Moricin, a Novel Type of Antibacterial Peptide Isolated from the Silkworm, Bombyx mori*

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A novel antibacterial peptide that shows antibacterial activity against Staphylococcus aureus was isolated from the hemolymph of the silkworm, Bombyx mori. The novel peptide consisted of 42 amino acids and was highly basic. This peptide indicated no significant similarity with other antibacterial peptides. The peptide showed antibacterial activity against several Gram-negative and -positive bacteria and had a higher activity against Gram-positive bacteria than cecropin B1, a major antibacterial peptide of B. mori. The novel peptide was inducible by bacterial injection. These results suggest that the peptide is responsible for the antibacterial activity in B. mori against Gram-positive bacteria. The effects of the peptide on bacterial and liposomal membranes showed that a target of the peptide is the bacterial cytoplasmic membrane. The results also suggest that the N-terminal portion of the peptide, containing a predicted α-helix, is responsible for an increase in the membrane permeability. We propose the name “moricin” for this novel antibacterial peptide isolated from B. mori.

Although insects do not have immune systems that involve antigen-antibody reactions, they do have efficient self-defense mechanisms against bacterial infection. For example, they can induce antibacterial proteins upon bacterial infection (Boman and Hultmark, 1987). To date, many antibacterial proteins have been isolated from different species of insects (Cociancich et al., 1994a) and can be classified into five major groups (Hultmark, 1993): cecropins, insect defensins, attacin-like (glycinrich) proteins, proline-rich peptides, and lysozymes. Cecropins and insect defensins belong to a small group of antibacterial proteins showing high antibacterial activity and rapid bactericidal effects. The antibacterial mechanisms of cecropins and insect defensins have also been studied (Okada and Natori, 1984, 1985; Christensen et al., 1988; Matsuyama and Natori, 1990; Cociancich et al., 1993; Yamada and Natori, 1994).

Cecropins are thought to be primarily responsible for the antibacterial activity in some insects since they show antibacterial activity against many kinds of Gram-negative and -positive bacteria. Although cecropins have been isolated from several species of lepidopteran and dipteran insects (Cociancich et al., 1994a), they have not been found in other orders of insects. This suggests that cecropins are not general antibacterial proteins in insects.

Insect defensins are highly effective against Gram-positive bacteria, including human pathogenic bacteria such as Staphylococcus aureus, whereas they do not exhibit strong activity against Gram-negative bacteria. Contrary to cecropins, insect defensins are more common in insects and have been isolated from several orders of insects such as dipteran, hymenopteran, coleopteran, trichopteran, hemipteran, and odonata (Hoffmann and Hetru, 1992; Cociancich et al., 1994b). However, insect defensins have yet to be observed in lepidopteran insects. Except for the insect defensins, all types of antibacterial proteins have been reported in lepidopteran insects (Boman et al., 1991; Hara and Yamakawa, 1995). All five antibacterial protein groups have been isolated from dipteran insects (Hultmark, 1993). Considering that all cecropins are not effective against some genera of Gram-positive bacteria, such as Staphylococcus and Bacillus (Hultmark et al., 1982; Qu et al., 1982; Teshima et al., 1987; Tu et al., 1989; Boman et al., 1989), we hypothesize that there is as yet unidentified antibacterial protein(s) in lepidopteran that shows a strong activity against Gram-positive bacteria.

Previously, we investigated the antibacterial activity of the hemolymph from Bombyx mori against Gram-negative bacteria (Yamakawa et al., 1990; Hara et al., 1994; Hara and Yamakawa, 1995). In the present work, we surveyed the antibacterial activity of the hemolymph against one of Gram-positive bacteria, S. aureus, and isolated a novel antibacterial peptide. The peptide showed antibacterial activity against both Gram-negative and -positive bacteria. This peptide indicated a higher activity against Gram-positive bacteria than cecropin B2, a major antibacterial peptide of B. mori. Our results also suggested that a target of the peptide was the bacterial cytoplasmic membrane. Moreover, the N-terminal half of the peptide, containing a predicted α-helix, was shown to be responsible for the increase in membrane permeability.

MATERIALS AND METHODS

Collection of Immune Hemolymph—B. mori (Tokai × Asahi) was reared on an artificial diet (Nihonnosanko) at 25 °C. The fifth instar larvae of B. mori were immunized with Escherichia coli HB101. The hemolymph was collected and heat treated as described previously (Yamakawa et al., 1990).

Purification Procedures—The heat-treated hemolymph was subjected to ammonium sulfate precipitation. The substances precipitating between 15 and 75% saturation with ammonium sulfate were gel filtered through a Sephadex G-50 column as described previously (Hara et al., 1994). A series of solutions was prepared containing 0.05 M ammonium acetate, pH 5.0 (buffer A), and 0.8 M ammonium acetate, pH 7.0 (buffer B), in the following ratios: 9:5:0.5, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, and 3:7 (v/v). An aliquot containing antibacterial activity was loaded onto a cation exchange column (CM-Sepharose FF, 2.5 × 4.4 cm, Pharmacia Biotech Inc.) equilibrated with buffer A. The column was first washed with 50 ml of buffer A, and substances were eluted stepwise with 50 ml of each buffer B solution described above in that order. The eluates...
monitored by a Bio-Rad model EG-1 Econo Gradient Monitor were fractionated according to their conductivity. One of the fractionated samples was applied to a reverse-phase HPLC^{1} column of Capcell Pak C8 SG300 (10 \times 250 mm, Shiseido) equilibrated with 12% acetonitrile containing 0.1% trifluoroacetic acid. Substances were eluted using a linear gradient of 12–60% acetonitrile containing 0.1% trifluoroacetic acid at approximately 60 min. Flow rate was 2.4 ml/min, and 2.4-ml fractions were collected consecutively. Fractions containing high antibacterial activity against S. aureus were subjected to reverse-phase HPLC again for further purification. During the second HPLC iteration, the high antibacterial fractions used a Capcell Pak C8 SG300 column (4.6 \times 250 mm, Shiseido) for approximately 35 min with a linear gradient of 30–60% acetonitrile containing 0.1% trifluoroacetic acid. Flow rate was 0.5 ml/min, and the absorbance was monitored at 220 nm. The purified peptide was quantified by the folin phenol method (Peterson, 1983).

Analysis for Amino Acid Composition and Sequence—The amino acid composition and sequence were determined using a Hitachi model 835–50 amino acid analyzer and an Applied Biosystems model 473A protein sequencer, respectively.

Proteolytic Enzyme Treatment—40 \mu g of the purified peptide was digested in 200 \mu l of 50 mM Tris-HCl, pH 7.5, at 37 °C for 24 h with 1 \mu g of endoproteinase, Asp-N, from Pseudomonas fragi var. (Takara). The resultant peptide fragments were isolated by reverse-phase HPLC. To determine the C-terminal structure of the peptide, 3 \mu g of the peptide sample was incubated at 37 °C for 20 h with 0.7 \mu g of carboxypeptidase A (Sigma, type II, phenylmethanesulfonyl fluoride-treated) in 40 \mu l of 0.2 M boric acid, 0.2 M KCl-NaOH, pH 9.2 (Ambler, 1972). The reaction mixture was analyzed by polyacrylamide gel electrophoresis in acidic conditions (Hultmark et al., 1980).

Assay for Antibacterial Activity—Throughout the purification steps of antibacterial peptides, antibacterial activity was assayed by measuring growth inhibition zones (Hultmark et al., 1982) on thin agarose plates of nutrient medium (Difco) with S. aureus ATCC6538P as an indicator. In some cases, plates containing 150 mM NaCl were used.

Assay for the Permeability of Bacterial Cytoplasmic Membrane—S. aureus ATCC6538P was grown at 30 °C for 16 h in LB medium containing 1% galactose as an inducer for phospho-beta-galactosidase, a cytoplasmic enzyme of S. aureus (Oskouian and Stewart, 1987). The cells were harvested from 0.5 ml of the broth, washed three times with 1 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 130 mM NaCl, and suspended in the same buffer (absorbance at 650 nm was adjusted to 0.13). An aliquot of the cell suspension (48 \mu l) was added to incremental amounts of the purified peptide dissolved in 6 \mu l of 10 mM sodium phosphate buffer, pH 7.0, containing 130 mM NaCl. The mixtures were then incubated at 30 °C for 20 min. 6 \mu l of 15 mM o-nitrophenyl-beta-D-galactopyranoside (Sigma), a chromogenic substrate of phospho-beta-galactosidase (Hengstenberg et al., 1976), was added to each mixture. After incubating the mixtures at 30 °C for an additional 90 min, the reaction was stopped by the addition of 0.8 ml of 0.1 M NaOH (10 \mu l). The production of o-nitrophenol was assayed by measuring the absorbance at 405 nm.

Assay for the Permeability of Liposomal Membrane—Liposome with trapped glucose was prepared according to the method of Yamada and Natori (1994). The phospholipid composition of the liposome was similar to that of the cytoplasmic membrane of S. aureus; the molar ratio of phosphatidylglycerol:cardiolipin was 7:2:5:2. Phosphatidylglycerol from egg yolk lecithin (Sigma) and cardiolipin from bovine heart (Sigma) were used for the liposome preparation. 2 \mu mol of the phospholipid mixture in chloroform was dried in a glassware test tube under nitrogen gas. After addition of 0.3 M glucose (0.2 ml), a multilamellar liposome was formed by vortex mixing. The liposome prepared in this way was washed three times with 1 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 130 mM NaCl and suspended in 0.6 ml of the same buffer. An aliquot of the liposome suspension (10 \mu l) was added to incremental amounts of peptides dissolved in 10 \mu l of 10 mM sodium phosphate buffer, pH 7.0, containing 130 mM NaCl. These mixtures were then incubated at 25 °C for 1 h. After incubation, the amount of glucose released from the liposome was analyzed with a Glucose C-test kit (Wako Chemical).

RESULTS

Antibacterial Activity of B. mori Hemolymph against S. aureus—We first investigated the antibacterial activity of B. mori hemolymph against a Gram-positive bacterium, S. aureus, by the growth inhibition zone assay (Hultmark et al., 1982). Our results demonstrated that the activity against S. aureus is induced by the injection of bacterial cells into the body cavity of B. mori larvae analogous to the activity induced against Gram-negative bacteria (data not shown). The efficiency of induced activity against S. aureus was compared between larvae immunized with E. coli and S. aureus. Induction was observed in all larvae injected with E. coli; however, only 60% of the S. aureus-injected larvae showed any activity. This result implies that S. aureus is inferior to E. coli as an inducer of the activity against S. aureus.

Next, a question if the activity against S. aureus derived from cercopins, the major antibacterial peptide in B. mori, was examined. Antibacterial substances from immunized hemolymph with E. coli and from non-immunized control hemolymph were fractionated by cation exchange column chromatography and subsequently by reverse-phase HPLC. The activity against S. aureus of each HPLC fraction was assayed. Fractions from the immunized hemolymph showed two peaks indicating antibacterial activity (Fig. 1a). The two peaks were not found in the fractions from non-immunized hemolymph, suggesting they are induced by bacterial infection (Fig. 1b). The first antibacterial peak (41 min) contained cercopin B as described below. The second peak (44 min) contained a previously unreported antibacterial substance(s).

Purification of the Novel Antibacterial Peptide—Antibacterial substance(s) were purified from 200 ml of the immunized hemolymph. The main absorbance peak exhibiting antibacterial activity against S. aureus was eluted with buffer A/buffer B (4:6) on a cation exchanger. The substance was further fractionated by reverse-phase HPLC.

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1 The abbreviation used is: HPLC, high performance liquid chromatography.
The peak fractions corresponding to the induced antibacterial activity (44 min) in the analytical HPLC were pooled (Fig. 1a). The pooled sample was again subjected to reverse-phase HPLC, and the peak fraction showing antibacterial activity was collected. A typical HPLC elution pattern is shown in Fig. 2. The unidentified substance showed a single stained band in SDS-polyacrylamide gel electrophoresis for low molecular weight proteins (Schägger and von Jagow, 1987), suggesting the presence of a peptide (data not shown). After the final purification step, about 150 μg of the antibacterial peptide was obtained.

In the first HPLC, the fractions containing antibacterial samples (41 min) were also collected and pooled (Fig. 1a). Three types of cecropin B (Teshima et al., 1986; Morishima et al., 1990) were purified by further HPLC. Namely, the amino acid sequences determined using a protein sequencer revealed that they were cecropin B1 (Lys-21 was hydroxylated), B2 (no Lys was hydroxylated), and a molecule having two hydroxylated Lys residues (Lys-21 and Lys-33), respectively (data not shown). We designated the third molecule as “cecropin B3.”

Primary Structure of the Peptide—The amino acid composition of the purified antibacterial peptide was determined (Table I). The amino acid composition was different from those of cecropins, and it contained Thr and His residues not found in B. mori cecropins (Teshima et al., 1987).

A sequence of 33 amino acid residues was initially obtained by the Edman degradation method (Fig. 3). To obtain additional sequence information, the peptide was digested with an endoproteinase Asp-N, which cleaves the peptide bond on the N-terminal side of Asp residue (Gabriel, 1980). The resultant fragments were purified by reverse-phase HPLC (the fragments were designated as D1 and D2 based on their elution), and their sequences were determined (Fig. 3). The combined sequences showed that the peptide consists of 42 amino acid residues as shown in Fig. 3. The amino acid composition calculated from the sequence was agreed well with the analyzed composition (Table I).

For further confirmation, the molecular mass of the peptide was measured by ion spray mass spectrometry on a Sciex model API-III triple quadrupole spectrometer. The obtained value, 4543.1 ± 0.6 Da, was coincident with the calculated value, 4543.5 Da, assuming that the C terminus of the peptide is unmodified.

The C termini of cecropins are generally amidated (Boman and Hultmark, 1987; Hara et al., 1994). Contrary to cecropins, the analysis of the peptide by mass spectrometry suggested that the C terminus was unmodified. To confirm the C terminus is unmodified, the peptide was digested with carboxypeptidase A, which can release amino acids from unmodified C termini (Neurath, 1960). The carboxypeptidase A cleaved the peptide, suggesting that the C terminus of the peptide is unmodified (data not shown).

Antibacterial Activity of the Peptide—The lethal concentrations of the peptide and cecropin B against some Gram-negative and -positive bacteria were determined by the growth inhibition zone assay (Hultmark et al., 1982). The results show that the peptide has antibacterial activity against both Gram-negative and -positive bacteria. When compared with cecropin B1, the novel peptide indicated a higher activity against Gram-positive bacteria (Table II). Notably, the peptide showed high activity against some pathogenic Gram-positive bacteria such as Bacillus cereus, S. aureus (IFO3083), S. xylosus, and Strep-tococcus pyogenes, whereas cecropin B1 was less effective. Furthermore, the addition of NaCl did not reduce the activity of the peptide against S. aureus, while it considerably reduced the activity of cecropin B1 (Table II).

Since S. aureus did not grow when more than 2 μg/ml (0.44 μM) of the peptide was present in a liquid medium of brain heart infusion, the time course of the viability of S. aureus cells incubated with 3 μg/ml of the peptide was examined. The results demonstrated that the reduction of the viability of S. aureus cells occurred within a few min after addition of the peptide (Table III), suggesting that the peptide has strong bactericidal activity against S. aureus.

Effect of the Novel Peptide on the Permeability of Bacterial Cytoplasmic Membrane—Since the peptide rapidly killed S. aureus (Table III) and the primary structure of the novel peptide showed the characteristics of an antibacterial protein (see “Discussion”), it was hypothesized that a target of the peptide was the bacterial membrane. Hence, the effect of the peptide on the permeability of S. aureus cytoplasmic membrane was investigated. The change in the permeability was assayed by measuring the level of the hydrolysis of o-nitrophenyl-β-D-
galactopyranoside-6-phosphate, a chromogenic substrate for phospho-β-galactosidase. As a result, the hydrolysis of O-nitrophenyl-β-D-galactopyranoside-6-phosphate was confirmed to occur when more than 5 μM of the peptide was in the incubation mixture (Table IV), suggesting that the peptide increases the permeability of the cytoplasmic membrane of S. aureus.

Effects of the Novel Peptide and Its Proteolytic Fragments on the Permeability of Liposomal Membrane—Whether or not the increase of the permeability of the cytoplasmic membrane was caused by the direct effect of the peptide on the membrane was examined using an artificial liposome membrane with entrapped glucose. The change in the permeability was assayed by measuring the level of glucose leaking into the buffer solution. The results demonstrated that the peptide caused the release of glucose from the liposome (Fig. 4), suggesting that the change in the permeability of cytoplasmic membrane is due to the direct effect of the peptide on the membrane. The effect of the endoproteinase Asp-N-digested fragments of the peptide, D1 and D2 (Fig. 3), on the permeability of liposomal membrane was also investigated. Fragment D2 released glucose from the liposome, and the releasing efficiency was about one-tenth of that of the intact peptide (Fig. 4). Conversely, fragment D1 did not result in the release of glucose from the liposome. These results suggest that the fragment D2 region of the peptide is responsible for the increase of the permeability of the mem-

**TABLE II**

Antibacterial activity of the novel peptide and cecropin B1

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Lethal concentration (\mu M)</th>
<th>Cecropin B1 (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli J M109</td>
<td>0.31</td>
<td>0.18</td>
</tr>
<tr>
<td>Acinetobacter sp. NIL5 B-4653</td>
<td>0.27</td>
<td>0.19</td>
</tr>
<tr>
<td>Pseudomonas fluorescens IAM1179</td>
<td>0.53</td>
<td>0.49</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa IAM15140</td>
<td>0.81</td>
<td>0.62</td>
</tr>
<tr>
<td>Bacillus subtilis IAM1107</td>
<td>0.19</td>
<td>0.50</td>
</tr>
<tr>
<td>Bacillus megaterium IAM1030</td>
<td>0.09</td>
<td>0.21</td>
</tr>
<tr>
<td>B. cereusIFO3457</td>
<td>0.38</td>
<td>33</td>
</tr>
<tr>
<td>S. aureus ATCC6538P</td>
<td>0.21</td>
<td>0.77</td>
</tr>
<tr>
<td>S. aureus ATCC6538P*</td>
<td>0.22</td>
<td>13</td>
</tr>
<tr>
<td>S. aureusIFO3083</td>
<td>0.46</td>
<td>16</td>
</tr>
<tr>
<td>Staphylococcus xylosus IAM1312</td>
<td>0.27</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Staphylococcus epidermidis IFO12993</td>
<td>0.18</td>
<td>0.49</td>
</tr>
<tr>
<td>S. pyogenes ATCC21547</td>
<td>0.25</td>
<td>6.9</td>
</tr>
</tbody>
</table>

* The plates contained additional 150 mM NaCl.

**TABLE III**

Effect of the novel peptide on viability of S. aureus cells

<table>
<thead>
<tr>
<th>Time</th>
<th>Colony-forming units ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>205 ± 13</td>
</tr>
<tr>
<td>2 min</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>5 min</td>
<td>30 ± 18</td>
</tr>
<tr>
<td>10 min</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>20 min</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>24 h</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

**DISCUSSION**

We isolated a novel type of antibacterial peptide from the hemolymph of B. mori by monitoring its antibacterial activity against S. aureus. We propose the name "moricin" for this novel peptide.

A search of the Protein Identification Resource data base yielded no peptides or proteins with significant similarity to moricin. Like other antibacterial peptides, moricin is highly basic, and the higher basicity tends to correlate with the higher antibacterial activities (Fink et al., 1989; Bevins and Zaslowsky, 1990; Hoffmann and Hetru, 1992; Cociancich et al., 1994a; Kagan et al., 1994; Gabay, 1994; Iwanaga et al., 1994). The basicity is thought to be responsible for the attachment of positively charged peptides to the negatively charged bacterial surface through the electrostatic interaction (Christensen et al., 1988; Gabay, 1994). The value of isoelectric point of moricin was calculated to be 12.0, higher than those of cecropins (pI = 8.2–9.6). Second, moricin has a predicted amphipathic α-helix. Generally, α-helical structures are responsible for the expression of antibacterial activity. For example, NMR analysis indicated that cecropins consist of two amphipathic α-helices (Holak et al., 1988; Iwai et al., 1993). Recently, the α-helical region of an insect defensin was found to be responsible for both antibacterial activity and the increase of permeability of liposomal membrane (Yamada and Natori, 1994). In the N-terminal half of moricin, charged amino acids appear at intervals of three or four amino acid residues, indicating a characteristic
structure in antibacterial proteins containing the amphipathic α-helix (Cociancich et al., 1994a; Krell, 1994). The α-helical wheel projection (Schiffer and Edmundson, 1967) of amino acid residues (5–22) of moricin resulted in a clear separation of hydrophobic and hydrophilic faces (Fig. 5), suggesting the existence of an amphipathic α-helix in this region.

The results indicated that a target of moricin is the bacterial membrane. The N-terminal fragment (fragment D2, 29 amino acid residues) seems to be partially responsible for the increase of the membrane permeability because the fragment itself increased the permeability of liposomal membrane (Fig. 4). Hence, the active center of moricin may be in the predicted α-helical region (residues 5–22). On the other hand, it is characteristic that basic amino acid residues cluster in the C-terminal region. Although the effects of the two fragments on the viability of S. aureus and on the permeability of cytoplasmic membrane require further investigation, the results suggest that the basic C-terminal region interact with the surface of bacterial membrane and then changes the permeability of the membrane by the N-terminal amphipathic α-helix.

Since moricin is induced by bacterial infection and shows a strong antibacterial activity against bacteria, we assume that moricin plays an important role in the self-defense against bacterial infection in B. mori. Our results suggest that the main target of moricin is bacteria, because moricin showed only slight antifungal activity against some strains of yeast and no hemolytic activity against murine erythrocytes (data not shown). Although both moricin and cecropins show antibacterial activity against several species of bacteria, moricin tends to have higher activity against Gram-positive bacteria. On the contrary, cecropins have higher activity against Gram-negative bacteria. This suggests that moricin and cecropins are simultaneously induced upon bacterial infection, and they can efficiently eliminate the wide variety of invading bacterial species. Furthermore, the existence of moricin in B. mori may be a reason why the defensin-type antibacterial peptides are missing in lepidopteran insects. To confirm this hypothesis, the presence of moricin-type antibacterial peptides in other lepidopteran insects should be investigated.

Interestingly, moricin has no modified amino acid residues such as the α-amidation of C termini in cecropins, the hydroxylation of Lys residues in B. mori cecropins, the O-glycosylation of Thr residues in proline-rich antibacterial peptides (Bulet et al., 1993; Cociancich et al., 1994b; Hara and Yamakawa, 1995), or formation of intramolecular disulfide bonds in defensins (Kuzuhara et al., 1990; Lepage et al., 1990). These unique properties of moricin provide a favorable condition for the production of the peptide by chemical synthesis or by biotechnology using suitable protein expression vectors. For this reason, we are presently expressing an artificial moricin gene in mass scale to obtain a sufficient quantity for further analysis of its precise role in self-defense system.
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