

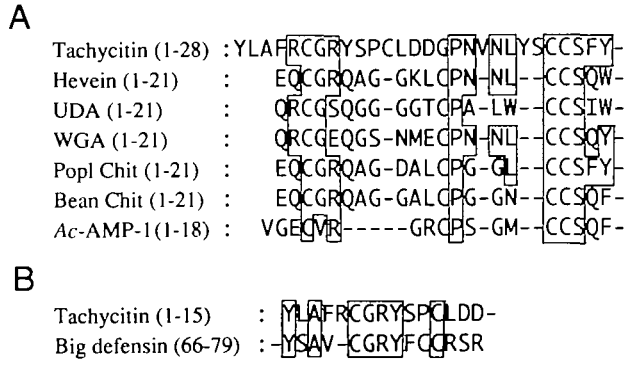

Fig. 5. **Alignment of the NH_2-terminal region of tachycitin with those of chitin-binding proteins and chitinases (A), and with the COOH-terminal part of big defensin (B).** Identical amino acids to tachycitin are boxed. Hevein, antifungal peptide from *Hevea brasiliensis* (*26*); UDA, lectin from *Urtica dioica* (*28*); WGA, wheat germ agglutinin (*29*); Popl Chit, acid endochitinase, win 6.2C, from poplar (*30*); Bean Chit, bean leaf chitinase (*31*); *Ac*-AMP-1, antimicrobial peptide 1 from *Amaranthus caudatus* (*27*).

TABLE IV. **NH_2-terminal sequences of Cys-containing peptides derived from tachycitin.**

Cycle No.	S2-T1	S2-T2	S2-T5-TL4	S2-T5-TL8
		[PTH-amino acids (yield, pmol)]		
1	C-C(nq)[a]	L(82), V(91)	V(31), Y(29), A(32)	Y(138), Y
2	G(119), L(104)	E(54)	N(16), S(6), G(32)	S(14), N(41)
3	R(57), A(97)	N(45), D(35)	K(26), C-C(nq)	P(41)
4	R(99)	C-C(nq), W(35)	E(14),	X
5		P(101), P	C-C(nq)	
6		K(27), S(11)	H(10)	
7		K(43)		
	CGR(6-8)	LENCPK(37-42)	YSCC(22-25)	YSPC(9-12)
	|	/	| \	/
	CLAR(33-36)	VCDWPSK(52-58)	AGC (59-61)	YNC(28-30)
			VNKECH(64-69)	

[a]nq, not quantitated.

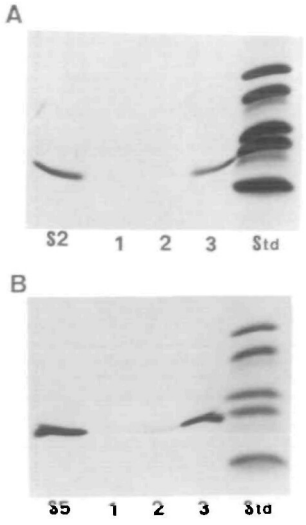

Fig. 6. **Chitin-binding abilities of tachycitin (A) and big defensin (B).** Tachycitin or big defensin (5 μg) was incubated with chitin (20 mg) in 200 μl of 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl at 25°C for 10 min and the supernatant was obtained by centrifugation. The precipitated chitin was eluted with 1 M NaCl in the same buffer and then 10% acetic acid. Each supernatant was treated with 10% trichloroacetic acid and the resulting precipitate was subjected to Tricine-SDS-PAGE. Lane 1, unbound supernatant with the equilibration buffer; lane 2, eluate with 1 M NaCl; lane 3, eluate with 10% acetic acid. S2, tachycitin; S5, big defensin; Std, molecular weight standards.

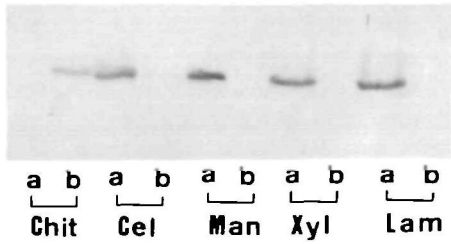

Fig. 7. **Binding specificity of tachycitin to polysaccharides.** Polysaccharides except for chitin were immobilized to Toyopearl as described under "MATERIALS AND METHODS." Tachycitin (5 μg) was applied to a small column packed with chitin (10 mg) or other polysaccharides (20 mg) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, and each column was eluted with 10% acetic acid. Each flow-through fraction (a) or eluate (b) was treated with 10% trichloroacetic acid and the resulting precipitate was subjected to Tricine-SDS-PAGE. Chit, chitin; Cel, cellulose; Man, mannan; Xyl, xylan; Lam, laminarin.

N-acetyl-D-glucosamine, a component sugar of chitin, and 100 mM chitotriose. In addition, tachycitin did not bind to *N*-acetyl-D-glucosamine-agarose and chitooligo-agarose, immobilized with the mixture of di- to pentasaccharides (data not shown). The NH_2-terminal 12 residues of tachycitin showed high sequence similarity with the COOH-terminal region of big defensin (Fig. 5B) and big defensin was also found to bind to chitin, under the same conditions (Fig. 6B). Chitin column chromatography, therefore, was effectively used for buffer exchange, concentration, or desalting of both protein samples. To test the binding specificity of tachycitin, we applied it to columns packed with several polysaccharides including cellulose, mannan, xylan, and laminarin, equilibrated with the same buffer. However, tachycitin did not bind to any of these columns and was detected only in the flow-through fractions (Fig. 7).

DISCUSSION

Tachycitin interacts with LPS as do other antibacterial peptides isolated from horseshoe crab hemocytes, such as anti-LPS factor (*12*), tachyplesin (*15, 32*), and big defensin (*17*), whereas there is no significant sequence homology between these peptides. Crystal structure of anti-LPS factor indicates that it has a single domain consisting of three α-helices packed against a four-stranded β-sheet to form a wedge-shaped molecule with a striking charge distribution and amphipathicity (*33*). The binding site for LPS probably involves the extended amphipathic loop, which represents an LPS-binding motif shared by two mammalian proteins, LPS-binding protein (*34*) and bactericidal/permeability-increasing protein (*35*). However, the binding affinity of tachycitin for LPS is considerably lower than that of anti-LPS factor. Tachycitin may contain a different three dimensional structural motif for LPS binding.

Antimicrobial activity of tachycitin is not so strong by itself and the IC_{50} values for various bacteria are 3 to 70-fold higher than those of big defensin (*17*). However, tachycitin and big defensin acted synergistically and the IC_{50} value of big defensin against *E. coli B* was decreased to 1/50 in the presence of small amount of tachycitin (Fig. 3). In physiological conditions, tachycitin may function as an antimicrobial protein in cooperation with other antibacterial substances stored in granules of hemocytes, which are secreted into the extracellular fluid in response to the external stimulation of LPS.

Tachycitin consists of 73 amino acid residues containing five disulfide bonds with no *N*-linked sugar and the COOH-terminal threonine is amidated (Fig. 4). This protein retains antimicrobial activity with treatment at 90°C for 1 h, but it loses the original activity after reduction and alkylation, indicating that disulfide bonds play an important role to hold the proper conformation required for the activity. The amino acid sequence of tachycitin showed some similarity with those of chitin-binding proteins isolated from plants. As expected, tachycitin bound to chitin, a polymer of β-1,4-*N*-acetyl-D-glucosamine. The sequence homology is particularly striking in the NH_2-terminal 28 residues, which may be important for the chitin-binding (Fig. 5A). Furthermore, the NH_2-terminal region of tachycitin has a significant sequence homology to the COOH-terminal portion of big defensin (Fig. 5B). Big defensin also shows chitin-binding activity (Fig. 6), suggesting that the sequence of -Cys-Gly-Arg-, also conserved in hevein-like domains of plant chitin-binding proteins, is the primary binding site for chitin. Although tachycitin interacts with chitin, it does not bind to other polysaccharides such as cellulose (poly-β-1,4-D-glucose), mannan (poly-β-1,4-D-mannose), xylan (poly-β-1,4-D-xylose), and laminarin (poly-β-1,3-D-glucose). In addition, tachycitin does not bind to chitooligo-agarose immobilized di- to peptasaccharides, suggesting that the binding requires longer units of β-1,4-*N*-acetyl-D-glucosamine, or a specific conformation of the polysaccharide. Tachycitin does not show hemagglutinating activity, unlike the chitin-binding lectin WGA

composed of four tandem repeats of hevein-like domains, probably because tachycitin does not recognize the mono- or oligosaccharides of *N*-acetyl-D-glucosamine. Interestingly, tachyplesin, the major component of the small granules with strong antimicrobial activity, binds to chitin in a similar manner, but lectin-like proteins derived from the large granules, L6 (tachylectin-1) (*13*) and L10 (tachylectin-2) (*14*), have no apparent binding ability to chitin (data not shown). Therefore, the chitin-binding property may be a common feature of the small granular components.

Chitin is a component of the cell wall of fungi, and chitin-binding proteins identified in plants such as antibacterial substances, lectins, and chitinases are considered to be defense molecules to kill fungal pathogens when recognizing cell wall component(s), perturbing the plasma membrane, or hydrolyzing the polysaccharides on the cell wall (*36, 37*). In *C. albicans*, attenuated virulence of the chitin-deficient mutants has been reported (*38*). On the other hand, chitin is the major structural component of arthropod exoskeletons. The physiological significance of chitin-binding activities of the small granular components found in the hemocytes is unknown but they probably recognize chitin exposed at the site of a lesion and they appear to serve not only as antibacterial molecules against invading microbes but also in wound healing, which may stimulate and accelerate biosynthesis of chitin at sites of injury.

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