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Activity and Purification of Linenscin OC2, an Antibacterial Substance Produced by *Brevibacterium linens* OC2, an Orange Cheese Coryneform Bacterium

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An orange cheese coryneform bacterium isolated from the surface of Gruyère of Comté and identified as *Brevibacterium linens* produces an antimicrobial substance designated linenscin OC2. This compound inhibits gram-positive food-borne pathogens including *Staphylococcus aureus* and *Listeria monocytogenes* but is not active against gram-negative bacteria. Linenscin OC2 caused viability loss and lysis of the test organism, *Listeria innocua*. Electron microscopy showed that linenscin OC2 induces protoplast formation and cell lysis. The native substance is resistant to proteolytic enzymes, heat, and organic solvents and stable over a wide range of pH. The molecular weight of the native linenscin OC2 was estimated by gel chromatography to be over 285,000. Linenscin OC2 was purified by ammonium sulfate precipitation, 2-propanol extraction, and reverse-phase chromatography. Direct detection of antimicrobial activity on a sodium dodecyl sulfate-polyacrylamide gel suggested an apparent molecular mass under 2,412 Da. Molecular mass was determined to be 1,196.7 Da by mass spectrometry. Amino acid composition analysis indicated that linenscin OC2 may contain 12 residues.

Listeria monocytogenes has been recognized as a major food-borne pathogen since the outbreaks in California and Switzerland (5) involving cheeses. *L. monocytogenes* has the ability to withstand various environmental conditions, such as low pH (7, 26) and salt concentration of up to 10% (24). It is, therefore, not surprising that *Listeria* organisms can survive in various kinds of fermented products such as cheeses (32). Moreover, they can resume growth during the ripening of surface-ripened soft and semihard cheeses as a result of a pH rise (30, 31). Thus, any studies on antagonistic systems that could prevent growth of this organism in cheese are of great interest.

Lactic acid bacteria produce several antagonistic systems such as hydrogen peroxide, diacetyl, organic acids, and bacteriocins (27). Only the latter substances seem to have a real practical interest. However, a few of them are active against food-borne pathogens such as *Listeria* organisms: nisin produced by *Lactococcus lactis* (15), mesenterocins or leucocins produced by *Leuconostoc mesenteroides* (9, 11–13), and pediocins produced by *Pediococcus acidilactici* (28). To date, nisin is the only one that has found practical applications in cheese technology, mostly for preventing growth of clostridia in processed cheese and cheese spreads (35). Recently, nisin was shown to substantially reduce the level of *L. monocytogenes* in Camembert cheese made with artificially contaminated milk but was unable to prevent the growth of this organism during ripening (22), presumably because nisin was degraded by proteolytic enzymes produced by lactic acid bacteria and mould and/or was bound on some cheese components. Thus, there are still no antagonistic systems which remain active during cheese ripening. In particular, there is a strong need for cheese surface flora exhibiting inhibitory properties against *L. monocytogenes*.

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It is well known that orange cheese coryneform bacteria play an important role in the ripening of red smear cheeses (33), in particular by producing methanethiol (8, 21), a sulfur compound which imparts a typical flavor to this kind of cheese (20). Some coryneform bacteria have already been isolated from surface-ripened cheeses (29, 38) for their ability to produce inhibitory substances active against *L. monocytogenes*. In a previous study (29), we have shown that the substance produced by an orange cheese coryneform identified as *Brevibacterium linens* OC2 was particularly active against a panel of 21 *Listeria* spp. including 14 strains of *L. monocytogenes*. It has also been shown that it is active against *Staphylococcus aureus* (34).

The present article deals with the antibacterial activity, the properties of the crude preparation, and the purification protocol of the antibacterial substance produced by *B. linens* OC2, which has been termed linenscin OC2.

MATERIALS AND METHODS

Bacterial culture and medium. *B. linens* OC2 was maintained at −80°C in TSBYE (tryptic soy broth [Difco] plus 0.6% yeast extract [Biomérieux, Marcy l'Etoile, France]) to which 15% (vol/vol) glycerol (Prolabo) was added. Before use, the strain was cultivated twice for 72 h at 27°C in TSBYE.

To produce linenscin OC2, a 1% inoculum of *B. linens* OC2 was grown in TSBYE for 72 h at 27°C in a rotary shaker. Stationary-phase cells were centrifuged at 10,000 × *g* for 10 min at 4°C. The culture supernatant was either sterilized with 0.45-μm-pore-size Millex-HV filters (Millipore SA) and stored at −20°C until being used for antimicrobial assays or concentrated by ammonium sulfate precipitation for purification.

Antimicrobial assays of linenscin OC2. A nonpathogenic *Listeria* strain, *Listeria innocua* LIN11 (Pasteur Institute, Paris, France) was used as the indicator organism for routine antimicrobial assays. A preliminary study (34) showed that this strain was representative of the sensitivity of *Listeria* organisms to linenscin OC2. The bacteriocin titer was determined by the serial twofold dilution assay previously described by Mayr-Harting et al. (23). Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per milliliter.

Broad antimicrobial spectrum of linenscin OC2. The serial twofold dilution method was used to assess the microbial activity of crude linenscin OC2 extracts towards several gram-positive and gram-negative bacteria (Table 1). All strains

TABLE 1. Activity spectrum of crude linenscin OC2

Indicator strain	Source[a]	Inhibition (AU/ml)
Gram positive		
Listeria innocua LIN11	CIP	12,800
Staphylococcus aureus S2	CIP	12,800
Staphylococcus aureus S7	CIP	25,600
Bacillus subtilis 202	CNRZ	25,600
Lactococcus lactis 1076	CNRZ	51,200
Lactococcus lactis 126	CNRZ	12,800
Lactococcus cremoris SD4	CNRZ	12,800
Leuconostoc mesenteroides 3437	CNRZ	12,800
Leuconostoc mesenteroides 1314	CNRZ	12,800
Leuconostoc mesenteroides 1231	CNRZ	12,800
Leuconostoc mesenteroides 1240	CNRZ	25,600
Gram negative		
Escherichia coli 2.2	CNRZ	—[b]
Escherichia coli 3.1	CNRZ	—
Escherichia coli 4.1	CNRZ	—
Escherichia coli 5.1	CNRZ	—
Hafnia alvei 711	CNRZ	—
Hafnia alvei 713	CNRZ	—
Hafnia alvei 13.2	CNRZ	—
Hafnia alvei 13.3	CNRZ	—
Pseudomonas fragi 4973	ATCC	—
Pseudomonas putida 12633	ATCC	—
Salmonella anatum 1	CIP	—
Salmonella dublin 2	CIP	—
Salmonella montevideo 3	CIP	—

[a] ATCC, American Type Culture Collection (Rockville, Md.); CIP, collection of Institute Pasteur (Paris, France); CNRZ, collection of Station de Recherches Laitières (Jouy-En-Josas, France).
[b] —, no inhibition.

were previously subcultured in TSBYE, except the lactic acid bacteria which were cultivated in TSBYE supplemented with 0.5% glucose and then inoculated in the soft-agar medium of the same composition.

Mode of action of linenscin OC2. An overnight culture of *L. innocua* LIN11 was centrifuged at 6,000 × *g* for 10 min, and the cell pellet was suspended in fresh TSBYE to ca. 2×10^8 cells per ml. The cell suspension was incubated at 30°C, and growth was monitored by optical density at 620 nm. Linenscin OC2 (final concentration, 2,560 AU/ml) was added when the culture was in the exponential growth phase. At appropriate time intervals the numbers of viable bacteria were also determined by diluting samples in 0.1% peptone water and plating them on TSAYE agar plates. The colonies were counted after 2 days of incubation at 30°C.

Extracellular ATP was measured by bioluminescence (36). Samples of the culture were filtered through 0.45-μm-pore-size filters HA (Millipore SA) and diluted in 10 mM morpholinopropane-NaOH (MOPS) buffer (pH 7.4). The preparations were assayed with an ATP biometer (model Biolumat LB 9500T; Berthold) and the enzyme substrate kit of Boehringer (Boehringer Mannheim, Mannheim, Germany). Known amounts of ATP (Sigma) diluted in 10 mM MOPS buffer plus 10 mM $MgSO_4$ (pH 7.4) were added to the extracts as internal standards in order to correct inhibition of the luciferase reaction by the extracts. Correction was made for background luminescence.

Electron microscopy. Crude linenscin OC2 (final concentration, 2,560 AU/ml) was added to a culture of *L. innocua* LIN11 in exponential growth phase (optical density at 620 nm, 0.6), and 2 h 30 min later 50-ml samples of cell suspensions (control and treated cells) were taken. The cells were prefixed with 2% (vol/vol) glutaraldehyde (Prolabo) and centrifuged at 6,000 × *g* for 10 min at 4°C. The pellet was dispersed in molten agar (3%, wt/vol) at room temperature and, after solidification, fixed first in glutaraldehyde (2.5% [vol/vol] in 10 mM sodium cacodylate, pH 7.2) for 15 h at 4°C and then in osmium tetroxide (1% [wt/vol] in the same buffer) for 15 h at 4°C before dehydration for 2 h at room temperature in acetone. Finally, it was embedded in Epon for 48 h at 60°C. Thin sections (microtome Ultracut; Reichert, Vienna, Austria) were mounted on grids, covered with collodion film, and poststained with lead citrate and uranyl citrate. All preparations were observed with a Zeiss EM10 electron microscope (Oberkochen).

Effects of heat, pH, hydrolytic enzymes, and organic solvents on the activity of linenscin OC2. Crude extracts of linenscin OC2 were subjected to (i) heating for either 30 min at 60°C at pH 7.0 or 10 min at 100°C at various pH values; (ii) incubation for 2 h at 37°C in the presence of the following enzyme solutions (5 mg/ml) in 10 mM sodium phosphate buffer (pH 6.8) and then boiling for 5 min: pronase E, proteinase K, trypsin, α-chymotrypsin, pepsin (diluted in 0.02 N HCl [pH 2.0]), papain, ficin, carboxypeptidase A, α-amylase, and lipase (all purchased from Sigma); and (iii) extraction for 2 h at room temperature with 2-propanol–acetone–chloroform (90% [vol/vol]). The organic phase was then separated, and the solvent was evaporated with a vacuum centrifuge (Savant Instruments, Inc., Farmingdale, N.Y.). The residue was resuspended in 10 mM sodium phosphate buffer (pH 7.0). Controls consisted of enzyme solutions, organic solvents without linenscin OC2, and linenscin OC2 in sodium phosphate buffer alone.

Ultrafiltration and gel filtration chromatography. The size of crude linenscin OC2 was first determined by supernatant ultrafiltration through Ultrafree MC units (Millipore SA). The titers of retentates and filtrates were determined with *L. innocua* LIN11 as the test organism. Crude linenscin OC2 was also sized by gel filtration chromatography using a 30-ml column bed (Superose 12HR 10/30; Pharmacia SA). The running buffer combined 50 mM sodium phosphate (pH 7.0) plus 50 mM NaCl. The fractions were collected every minute and assayed for antimicrobial activity. The fractions containing linenscin OC2 were sized by plotting the elution volume of linenscin OC2 as a function of log molecular weight, by using protein standards (670, 158, 44, 17, and 1.35 kDa) (Bio-Rad SA) separated by gel filtration under the same conditions.

Purification of linenscin OC2. Solid ammonium sulfate (Prolabo) was slowly added to the culture supernatant up to 80% saturation (wt/vol), and the mixture was stirred overnight at 4°C. After centrifugation (12,000 × *g*, 30 min, 4°C), the pellet was resuspended in 10 mM sodium phosphate (pH 9.0). The precipitate was concentrated by freeze-drying and then extracted with 90% (vol/vol) 2-propanol (Prolabo). After evaporation of the upper phase containing the 2-propanol with a vacuum centrifuge (Savant Instruments), the residue was loaded on a C_{18} Sep-Pak cartridge (Millipore SA) and washed with 20% (vol/vol) 2-propanol in 0.1% (vol/vol) trifluoroacetic acid (TFA) (Sigma), and then linenscin was eluted with 50% (vol/vol) 2-propanol in 0.1% (vol/vol) TFA. The 2-propanol was evaporated with the vacuum centrifuge, and linenscin was further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) (Waters SA) on a 5 μm nucleosil C_{18} column (250 by 4.6 mm) (SFCC, St. Quentin-En-Yvelines, France) using a 60-min linear A/B gradient from 80/20% to 50/50%.

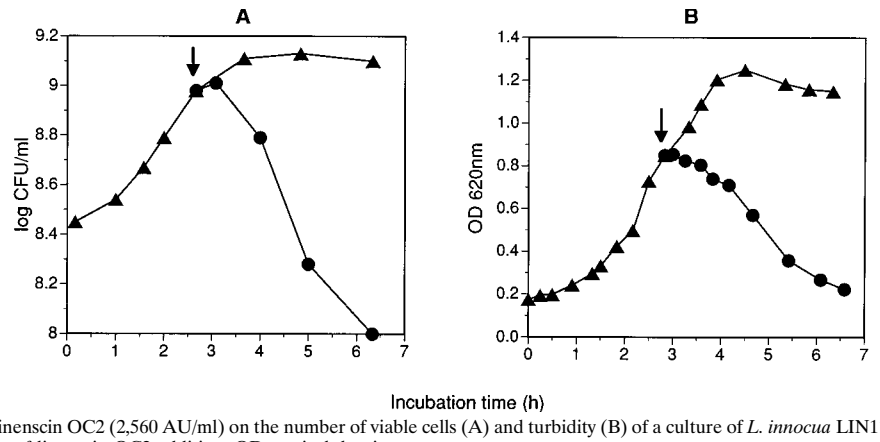

FIG. 1. Effect of crude linenscin OC2 (2,560 AU/ml) on the number of viable cells (A) and turbidity (B) of a culture of *L. innocua* LIN11 growing at 30°C in TSBYE (●). ▲, control. Arrow, time of linenscin OC2 addition. OD, optical density.

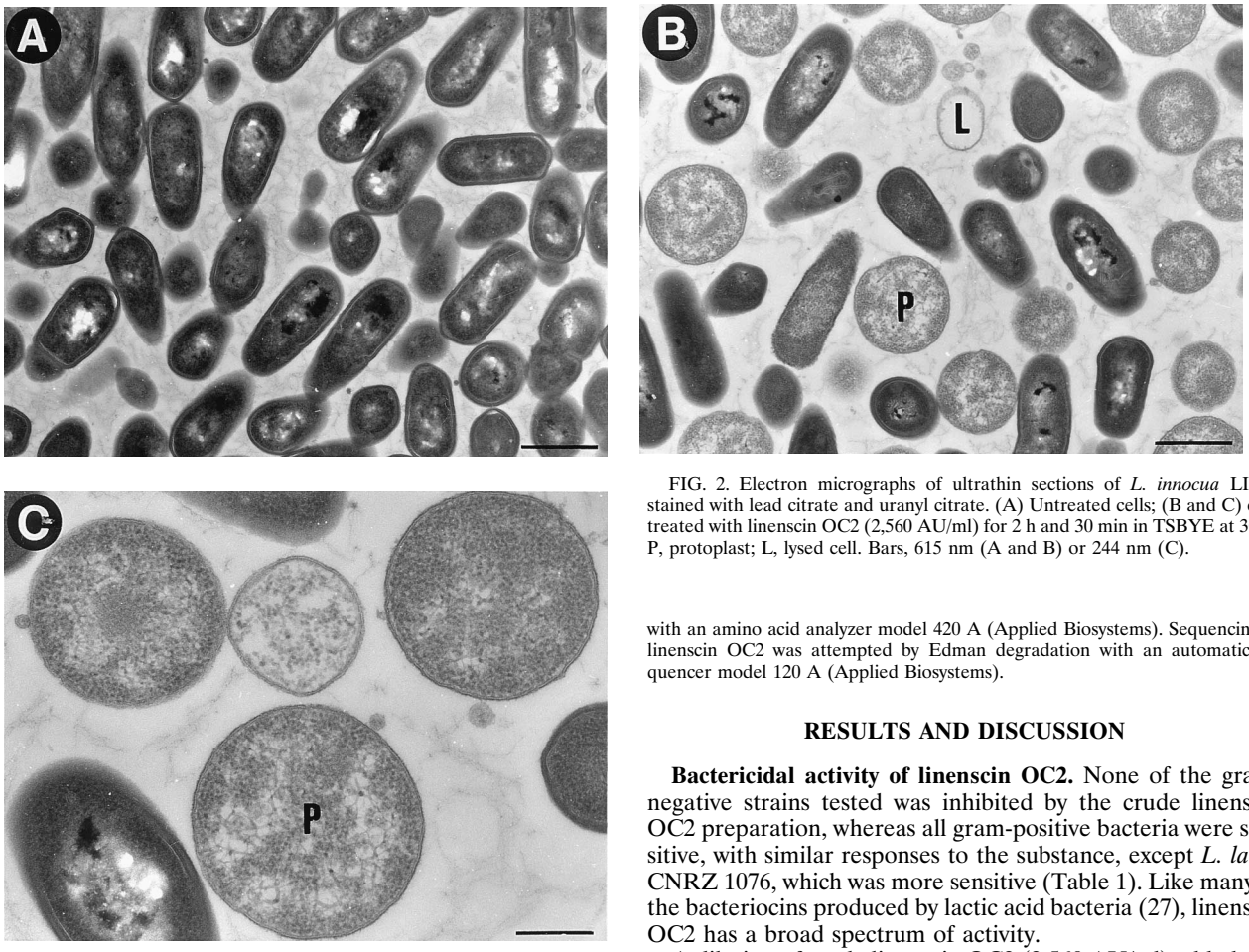

FIG. 2. Electron micrographs of ultrathin sections of *L. innocua* LIN11 stained with lead citrate and uranyl citrate. (A) Untreated cells; (B and C) cells treated with linenscin OC2 (2,560 AU/ml) for 2 h and 30 min in TSBYE at 30°C. P, protoplast; L, lysed cell. Bars, 615 nm (A and B) or 244 nm (C).

Buffer A was made up of 10% (vol/vol) 2-propanol in 0.1% (vol/vol) TFA; buffer B was composed of 95% (vol/vol) 2-propanol in 0.1% (vol/vol) TFA. The flow rate was 0.4 ml/min, and the fractions were collected every 2 min. These fractions were evaporated in the vacuum centrifuge and resuspended in 10 mM sodium phosphate buffer (pH 9.0). Linenscin OC2 activity was determined for each fraction.

Protein concentration determinations. Protein concentrations were determined with 50-ml samples by the method of Bradford (4) with Coomassie protein assay reagent according to the manufacturer's instructions (Pierce, Rockford, Ill.). Bovine serum albumin was used to construct a standard curve.

SDS-PAGE. To estimate the molecular weight of purified linenscin OC2, sodium dodecyl sulfate (SDS)-polyacrylamide gel (0.75 mm) electrophoresis (PAGE) was performed on 20% uniform-pore gel (Hoefer Scientific). Ten microliters of the sample and molecular weight standards (with molecular weights in brackets) (myoglobin [16,949], myoglobin I and II [14,404], myoglobin I [8,159], myoglobin II [6,214], and myoglobin III [2,512]; Pharmacia, LKB) was applied to the gel. The samples were prepared by mixing equal parts of the linenscin sample and buffer and boiling the mixture at 100°C for 5 min. Migration was performed in a vertical slab gel apparatus (Protean Cell II; Bio-Rad) at a constant voltage (100 V) for 2 h. Half of the gel was stained with a silver stain kit (Bio-Rad) as directed by the manufacturer, while the other half was assayed for antimicrobial activity by the direct test described by Bhunia et al. (2). This part of the gel was fixed in 2-propanol and acetic acid and soaked for at least 4 h in sterile deionized water. It was then aseptically placed in a sterile petri dish containing 15 ml of TSAYE and covered with 10 ml of soft agar containing 10^6 cells of *L. innocua* LIN11 per ml as the indicator. The plate was incubated at 30°C for 18 h and examined for zones of inhibition.

Mass spectrometry analysis. Mass spectrometry analysis of linenscin OC2 was performed by using a PESCIEX triple quadripole instrument fitted with an electrospray ionization source. RP-HPLC-purified linenscin OC2 was dissolved in 0.1% (vol/vol) TFA containing 80% (vol/vol) of acetonitrile. Data were accumulated for 10 min at 80 V.

Amino acid composition. HPLC-purified linenscin OC2 was first hydrolyzed in 4.7 N HCl under vacuum at 110°C for 24 h. The amino acids were then derivatized with phenylisothiocyanate and separated by HPLC before being analyzed with an amino acid analyzer model 420 A (Applied Biosystems). Sequencing of linenscin OC2 was attempted by Edman degradation with an automatic sequencer model 120 A (Applied Biosystems).

RESULTS AND DISCUSSION

Bactericidal activity of linenscin OC2. None of the gram-negative strains tested was inhibited by the crude linenscin OC2 preparation, whereas all gram-positive bacteria were sensitive, with similar responses to the substance, except *L. lactis* CNRZ 1076, which was more sensitive (Table 1). Like many of the bacteriocins produced by lactic acid bacteria (27), linenscin OC2 has a broad spectrum of activity.

A dilution of crude linenscin OC2 (2,560 AU/ml) added to a cell suspension of *L. innocua* LIN11 in the exponential growth phase resulted in a reduction of viable counts of ca. 1 log cycle in 3 h (Fig. 1). At the same time, a drop in optical density was observed, indicating that the effect of linenscin OC2 was also bacteriolytic. No extracellular ATP was detected at linenscin concentrations below that causing lysis (data not shown). This indicates either that this substance does not create pores in the cytoplasmic membrane, as is usually the case with the bacteriocins produced by most gram-positive bacteria (3, 6, 14, 37, 40, 41), or that the pores are not large enough to allow the escape of ATP.

After 2 h and 30 min of contact with crude linenscin OC2 at 2,560 AU/ml, the optical density decreased by 0.4 (Fig. 1). At that stage, as seen by electron microscopy, the cell suspension was composed of approximately equal percentages of protoplast-like cells and apparently intact cells (Fig. 2). The protoplast-like cells appear in different states from good to degraded forms. As these cells were in TSBYE, i.e., a nonhypertonic medium, it was not surprising that they were prone to lysis. Linenscin OC2 seems to have a mode of action similar to that of certain bacteriocins produced by gram-negative bacteria such as colicin M and pesticin (10, 19) and some antibiotics such as cycloserine and bacitracin (25), whose effect is to inhibit peptidoglycan formation.

Effects of heat, hydrolytic enzymes, and organic solvents on linenscin OC2 activity. Crude linenscin OC2 was little affected by heating for 10 min at 100°C except at pH 2.0, indicating that the active substance is relatively heat stable (Table 2). Also, no change in activity was noticed after 1 month of incubation at

TABLE 2. Effects of heat, enzyme,[a] and solvent treatments on the activity of crude linenscin OC2

Treatment	Residual activity (AU/ml)[b]
pH 2.0	
Control	12,800
100°C for 10 min	1,600
pH 7.0	
Control	12,800
100°C for 10 min	6,400
60°C for 30 min	6,400
pH 11.0	
Control	12,800
100°C for 10 min	6,400
Control (phosphate buffer)	12,800
Pronase E (type XIV)	6,400
Proteinase K (type XI)	6,400
α-Chymotrypsin (type II)	6,400
Trypsin (type I)	6,400
Ficin	6,400
Papain	3,200
Pepsin	6,400
Carboxypeptidase A	12,800
α-Amylase	12,800
Lipase (type I)	3,200
Chloroform[c]	0
Acetone[c]	6,400
2-Propanol[c]	12,800

[a] All enzymes from Sigma were used at 5 mg/ml in the reaction mixture.
[b] The indicator strain was *L. innocua* LIN11.
[c] 90% (vol/vol) extract.

30°C or after 1 year during storage at −20°C (data not shown). The inhibitory activity of crude linenscin OC2 was diminished but not eliminated by proteolytic enzymes α-amylase and lipase. These results prove the great resistance of the biological moiety of linenscin OC2 and suggest that it is not a bacteriocin. This reduction may only be due to binding on the proteins. On the other hand, the active substance was extracted from the supernatant by acetone and 2-propanol but not by chloroform, indicating that linenscin OC2 is a very hydrophobic substance but not a lipid.

Molecular weight of crude linenscin OC2. All of the linenscin OC2 activity was retained by the 100- and 10-kDa membranes during ultrafiltration of culture supernatants (data not shown). With the cutoff of 300 kDa, 6% of the activity passed through the membrane. These data suggest that the native linenscin OC2 has a high molecular weight or that it occurs in large aggregates. This was confirmed by gel chromatography using a Sepharose column. The fractions were collected every minute and assayed for antimicrobial activity. By comparing elution volumes with those of protein standards, the molecular weights of the active fractions were determined to be greater than 285,000.

Purification of linenscin OC2. To allow further characterization, linenscin OC2 was purified. The substance was first concentrated by ammonium sulfate precipitation from the supernatant of a 72-h culture at 27°C. It was then extracted by 90% (vol/vol) of 2-propanol and finally separated by RP-HPLC. The purification steps and recoveries of linenscin OC2 are given in Table 3. The final specific activity of pure linenscin OC2 was ca. 47,700-fold greater than that in the culture supernatant, with a final recovery of 2%. The C_{18} RP chromatographic elution profile of linenscin OC2 is shown on Fig. 3. The linenscin was eluted as a fairly symmetrical peak of activity at about 40% of solvent B, which coincides with an absorbancy peak at 220 nm. The strong binding of linenscin OC2 to the C_{18} column packing at relatively high 2-propanol concentrations is indicative of a highly hydrophobic molecule.

SDS-PAGE and mass spectrometry. When fraction IV (Table 3) of the linenscin OC2 preparation underwent electrophoresis on 20% polyacrylamide gel in the presence of 0.1% SDS, one weak band was revealed by silver staining, although the molecular weight standards were easily detected. The other half of the gel overlaid with TSAYE (0.75%) seeded with *L. innocua* LIN11 demonstrated a clear zone of inhibition (Fig. 4) which corresponds to a molecular weight under 2,512. The same result was obtained with the substance after 2-propanol extraction (fractions II and III). Mass spectrometry analysis of fraction IV containing linenscin OC2 revealed only one peak, whose molecular weight was estimated at 1,196.7 ± 0.1 (data not shown).

Upon gel filtration crude linenscin OC2 was eluted with a molecular weight of more than 285,000, whereas after the first steps of purification this substance had a molecular weight under 2,512, as determined by direct detection of antimicrobial activity in SDS-PAGE. The high apparent molecular weight of crude linenscin OC2 may be due to polymer formation and/or association of linenscin with macromolecules present in the culture medium. Some bacteriocins produced by lactobacilli (e.g., lactocin 27, lacticin B, and helveticin J) also associate with other substances under nondissociating conditions (1, 16, 37).

Amino acid composition. The amino acid composition of fraction IV containing linenscin OC2 obtained after the final RP-HPLC step is presented in Table 4. On the basis of one residue of Phe per molecule, it can be calculated that the active sample contains a majority (90%) of hydrophobic and uncharged amino acids and is particularly rich in proline residues. The hydrophobic character they may impart to the molecule is consistent with its relatively high affinity to 2-propanol. Composition analysis indicated that linenscin OC2 may contain 12 amino acid residues, leading to a molecular weight of 1,199.

TABLE 3. Purification of linenscin OC2

Sample [ml]	Total activity[a] (AU)	Protein concn (mg/ml)	Sp act (AU/mg)	Activity recovered (%)	Fold purification
I. Culture supernatant [1,140]	233,472,000	42.0	4,881	100	1
II. Ammonium sulfate precipitation + 2-propanol extraction [3.3]	21,626,880	729.3	8,986	9.3	1.8
III. Sep-Pak [3.3]	10,813,440	733.3	4,469	4.6	0.9
IV. HPLC (RP C_{18}) [3.3]	4,662,240	0.02	2.33×10^8	2.0	47,737.7

[a] *L. innocua* LIN11 was used as the test organism.

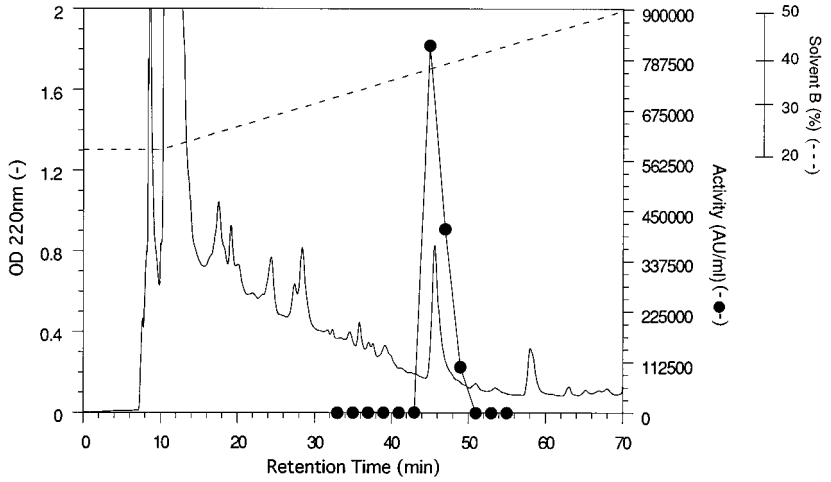

FIG. 3. RP-HPLC of fraction III (Table 3) of linenscin OC2 loaded on a C_{18} column. The amount applied represents 1/66 of that obtained from an approximately 1.1-liter culture. OD, optical density.

This molecular weight is extremely close to that obtained by mass spectrometry. Furthermore, this means that there is no room for the presence of other chemical compounds such as carbohydrates in the molecule. One other line of evidence indicates that linenscin OC2 probably does not contain a glycan moiety: in a peptidoglycan or a glycopeptide, the carbohydrate side chains are attached to hydroxyl groups of serine and threonine or to the side chain amide of asparagine. As shown in Table 4, none of these amino acids are present in the molecule. Reductions in numbers of amino acids could result from contaminating peptides, although one band was revealed by SDS-PAGE and only one peak was observed by mass spectrometry.

No amino acid residue was detected upon direct N-terminal amino acid sequencing of linenscin OC2, indicating that the N-terminal amino acid was blocked or it may be a cyclic molecule. Incidentally, like SDS-PAGE and mass spectrometry analysis, this may have served to confirm the purity of the linenscin OC2 preparation since a contaminating peptide would have generated a residue at the first cycle, provided it was not end blocked as well and/or it is in a sufficient concentration to be detected. Further work should be directed to find an enzyme (such as peptidases) able to cleave linenscin OC2 into fragments amenable to sequential degradation.

TABLE 4. Amino acid composition of fraction IV containing linenscin OC2

Amino acid	Molar ratio/fraction[a] (no. of residues)
Asp	0.57 (0 or 1)
Glu	0.88 (1)
Ser	0.14 (0)
Gly	0.75 (1)
His	0.15 (0)
Thr	0.18 (0)
Ala	0.15 (0)
Pro	4.85 (4 or 5)
Tyr	0.28 (0)
Val	1.35 (1)
Cys	0.15 (0)
Ile	0.91 (1)
Leu	1.95 (2)
Phe	1.00 (1)
Lys	0.18 (0)

[a] Triplicate measurements were obtained for two independently purified samples of linenscin OC2. The number of amino acid residues was determined from the molecule ratio relative to phenylalanine.

Conclusions. Some orange cheese coryneform bacteria produce antibacterial substances such as bacteriocins, but to date only two, linencin A and linocin M18, have been purified and identified (17, 18, 39). Linencin A, a 95-kDa protein, was active only against strains of *B. linens* (17), whereas linocin M18 which consists of a 31-kDa protein was active against several gram-positive bacteria (39).

Linenscin OC2, described in this study, is different from linencin A and linocin M18. It is not a bacteriocin sensu stricto but possesses several advantages over the bacteriocins produced by gram-positive bacteria. As *B. linens* is used in the surface-ripening flora of cheese, a metabolite secreted by this bacterium should be acceptable. The spectrum of activity of linenscin OC2 is interesting (among the bacteria tested, the pathogens *L. monocytogenes* and *S. aureus* were sensitive), but further characterization of linenscin OC2, i.e., complete structure, toxicity, and efficacy in situ, are necessary for its possible application to red smear surface-ripened soft-cheese production. If not used in food, another application of linenscin OC2 could be as an antibiotic in human therapy, if it retains its activity in vivo. Linenscin OC2 efficacy against clinical bacteria

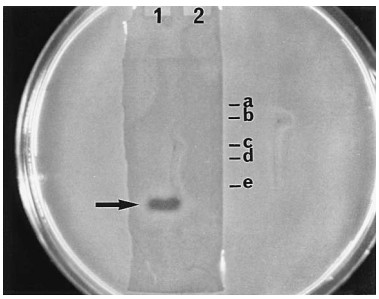

FIG. 4. Direct detection of linenscin OC2 (fraction IV) after SDS-PAGE. The gel was overlaid by soft agar seeded with *L. innocua* LIN11. Arrow, inhibition spot. Lane 1, active fraction containing linenscin OC2; lane 2, molecular size marker (a to e, 16,949, 14,404, 8,159, 6,214, and 2,512 Da, respectively).

should be tested, especially against multiresistant strains of *Enterococcus faecalis* and *S. aureus.*

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