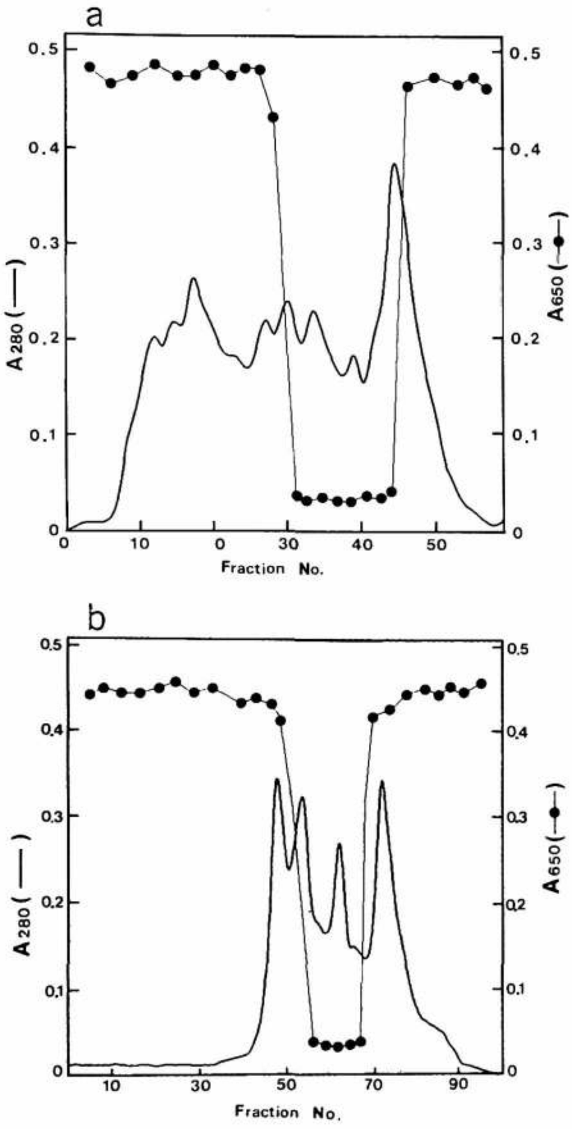

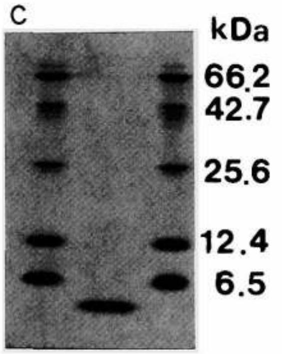

Fig. 1. **(a) First reverse-phase (C_{18}) open column chromatography of the heat-treated crude hemolymph solution.** A C_{18} open column was packed with ODS-A120 (1.0×15 cm, YMC-Gel, Japan). Fractions of 5 ml were eluted with a linear gradient of 5–35% trifluoroacetic acid, and their antibacterial activities were tested using *S. aureus*. Antibacterial activity was measured as inhibition of bacterial growth. —, absorbance at 280 nm; —●—, bacterial growth (A_{650}). **(b) Second reverse-phase (C_{18}) open column chromatography of material from the first reverse-phase column.** The active fraction from the first open column was fractionated in an open column system with ODS-A120 (C_{18}, YMC-Gel). Material was eluted with a linear gradient of 5–35% acetonitrile. —, absorbance at 280 nm; —●—, bacterial growth (A_{650}). **(c) SDS–polyacrylamide gel electrophoresis of purified tenecin 1.** Electrophoresis was carried out on 2 μg of purified protein under denaturing conditions. The marker proteins at both sides are bovine serum albumin (66,200), egg albumin (42,700), α-chymotrypsinogen (25,600), cytochrome *c* (12,400), and aprotinin (6,500).

TABLE I. **Antibacterial activities of hemolymph of *T. molitor* larvae immunized with *E. coli*.**

Assay conditions	No. of colonies
Positive control[a]	500
Negative control[b]	0
Normal hemolymph	495
Hemolymph after *E. coli* injection	
Without dilution	0
Diluted 1/2	0
Diluted 1/4	14

[a]Assay without added material, [b]assay without bacteria.

TABLE II. **Antibacterial specificities of tenecin 1.** Antibacterial activities were measured as described in the text and are expressed as concentrations causing 50% inhibition of bacterial growth relative to control.

Bacterial strain	Antibacterial activity (μg/ml)
Bacillus subtilis ATCC 1768	2.0
Bacillus subtilis ATCC 6633	1.0
Staphylococcus aureus ATCC 6538	3.7
Staphylococcus aureus SG 501	0.8
Staphylococcus epidermidis ATCC 12228	8.0
Staphylococcus pyrogen 77A	1.0
Staphylococcus pyrogen 308A	1.6
Micrococcus luteus ATCC 9341[a]	8.0
Micrococcus luteus ATCC 10240[a]	5.0
Corynebacterium diphtheriae ATCC 8024[b]	8.0
Corynebacterium diphtheriae ATCC 8032[b]	6.0
Escherichia coli ATCC 2592	>30
Shigella flexneri ATCC 203	>30
Pseudomonas aeruginosa ATCC 9027	>30
Proteus vulgaris OX-19 ATCC 6380	>30

[a]Culture at 37°C for 12 h. [b]Culture at 37°C for 20 h.

1 was further fractionated on a second C_{18} reverse open column, which gave almost a single distinct peak of antibacterial activity against *S. aureus* coinciding with a peak of A_{280}, as shown in Fig. 1b. For further purification, this material was subjected to reverse phase HPLC and active fractions were collected and lyophilized. On SDS-PAGE, this purified tenecin 1 gave a single band (Fig. 1c). From this result, we concluded that tenecin 1 has a molecular mass of about 4 kDa and was purified to near homogeneity by this procedure.

Antibacterial Activities of Tenecin 1—We examined the antibacterial activities of the tenecin 1 against various bacteria. As shown in Table II, tenecin 1 inhibited the growth of various Gram-positive bacteria, but did not affect on the growths of Gram-negative bacteria at a concentration of 30 μg/ml. This spectrum of antibacterial activities is similar to those of sapecin family proteins.

Isolation and Characterization of a cDNA Clone for Tenecin 1—Using synthetic DNA probes, we isolated two hybridization-positive clones by screening about 50,000 clones in a cDNA library. Both clones were found to contain inserts corresponding to full length cDNA for tenecin 1 mRNA. Since they had the same mobility on agarose gel electrophoresis, we analyzed the nucleotide sequence of only one of them, T1A. This clone contained a cDNA insert of about 0.2 kilobase pairs (kb) including a poly(A) tail. Its nucleotide sequence is shown in Fig. 2 with the deduced amino acid sequence. This cDNA was that of tenecin 1, because it contained amino acid sequences of three frag-