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A Potent Antibacterial Protein in Royal Jelly

PURIFICATION AND DETERMINATION OF THE PRIMARY STRUCTURE OF ROYALISIN*

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A new potent antibacterial protein, for which we propose the name royalisin, was found in royal jelly of the honeybee *Apis mellifera* L. and purified to homogeneity for the first time by acid extraction, gel filtration, and reverse-phase high pressure liquid chromatography. The primary structure of royalisin was determined to consist of 51 residues, with three intramolecular disulfide linkages, having a calculated molecular mass of 5523 Da. Royalisin is an amphipathic protein, with the C-terminal half of the molecule being rich in charged amino acids; and it showed extensive sequence homology to two other antibacterial proteins, sapecin from embryonic *Sarcophaga peregrina* cells and phormicins from *Phormia terranovae* larvae. Royalisin was found to have potent antibacterial activity against Gram-positive bacteria at low concentrations, but not against Gram-negative bacteria. Royalisin may be involved in a defense system active against bacterial invasion of the honeybee.

Various insects are known to produce humoral antibacterial proteins in response to pathogens which participate in the host defense system against invading microorganisms. The isolated antibacterial proteins from certain moths and flies are cecropins (1-5) and attacins (6-8) from *Hyalophora cecropia* pupae, sarcotoxins (9) from *Sarcophaga peregrina* larvae, sapecin (10) from embryonic *S. peregrina* cells, and diptericins (11) and phormicins (12) from *Phormia terranovae* larvae. Recently, apidaecins have been isolated from lymph fluid of the honeybee *Apis mellifera* L. and are highly active against Gram-negative bacteria (13). On the other hand, the presence of antibacterial properties in royal jelly secreted from the pharyngeal glands of the honeybee has been known for many years. The inhibitory activity of royal jelly against both Gram-positive and Gram-negative bacteria has been demonstrated (14). Later studies (15-18) reported that royal jelly exhibited antibiotic activity against a variety of microorganisms including some actinomycetes and certain species of molds and other fungi. It is evident that the antibacterial spectrum of royal jelly differs significantly from that of apidaecins. With respect to the active substances, the only antibiotic component previously identified in royal jelly was the fatty acid 10-hydroxy-Δ^2-decenoic acid (19). Our interest in further defining antibacterial properties of royal jelly led to a search for other antibacterial constituents.

This report describes for the first time a potent antibacterial protein, named royalisin, found in royal jelly. The complete primary structure of royalisin was investigated and found to be distinct from that of all other known antibacterial proteins or peptides of insects or invertebrates so far reported. The antibacterial activity of purified royalisin was examined against a number of bacterial species, and the results indicate selective growth inhibition against Gram-positive bacteria such as *Lactobacillus, Bifidobacterium,* and *Leuconostoc* at effective concentrations below 1 μM.

MATERIALS AND METHODS[1]

RESULTS

Purification of Royalisin—To confirm earlier observations of the antibacterial effects of native royal jelly, its influence on the growth of nine species of Gram-positive bacteria and seven species of Gram-negative bacteria was examined. As shown in Table 1, growth of the Gram-positive bacteria was strongly suppressed by addition of royal jelly at concentrations of 10 μg/ml or less. Among royal jelly-sensitive bacteria, *Lactobacillus helveticus* ss. *jugurti* was the most sensitive and therefore was chosen as the indicator strain for use in our search for the antibacterial substance in royal jelly. In contrast, royal jelly only slightly inhibited the growth of the Gram-negative bacteria tested, and this effect was observed only at high concentrations (10 μg/ml) of royal jelly.

The antibacterial protein royalisin was partially purified from royal jelly by acid extraction followed by gel filtration on Sephadex G-100. Fig. 1 shows the elution profile of the antibacterial activity of royalisin together with the A_{254} reading and the A_{595} reading which indicates protein concentration. The antibacterial activity eluted as a single peak (fractions at 380-430 ml) together with some UV-absorbing material and appeared in the low molecular mass fractions. Further purification of the partially purified protein was achieved by reverse-phase HPLC.[2] As shown in Fig. 2, a single well-defined peak of potent antibacterial activity was obtained which eluted at 32% (v/v) CH_3CN. The yield of royalisin was 0.3 mg/g of royal jelly. When the purity of the final preparation of royalisin was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2), a single homoge-

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[1] Portions of this paper (including "Materials and Methods," Figs. 1-6, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviation used is: HPLC, high pressure liquid chromatography.

neous protein band was observed under reducing conditions with an apparent molecular mass of 3500–4000 Da. A similar molecular mass estimate of 3900 Da was obtained by gel filtration on a GCL-90 column (data not shown).

Primary Sequence of Royalisin—To determine the amino acid sequence of royalisin, the purified protein was subjected to amino acid analysis using an automated gas-phase sequenator. From data obtained by amino acid sequencing, 45 of the 51 residues of royalisin were identified. Since cysteine is known to present an obstacle to structural determination (20), alkylation of the cysteine residues of royalisin with 4-vinylpyridine was performed. Six residues of *S*-pyridylethylated cysteine in royalisin were found after amino acid analysis, indicating that all of the previously unidentified residues of royalisin were cysteine. This result was independently verified by amino acid composition analysis after acid hydrolysis and by fast atom bombardment mass spectroscopic analysis (data not shown). Based on these results, the complete primary sequence of royalisin was determined, as given in Fig. 3. The molecular mass of royalisin is 5523 Da as calculated from the amino acid sequence.

Assignment of Disulfide Linkages in Royalisin—The absence of free sulfhydryl groups in royalisin was demonstrated upon its failure to react with 4,4′-dithiodipyridine. Therefore, all cysteine residues present in royalisin must be linked with disulfide bridges. To elucidate the location of all disulfide bridges in royalisin, sequence analysis of proteolytic peptides derived from purified royalisin was performed as follows. As shown in Fig. 4, lysyl endopeptidase cleavage of royalisin resulted in the formation of five new peptide peaks (I–V). The peptide of peak IV was found to have amino acids corresponding to positions 1–9 and 27–33 and was assigned the position of a disulfide bridge between residues 3 and 31. The peptide of peak V was found to have amino acids corresponding to positions 10–26 and 34–40. This peptide, however, contained 4 cysteine residues; and hence, it was further digested enzymatically with thermolysin, and the peptides generated were analyzed similarly. One peptide corresponding to positions 18–21 and 37–40 and another corresponding to positions 16–17 and 35–36 were isolated separately. This result indicated the presence of disulfide bridges between residues 21 and 38 and residues 17 and 36, respectively. This assignment of the positions of three disulfide bridges in royalisin was further confirmed by the results of protease V8 digestion (data not shown).

Hydrophilicity and Homology of Royalisin—The hydrophilicity index of royalisin as shown in Fig. 5 indicates that it is an amphipathic molecule. The N-terminal half of the sequence (positions 1–22) is relatively hydrophobic, whereas the C-terminal half (positions 23–45) is mainly hydrophilic and contains an array of highly positively charged residues, particularly lysine at positions 26, 33, 39, 44, and 49. As shown in Fig. 6, a computer search of the Genetyx Protein Sequence Data Banks (Software Development Co., Tokyo) revealed that royalisin exhibits substantial homology to two other antibacterial proteins, sapecin and phormicin, but not to other known antibacterial proteins or peptides from insects or other invertebrates. In addition, royalisin was found to show 52–62% similarity to several other membrane-bound proteins including membrane antigen p140, phospholipase A_2, and a noncapsid protein of a murine virus.

Antibacterial Activity of Royalisin—The antibacterial effects of royalisin were tested against 18 species of Gram-positive bacteria and seven species of Gram-negative bacteria. As summarized in Table 2, royalisin had a narrower antibacterial spectrum than native royal jelly and strongly inhibited the growth of the Gram-positive bacteria tested including *Clostridium, Corynebacterium, Leuconostoc, Staphylococcus,* and *Streptococcus* at the effective concentration of 1 μM. The antibacterial potency of royalisin at a concentration of 1 μM was comparable to that of native royal jelly at 10 μg/ml (Table 1). However, royalisin showed no antibacterial activity against the Gram-negative bacteria tested including *Escherichia coli, Bacteroides, Klebsiella,* and *Salmonella* at the concentrations used. The antibacterial activity was relatively heat-stable since activity was retained after heating for 15 min at 100 °C. The activity was completely lost, however, after cleavage with either endoprotease Arg-C or lysyl endopeptidase.

DISCUSSION

This paper describes for the first time the purification and complete primary sequence of a new potent antibacterial protein, royalisin, in royal jelly. It has been reported (19, 21) that low pH and the presence of 10-hydroxy-Δ^2-decenoic acid are factors partially responsible for the ability of royal jelly to inhibit bacterial and fungal growth, but these observations have been questioned (22). So far, the major components of royal jelly responsible for its remarkable antimicrobial properties remain unidentified. Therefore in this study, attempts were made to isolate the active substance. After acid extraction and further fractionation of royal jelly, the antibacterial properties observed were attributed to the presence of a protein, royalisin. No fatty acid was found in the final soluble fractions eluted by HPLC. The new antibacterial protein was purified ~2000-fold as estimated from the potency of royalisin (Tables 1 and 2). Royalisin was found to be composed of 51 residues, being a cationic protein with a net charge of +2 as calculated from the amino acid composition (Fig. 3). The structural novelty of royalisin is in its high cysteine content (6 residues) and connection by three intramolecular disulfide linkages, which may confer a compact globular structure exhibiting high stability at low pH and high temperature as demonstrated in the study. The observed difference between the measured molecular mass of ~4000 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the calculated molecular mass of 5523 Da may be interpreted as evidence of a compact molecular structure. Based on the primary sequence, royalisin is distinctly different from both the humoral antibacterial peptide apidaecin (13) and the venom toxin melittin of the honeybee (23). A computer search for homology to other known antibacterial proteins, however, revealed that royalisin has extensive sequence homology to sapecin and phormicin, which are active against only Gram-positive bacteria (Fig. 6). Sapecin has a primary structure almost identical to that of phormicins, differing only by a single amino acid substitution of glycine for alanine at position 34. It was found that 24 of the 51 residues of royalisin are identical to sapecin and phormicin, including the sequence of all cysteine residues which have been highly conserved during evolution. No significant similarities were found between royalisin and any other antibacterial peptides such as defensins from guinea pig neutrophils (24–26), bactenecin from bovine neutrophils (27), magainins from clawed frog *Xenopus* skin (28), and tachyplesin and polyphemusin from the horseshoe crab (29, 30).

In this study, the specificity of royalisin was found to be limited to activity against various Gram-positive bacteria. *Lactobacillus, Bifidobacterium, Corynebacterium, Leuconostoc, Streptococcus,* and *Staphylococcus* were extremely sensitive to royalisin, and its minimum inhibitory concentration for certain bacteria in this study was ~1 μM, which is comparable with the effective concentrations of various antibiotics. Al-

though royalisin is structurally and functionally related to sapecin and phormicin, royalisin showed a somewhat different antibacterial specificity. Sapecin is especially inhibitory to *Staphylococcus, Micrococcus,* and *Corynebacterium* (10), whereas phormicin is active mainly against *Micrococcus* and *Bacillus* (12). In contrast to royalisin, native royal jelly exhibited some inhibitory effects against Gram-negative bacteria also. This difference could be explained by the presence of other factors such as 10-hydroxy-Δ^2-decenoic acid in native royal jelly which were completely lost during fractionation. Because of its antibacterial potency, royalisin looks attractive for future applications as an antibacterial compound, especially for the preservation of food.

Nothing is known about the mechanism of the antibacterial action of royalisin. Comparison of computer hydropathy profiles indicated that the known antibacterial proteins each display an amphipathic sequence. In the case of royalisin, the N-terminal half of the molecule is predominantly hydrophobic, whereas the C-terminal half is strongly basic and hydrophilic. An analogous profile was also found in various other antibacterial peptides such as sapecin, phormicin, tachyplesin, and polyphemusin, all of which have essential disulfide bonds and are active against Gram-positive bacteria. In contrast, melittin, which has a similar polarity profile but no disulfide bonds, exhibits potent lytic activity against Gram-negative bacteria such as *E. coli* (23). Thus, judging from the primary structure and antibacterial specificity, it seems that a general feature of the proteins active against Gram-positive bacteria is a high content of cysteine residues and a compact structure owing to intramolecular disulfide cross-linking. This notion is supported by the fact that two microbicidal peptides, MCP1 and MCP2, from macrophages and leukocytes (31, 32) also have three disulfide bonds in each molecule and exhibit selective inhibition against Gram-positive bacteria. Another structural requirement for cytotoxic properties appears to reside in a cluster of charged residues located in the C-terminal sequence of royalisin. A computer homology search showed that the hydrophilic C-terminal region of royalisin at positions 25–40 has significant homology to regions within cecropins and sarcotoxins, although these peptides have a basic N terminus, whereas it is the C terminus in royalisin that is basic. It has been reported (9, 33) that the sequences of colicin E_1 and sarcotoxin have a similar lysine-rich region located in the N-terminal half of the molecule that is essential for the disruption of the membrane potential of *E. coli.* It is possible that royalisin acts by a similar mechanism. Although it appears that the biological activity is likely to be related to its ability to interact with the membrane, we have been unable to detect the release of lactate dehydrogenase or visible evidence of bacterial membrane lysis by phase-contrast electron microscopy after royalisin treatment (data not shown).

The biological role of royalisin in royal jelly is still uncertain. Royalisin may participate in host defense by protecting the gut of the honeybee against invasion of various bacteria and may help in preservation of royal jelly. In addition, royalisin may have a hormonal function. It has been reported (34) that royal jelly contains insulin-like polypeptides; and recently, it has been demonstrated (35) that purified extracts from royal jelly displace porcine insulin from rat liver insulin receptors *in vitro.* This is consistent with a report (36) that intravenous injection of royal jelly causes a transient fall of blood pressure in rats. In preliminary experiments[3] using a radioligand assay, we have found that purified royalisin binds to the rat liver insulin receptor, with an inhibition constant of 45%. This observation suggests that royalisin may have structural and functional similarity to the insulin-like polypeptides in royal jelly. Furthermore, royalisin seems to have some structural similarity to a mammalian epidermal growth factor which also consists of 53 residues with three intramolecular disulfide linkages. The functions of royalisin in royal jelly as a growth factor need to be further examined, especially with regard to its possible role in promoting growth and differentiation in the honeybee. The origin of royalisin is not known; but at present, it is assumed to be derived from the honeybee. This is also under investigation.

[3] K. Kobayashi, unpublished data.

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SUPPLEMENTARY MATERIAL

TO

A Potent Antibacterial Protein in Royal Jelly: Purification and Determination of the Primary Structure of Royalisin

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MATERIALS AND METHODS

Materials

Royal jelly of *Apis mellifera L.* collected in Cheng-Mai, Thailand, was obtained from Akitaya Honten, Gifu, Japan. Upon receipt, the royal jelly was stored at -20°C until used. *Staphylococcus aureus* V8 protease and lysyl endopeptidase were obtained from Seikagaku Kogyo Co., Tokyo, Japan. Thermolysin was purchased from Wako Pure Chemical Ind., Tokyo, Japan. HPLC-grade acetonitrile was obtained from Kanto Chemical Ind., Tokyo, Japan. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo, USA, and Wako Pure Chemical Ind., Tokyo, Japan, and were analytical grade or better.

Purification of Royalisin.

Step 1: Extraction

The native royal jelly (5g) from *Apis mellifera L.* was dissolved in 100ml of distilled water at 4°C and the pH of the suspension was adjusted with NH_4OH (10%) to pH 8.5. After 30 min of sedimentation, the alkaline suspension was centrifuged at 10,000xg for 10 min at 4°C. The precipitate was collected and resuspended in 100ml of distilled water at 4°C and the pH of the suspension was lowered with 0.2N HCl to pH 2.0. The supernatant obtained after centrifugation at 10,000xg for 10 min was adjusted with NH_4OH (10%) to pH 5.0. After another centrifugation at 10,000xg for 10 min, the clear supernatant was retained and lyophilized, yielding about 170mg of crude protein from royal jelly.

Step 2: Gel filtration

The crude protein (170mg) was dissolved in 20ml of 10mM ammonium phosphate buffer, pH 5.0, containing 20mM NaCl and applied to a gel filtration column of Sephadex G-100 (2.6cmx90cm; Pharmacia, Uppsala, Sweden) that had been equilibrated previously with the same buffer. The column was then eluted at a flow rate of 3 ml/min with 10mM ammonium phosphate buffer, pH 5.0, and the protein elution pattern was monitored at 254nm. Aliquots of each fraction (0.5ml) were assayed for protein concentration and antibacterial activity. Protein concentration was determined using a commercial protein assay kit (Bio-Rad Lab., Richmond, CA, USA). Those fractions found to contain antibacterial activity were pooled, dialyzed, and lyophilized, yielding 2mg of protein.

Step 3: Reverse-phase HPLC

For further purification by HPLC, 40μg of the lyophilized protein was dissolved in 20μl of distilled water and applied to a reverse phase HPLC column of Aquapore RP-300 (C8) (4.6mmx10mm; Brownlee Labs, Santa Clara, CA, USA) connected to a TriVI HPLC system (JASCO, Tokyo, Japan). The column was eluted at a flow rate of 0.7 ml/min with a linear gradient of 35% (v/v) solvent B (90% (v/v) CH_3CN containing 0.05% (v/v) trifluoroacetate) in solvent A(0.05% (v/v) trifluoroacetate) over a total time of 50 min. The elution of protein from the column was monitored by measuring the absorbance at 220nm using a Uvidec 100VI spectrophotometer (JASCO, Tokyo, Japan) and a Chromatocorder 11 recorder (System Instrument Co., Tokyo, Japan). Antibacterial activity was determined using aliquots of the eluted fractions that had been vacuum-dried to remove acetonitrile.

SDS/Polyacrylamide Gel Electrophoresis

Electrophoresis on SDS/polyacrylamide slab gels was performed according to the method of Swank and Munkres (37) using a 12.5% acrylamide gel containing 8M urea. Aliquots of purified protein (1μg) were reduced by addition of 2-mercaptoethanol and were subjected to electrophoresis at 40V for 15 hours at 25°C. After electrophoresis, the gels were stained for proteins using 0.2% (w/v) Coomassie blue R-250 (Bio-Rad, Richmond, CA, USA). Pharmacia reference markers were used. Myoglobin III(2512), Myoglobin II(6214), Myoglobin I(8159), Myoglobin I&II(14404), and Myoglobin (16949).

Primary Structure Determination

Amino acid sequences of the purified protein, S-pyridylethylated protein, and peptide fragments were determined using a gas phase Model 470A sequencer (Applied Biosystems Inc., Foster City, CA, USA). Approximately 1μg of sample was used for each analysis. The S-pyridylethylated protein was obtained after reduction and pyridylethylation of cysteine residues in the purified protein. For determination of disulfide bridge location, peptide fragments were generated by enzymatic digestion of the purified protein with lysyl endopeptidase or thermolysin (from *Bacillus thermoproteolyticus*). For lysyl endopeptidase digestion, the purified protein (0.4mg) in 250μl of 50mM Tris/HCl (pH 9.0) was incubated at 37°C for 30 min with 30μg of lysyl endopeptidase (0.15U). The treatment of the purified protein with thermolysin (50μg) was carried out at 37°C for 2 hours in 50μl of 0.2N N-ethylmorpholine acetate buffer (pH 8.0). Each of these reaction mixtures was directly subjected to reverse-phase HPLC and the isolated peptide fragments were further applied to an automated amino acid sequencer.

Pyridylethylation of Royalisin

Pyridylethylation of cysteine residues in the purified protein was performed essentially as described by Fullmer (20). The purified protein (100μg), dissolved in 50μl of 0.2N N-ethylmorpholine acetate buffer, pH 8.0, was reduced by addition of 2 mercaptoethanol (1μl) and held for 30 min at room temperature. Subsequently, alkylation was performed by addition of 2μl of 4-vinylpyridine and the reaction mixture was held for 90 min at room temperature with stirring. The S-pyridylethylated protein was desalted by applying the reaction products to reverse-phase chromatography and subjected to protein sequence analysis.

Antibacterial Assay

All cultures were maintained as frozen stocks at -78°C. Before experimental use, cultures were propagated twice at 37°C in MRS broth (for *Lactobacillus helveticus ss jugrurti*) or other nutrient media suitable for a given bacterial strain (Difco Lab. Detroit, Mich, USA). Bacteria in the exponential phase of growth were collected by centrifugation at 1300xg for 10 min and resuspended in physiological saline at a cell concentration of 10^8 per ml. The indicator organism used for assay of antibacterial activity during purification was *Lactobacillus helveticus ss. jugurti*. The antibacterial activity and specificity of purified royalisin was tested with various other bacterial strains also. The protein samples to be tested were added to 3ml of culture medium and the medium was filter-sterilized using a Millex GV filter (0.22μm, Millipore Limited, Tokyo, Japan). The test medium was inoculated with 90μl of the bacterial suspension in saline. After incubation at 37°C for 16 hours with various concentrations of the protein samples, each culture was rapidly chilled and its turbidity was measured at 660nm using a UVDEC-660 spectrophotometer (JASCO, Tokyo, Japan). For determination of antibacterial activity, three replicates were performed for each microbial strain.

Table 1 Antibacterial activity of native royal jelly.

Bacterial growth in the presence of various concentrations of royal jelly is expressed as a percentage of that obtained with a control culture grown in the absence of royal jelly.

Bacterial strains		Royal jelly (μg/ml) 0.1	1.0	10
Gram (-)				
Bacteroides	fragilis	-	-	-
Bacteroides	vulgatus	-	-	-
Escherichia	coli IID861	-	-	33
Escherichia	coli IID5208	-	-	-
Klebsiella	pneumoniae IFO-3321	-	-	-
Salmonella	infantis	-	-	16
Salmonella	typhimurium	-	-	19
Gram(+)				
Bifidobacterium	adolescentis ATCC15703	-	82	90
Bifidobacterium	bifidum ATCC15696	-	-	33
Bifidobacterium	breve ATCC15700	-	64	86
Bifidobacterium	infantis ATCC15697	-	-	73
Bifidobacterium	longum ATCC15707	-	25	93
Eubacterium	aerofacience	36	102	87
Lactobacillus	acidophilus ATCC314	-	-	52
Lactobacillus	acidophilus ATCC4356	-	-	64
Lactobacillus	helveticus ss. jugurti	-	97	96

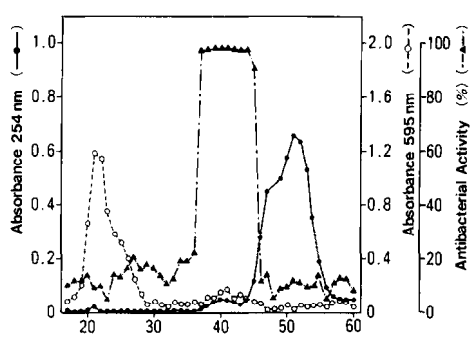

Figure 1 Sephadex G-100 gel filtration chromatography of proteins extracted from royal jelly.

The column was eluted with 10mM ammonium phosphate buffer, pH 5.0, containing 20mM NaCl and fractions (10ml) were collected. Each fraction was assayed for protein concentration and antibacterial activity. Triangles indicate the antibacterial activity as determined by inhibition of growth of *Lactobacillus helveticus ss. jugurti*. Closed circles indicate the absorbance at 254nm and open circles indicate the absorbance at 595nm after treatment with protein assay reagents.

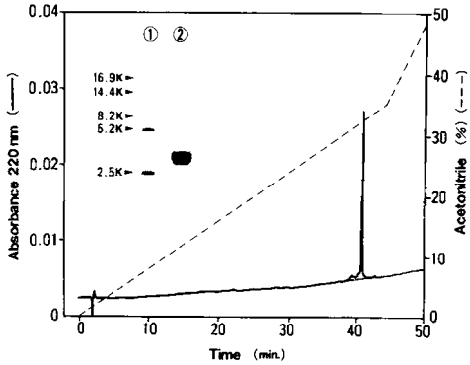

Figure 2 Reverse-phase HPLC analysis and SDS/polyacrylamide gel electrophoresis of royalisin.

Royalisin was analyzed in a TriVI HPLC system using a column of Aquapore RP-300(C8) (flow rate and gradient, see Materials and Methods). The solid line represents the absorbance at 220nm. The dotted line represents the percentage of CH_3CN. Electrophoretic analysis of purified royalisin is shown at the top left of the figure. SDS-polyacrylamide gel electrophoresis was performed using a 12.5% acrylamide gel containing 8M urea. Approximately, 1μg of royalisin was applied to each lane. The left lane (1) and right lane (2) represent molecular weight standards and royalisin, respectively. The molecular mass of standard proteins in kDa is shown at the left of lane 1.

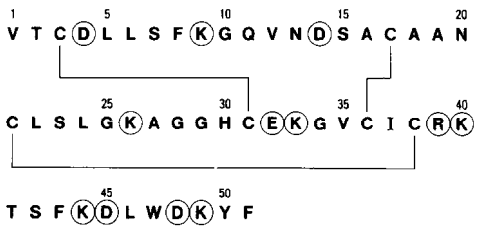

Figure 3 Amino acid sequence of royalisin.

The primary sequence of purified royalisin was determined using an automatic amino acid sequencer. Cysteine residues were identified as the S-pyridylethyl derivative after alkylation with 4-vinylpyridine. The presence and positions of the disulfide bridges were elucidated by peptide mapping and by sequencing of peptides generated by treatment with lysyl endopeptidase. Amino acid residues are indicated by one-letter symbols. Sequence positions and charged amino acid residues are marked by numbers and circles, respectively.

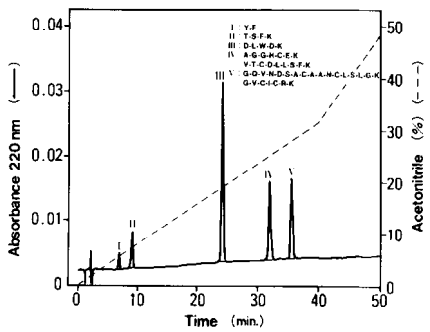

Figure 4 Peptide mapping of royalisin after lysyl endopeptidase digestion and the amino acid sequences of its peptide fragments.

Peptides generated by lysyl endopeptidase digestion of royalisin were purified by reverse-phase HPLC. The purified peptides (I-V) were then analyzed using a gas phase sequencer. The amino acid residues of each peptide, represented by the one-letter code, are shown at the top of the figure.

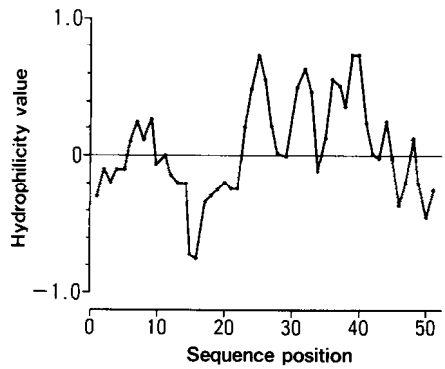

Figure 5 Hydrophilicity profile of royalisin.

The hydrophilicity profile was computed according to the method of Hopp and Woods (38). The average hydrophilicity values of adjacent hexapeptides are plotted against sequence positions. The hydrophilicity values are above and hydrophobicity values are below the horizontal line. The secondary structure prediction by the method of Chou and Fasman (39) revealed that royalisin contains two random coil domains at positions 14-20 and 26-35, and β-sheet domains at positions 1-13, 21-25, and 36-51.

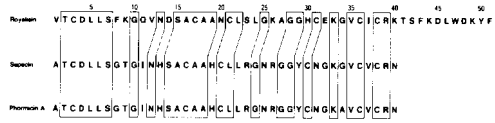

Figure 6 Comparison of amino acid sequences of royalisin, sapecin, and phormicin A.

Homology between three antibacterial proteins was found by a computer search. Identical amino acid residues indicated by one-letter symbols are boxed. Numbers indicate the amino acid positions.

Table 2 Antibacterial activity of royalisin.

Bacterial growth in the presence of various concentrations of royalisin is expressed as a percentage of that obtained with a control culture grown in the absence of royalisin.

Bacterial strains		Royalisin (μM) 0.01	0.1	1.0
Gram(-)				
Bacteroides	fragilis	-	-	
Bacteroides	vulgatus	-	-	
Escherichia	coli IID861	-	-	
Escherichia	coli IID5208	-	-	-
Klebsiella	pneumoniae IFO-3321	-	-	-
Salmonella	infantis	-	-	
Salmonella	typhimurium	-	-	
Gram(+)				
Bifidobacterium	adolescentis ATCC15703	15	43	59
Bifidobacterium	bifidum ATCC15696		-	91
Bifidobacterium	breve ATCC15700		67	87
Bifidobacterium	infantis ATCC15697		20	71
Bifidobacterium	longum ATCC15707	29	81	81
Clostridium	perfringens ATCC13124	-	-	75
Corynebacterium	pyogenes	-	31	96
Eubacterium	aerofacience	-	-	17
Lactobacillus	acidophilus ATCC314	-	-	44
Lactobacillus	acidophilus ATCC4356		-	47
Lactobacillus	bulgaricus ATCC11841	21	87	84
Lactobacillus	helveticus ss. jugurti	33	90	72
Lactobacillus	lactis ATCC8000	59	90	88
Lactobacillus	leichmannii ATCC7830	14	88	89
Leuconostoc	cremoris ATCC19254	16	84	88
Staphylococcus	aureus SC-D	17	22	82
Streptococcus	cremoris SCR-812	10	18	83
Streptococcus	thermophilus ATCC19258	9	14	86