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Characterization of Salivaricin B, A Protein Expressed By *Lactobacillus salivarius* M7

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Abstract: Salivaricin B (SalB) is a protein encoded by *Lactobacillus salivarius* M7. This protein is insensitive to proteolytic enzymes, such as pepsin, trypsin, and chymotrypsin, but is partially sensitive to proteinase K. SalB is also stable and maintains its activity over a range of pHs, from 3 to 9. SalB was found to be effective against many closely related lactic acid bacteria such as, *L. fermentum*, *L. plantarum, L. acidophilus* and distantly related gram-positive bacteria, including *Listeria monocytogenes, Streptococcus faecalis, Staphylococcus aureus* and *Staph. epidermidis*. The mode of action of SalB was obviously bactericidal, and death occurs without bacterial lysis. The gene responsible for SalB production in *L. salivarius* M7 had previously been characterized.

Key Words: Bacteriocin, Salivaricin B, *Lactobacillus salivarius* M7

Lactobacillus salivarius M7 Tarafından İfade Edilen Salivarisin B Adlı Proteinin Karakterizasyonu

Özet: Salivarisin B (Sal B) *Lactobacillus salivarius* M7 tarafından kodlanan bir proteindir. Bu protein pepsin, tiripsin, kimotiripsin gibi protein parçalayan enzimlerden etkilenmemelerine rağmen, diğer bir protein parçalayıcı enzim olan proteinaz K'ya karşı kısmen duyarlıdırlar. Sal B 3 ile 9 arası pH yelpazesinde aktiftir. Yapılan çalışmalarda, Sal B'nin *Lactobacillus fermentum, Lactobacillus plantarum,* ve *Lactobacillus acidophilus* gibi bir çok yakın akraba bakteriler ve *Listeria monocytogenes, Streptococcus faecalis, Staphylococcus aureus*, ve *Staphylococcus epidermidis* gibi akraba olmayan bakteriler üzerine etkili olduğu bulunmuştur. Sal B'nin etki şekli bakterisidal olup ölüm hücre parçalanmadan meydana gelmektedir. *Lactobacillus salivarius* M7'den Sal B kodlanması ve ifadesinden sorumlu genin yeri daha önce karakterize edilmiştir.

Anahtar Sözcükler: Bakteriosin, Salivarisin B, *Lactobacillus salivarius* M7

Introduction

The role of lactic acid bacteria (LAB) in the production of stable fermented food products has generated an interest in their ability to prevent bacterial spoilage. Many LAB species produce bacteriocins or bacteriocin-like compounds (1-4). Like nicin, they are proteinaceous compounds that kill closely related bacteria, but may also elicit bactericidal activity against more distantly related bacteria (5,6). Many researchers have concentrated on their industrial applications for the following reasons: firstly, they or their products can be utilized as natural food preservatives. Secondly, since they are easily adapted to living in the gastrointestinal system, their products can be used as probiotics. Thirdly, the genetic determinants for bacteriocin production and immunity can be used for strain improvement or for constructing a vector. *Lactobacillus salivarius* M7 colonizes the gastrointestinal tract of man and animals, and is responsible for acidic changes in the digestive tract. In this paper, we report the physiological characterization of the Salivarisin B (SalB) protein of *L. salivarius* M7. The gene responsible for SalB production in *L. salivarius* M7 had previously been characterized (7).

Materials and Methods

Bacterial strains and growth conditions

L. salivarius M7, *L. plantarum* ATCC 8014, *L. fermentum* ATCC 9338 and *L. acidophilus* ATCC 4357 were grown anaerobically in de Man Rogosa Sharpe

(MRS) broth (Difco, Detroit, Mich.), overnight at 37 °C. *Bacillus, Listeria, Staphylococcus* and *Streptococcus* strains were grown in Tryptic Soy Broth (TSB) and on TS agar (Difco). The *E. coli* Dh 5α strain was grown in Luria-Bertani broth at 37 °C with vigorous agitation. The strains used are listed in Table 1.

Table 1. Bacterial strains used.

Bacterial strains	Relevant properties	Source
L. salivarius M7	Wild type	Gaziantep/TNO
L. plantarum	Indicator strain	ATTC 8014
L. fermentum	Indicator strain	ATTC 9338
L. acidophilus	Indicator strain	ATTC 4357
L. monocytogenes	Indicator strain	L2
Strep. faecalis	Indicator strain	Field Str.
Staph. aureus	Indicator strain	ATTC 6538
Staph. *epidermidis*	Indicator strain	Field Str.
E. coli	Indicator strain	Dh 5a

Analysis of SalB production and sensitivity

Bacteriocin activity was tested by agar-well diffusion tests (7,8). Culture supernatants of overnight grown *L. salivarius* M7 cultures were adjusted to pH 7.0 and filtered through a 22 µm filter (Sartorius). Lawns of each strain were prepared by inoculating 100 µl of freshly grown cells in 35 ml of soft MRS agar at 40 °C and were then poured into plates. The plates were allowed to solidify for 15 min in a drying hood, and were then incubated anaerobically for 4 h at 37 °C. Two wells were punched in the agar, and the bottom of the wells was sealed with a drop of soft agar. Ten microliters of the *L. salivarius* M7 supernatant were dropped into the wells. The plates were incubated anaerobically at 37 °C for at least 18 h and were subsequently examined for a zone of growth inhibition. Two-fold serial dilutions of the supernatant were used for a semiquantitative assay of bacteriocin. The titer of bacteriocin activity was defined as the reciprocal of the highest dilution, which did not show inhibition of the indicator strain and was expressed in activity units per milliliter (9).

Sensitivity of SalB

Supernatants from log-phase (10 h culture) to late-stationary-phase (35 h culture) were collected from batch (non-pH-regulated) cultures of *L. salivarius* M7 in MRS broth. The pH of the supernatants was initially adjusted to various values, and subsequently heated to 100 °C. From each sample, a 10 µl aliquot of two-fold serial dilutions was used to determine the bacteriocin titer.

To test sensitivity to proteolytic enzymes, the log-phase and late-stationary-phase *L. salivarius* M7 culture supernatants were also used. Each aliquot of 1 ml containing 10,200 AU/ml was treated with proteinase K, pepsin, trypsin, chymotrypsin, catalase, lipase A, dextranase and lysosyme, respectively, at final concentrations of 500 µg/ml (Sigma). To exclude potential bacterial inhibition by hydrogen peroxide, the culture supernatants were treated with catalase at a final concentration of 100 U/ml (10).

Mode of Action of SalB

In order to analyze the mode of action of SalB and the effect of varying pHs, cell-free, filter-sterilized and log-phase *L. salivarius* M7 culture supernatants (5 ml aliquots) containing 10,200 AU/ml were adjusted to pH 3.0, 4.0, 5.0, 6.5 or 7.0, and incubated at 37 °C for 30 min. A total of 200 µl of log-phase cultures of the *L. fermentum* ATCC 9338, *L. plantarum* ATCC 8014 and *L. acidophilus* ATCC 4357 were centrifuged (5000 x g, 10 min), washed in saline (0.9 NaCl, wt/vol) and resuspended in 500 µl MRS broth. After adjusting the pH of each aliquot of *L. salivarius* M7 culture supernatant to 7.0, a 25 µl aliquot containing 10,200 AU/ml was added to each cell suspension and then incubated at 37 °C for 30 min. A 100 U/ml catalase was added to each mixture to prevent the inhibitory effect of hydrogen peroxide. After incubation, cells were cetrifuged (5000 x g, 10 min), washed in saline (0.9 NaCl, wt/vol) and plated on MRS agar. As a control, 100 µl of log-phase cells of *L. fermentum* ATCC 9338, *L. plantarum* ATCC 8014 and *L. acidophilus* ATCC 4357 were inoculated into MRS broth lacking bacteriocin activity, and the tubes were incubated at 37 °C for 16 h (5-7).

For dose-response studies, serial dilutions of SalB were added to 100 µl of log-phase suspensions of the *L. fermentum* ATCC 9338, *L. plantarum* ATCC 8014 and *L. acidophilus* ATCC 4357 strains and incubated for 30 min at 37 °C (7). The samples were then centrifuged (5000 x g, 10 min), washed in saline (0.9 NaCl, wt/vol) and spread on MRS agar. The percentage of SalB-induced cell death was calculated from the decrease in the number of viable cells.

Stability of SalB

Supernatants from log-phase *L. salivarius* M7 cultures were adjusted to varying pH values and heated to 100 °C for 15 min. The treated supernatants were stored at –20

°C, 4 °C and room temperature. At different time intervals aliquots were taken and used to determine the inhibitory activity.

Results

Production of SalB

Broth conditions for the production of SalB and the growth of *L. salivarius* M7 were evaluated in non-pH-regulated culture environments. Different initial pHs were tested for broth conditions for the production of SalB. Weak acidic conditions seemed to favor optimal growth of *L. salivarius* M7.

When the pH of the broth was initially adjusted to 6.5, maximum production of SalB (10,240 AU/ml) was observed (Table 2).

Table 2. SalB production at various pH values.

PH	AU/ml
5.0	640 AU/ml
5.5	640 AU/ml
6.0	6400 AU/ml
6.5	10,240 AU/ml
7.0	5220 AU/ml
7.5	5220 AU/ml

Effect of pH heat, and enzyme treatment on bacteriocin activity

The effect of pH and temperature on the stability of SalB was assessed on filtered, neutralized and heat-treated active supernatants containing 10,240 AU/ml SalB activity. At all pH values above pH 2, the activity of the supernatant remained stable (Table 3). Storing the supernatant under pH 3.0 for 30 min did not change the activity. Storing it in a refrigerator for up to 3 months and for over 20 h at pH 5.0 at room temperature also had no effect. SalB activity was partially sensitive only to treatment with proteinase K (Table 4). In comparison to the activity of proteinase K, SalB activity was tested both with phosphate buffer saline (15-20 mm of zone of inhibition), and with proteinase K: the latter was found to decrease the SalB activity by 15% (8 mm of zone of inhibition). SalB was also found to be stable under heat treatment (Table 5).

Table 3. Effect of pH on the activity of SalB at 37 °C.

pH	Activity development (inhibition zone against *L. fermentum* in mm)
9	9
8	9
7.5	11
7.0	13
6.5	20
6.0	15
5.5	8
5.0	8
4.5	6
4.0	6
3.5	6
3.0	6

Table 4. Sensitivity of SalB to enzymes at 37 °C.

Treatment	Diameter (mm) of zone of inhibition against *L. fermentum*
Control (phosphate buffer)	15
Trypsin	15
Chymotrypsin	14
Pepsin	14
Trypsin	14
Proteinase K	9
Lipase A	15
Lysozyme	14
Catalase	17

Table 5. Sensitivity of SalB to temperature.

Treatment	Diameter of the inhibition zone (in mm)activity	Proteinase K (%)
Control (no heat)	15	0
60 °C for 30 min	15	0
60 °C for 60 min	8	15
75 °C for 30 min	15	0
75 °C for 60 min	14	10
100 °C for 15 min	14	5
121 °C for 15 min	12	5

*The diameter of the well is not included in the inhibition zone.
*The data represent the diameter of the inhibition zone (in mm) obtained with the agar diffusion test.

Inhibition spectrum

Analysis of the inhibition spectrum of SalB using the agar-well diffusion test showed that this bacteriocin inhibited the growth of a variety of closely related bacteria (Table 6). These include *L. fermentum* ATCC

Table 6. Inhibition of lactobacilli by culture supernatant fluid of *L. salivarius* M7.

Target organism	Code numbers	Zone diameter
L. acidophilus	ATCC 4357	> 15
L. fermentum	ATCC 9338	> 15
L. plantarum	ATCC 8014	> 15
L. monocytogenes	L2*	> 15
Staph. aureus	ATCC 6538	3-5
Staph. epidermidis	*	15
Strep. faecalis	*	10

* Strain from field isolates.
The diameter of the well is not included in the inhibition zone.
The data represent the diameter of the inhibition zone (in mm) obtained with the agar diffusion test.

9338, *L. plantarum* ATCC 8014, *L. acidophilus* ATCC 4357 and some more distantly related bacteria such as, *L. monocytogenes* L2, *Strep. faecalis, Staph. epidermidis* and *Staph. aureus* ATCC 6538. SalB was very effective against all the *L. monocytogenes* strains tested. *Staph. aureus* was not affected when the amount of active SalB was less than 10,000 AU/mL.

Bactericidal action of SalB

To characterize the mode of action of SalB, the viability of the indicator strains *L. fermentum* ATCC 9338, *L. plantarum* ATCC 8014, *L. acidophilus* ATCC 4357 and *L. monocytogenes* L2 were monitored in the presence of different concentrations of SalB (Table 6). Starting from 1 µl of 50 AU/ml supernatant, a rapid decline in the viability of LAB strains was observed in the first minutes of contact with SalB at all pH values > 2.0. The optical density at 590 nm did not change at any pH values for LAB strains and *L. monocytogenes* during 20 h of incubation. At the end of each trial, a 100 µl sample of the indicator strains was plated on agar and allowed to grow at 37 °C for 16 h. Plate counts were performed, and the number of viable cells at each trial was found to decrease substantially (Table 7). A zone of growth inhibition was observed for all LAB strains and *L. monocytogenes* L2 starting from 1 µl of supernatant containing 50 AU/mL. However, when the same trials were applied to *Strep. faecalis*, there was a slow decline in OD, and there was also growth on the agar. When the concentration of SalB in the medium was ≥ 5000 AU/ml, the growth of *Strep. faecalis* was substantially impaired. A concentration of 10,000 AU/ml or more was insufficient to kill *Staph. epidermidis* and *Staph. aureus* ATCC 6538.

The decrease in the number of viable cells indicated that no lysis of the indicator strain occurred since there was no change in the OD of the medium via UV spectroscopy. Plate counting of the indicator strains treated with the various doses of SalB also clearly indicated that as the dose of bacteriocin increased, the number of dead indicator cells also rose.

Discussion

SalB, a bacteriocin produced by *L. salivarius* M7, was physiologically characterized. Its genetic characterization

Table 7. Effect of SalB on the death of LAB species and L. monocytogenes.

SalB (AU)	Colony count	Cell death (%)	Strain
0	2.6×10^9	0	*L. acidophilus* ATCC 4357
0	2.9×10^8	0	*L. monocytogenes* L2
50	2.7×10^8	68.9	*L. monocytogenes* L2
50	2.9×10^8	88.84	*L. acidophilus* ATCC 4357
5.00	2.1×10^8	91.92	*L. acidophilus* ATCC 4357
	2.2×10^8	24.13	*L. monocytogenes* L2
2600	3.3×10^6	99.87	*L. acidophilus* ATCC 4357
2.600	3.7×10^7	87.24	*L. monocytogenes* L2
10.240	1.2×10^2	99.99	*L. acidophilus* ATCC 4357
10.240	1.8×10^6	99.03	*L. monocytogenes* L2

had been completed previously (11). Neither the production of SalB nor the activity of the protein were found to be affected by the pH of the medium. *L. salivarius* M7 grew better under weak acid conditions, although it also grew and produced SalB between pH 3.0 and 9.0. Production, depending on cell density, started as early as 4 h and continued to 10 h of growth. The activity of the protein did not seem to change over 20 h after storage at room temperature, or 3 months at 4 °C. SalB was highly stable during heating, and during repeated freezing and thawing cycles was resistant to 100 °C for 30 min. and 121 °C for 15 min. The stability properties were similar to those of the bacteriocins, such as lactacin B and F, produced by *L. acidophilus* (12,13). SalB was active in the presence of many proteolytic enzymes including trypsin, chymotrypsin and lysozyme, lipolytic enzymes such as lipase A, and glucolytic enzymes such as dextranase, but was partially sensitive to proteinase K. These results suggest that the antibactericidal compound was indeed a protein in nature and that lipidic and glucidic moieties are absent in its structure or are not required for its biological activity. SalB was active against many closely related LAB strains, including *L. fermentum* ATCC 9338, *L. plantarum* ATCC 8014 and *L. acidophilus* ATCC 4357, and against many more distantly related Gram-positive bacteria such as *L. monocytogenes* L2, *Strep. faecalis*, *Staph. epidermidis* and *Staph. aureus* ATCC 6538. SalB has no inhibitory activity on Gram-negative bacteria and *B. cereus*. The time course of SalB activity against *L. fermentum* ATCC 9338, *L. plantarum* ATCC 8014 and *L. monocytogenes* L2 was not always parallel in non-pH-regulated MRS broth cultures. For example, the MRS broth culture of *L. salvarius* M7 initially adjusted to pH 6.5 and non-regulated throughout the experiment provided a higher titer than the culture of MRS broth adjusted to 7.0, 7.5 or 6.0 initially. With subcultures of *L. salivarius* M7 in MRS agar instead of in broth, there was no bacterial growth, which may indicate an intracellular induction factor. As described for most bacteriocins produced by LAB (11-16), SalB exhibits a bactericidal mode of action against sensitive cells in the log-phase of growth. This is reminiscent of ion-channel-forming bacteriocins, which require the presence of a transmembrane potential or a pH gradient to be active. Therefore, they are far more active on log-phase cells than on stationary-phase cells. The kinetic data have indicated that bacteriocins behave as a single lethal entity in a single hit process. Killing of sensitive cells by SalB occurred within a few minutes (for all LAB strains, *L. monocytogenes* L2 and other *L. monocytogenes* field strains), and the threshold dose against LAB strains and *L. monocytogenes* L2 was 50 AU/mL. For *Staph. epidermidis* and *Staph. aureus*, the threshold dose is over 5000 AU/ml, and for *Strept. faecalis* it is over 300 AU/mL. These differences reinforced suggestions that SalB may act in a single hit fashion (4, 9) since treatment of the exponential phase indicator cells with SalB resulted in the death of cells, with the percentage of cell death increasing with the amount of bacteriocin added. Interestingly, the bactericidal effect and stability of SalB occurred at pH 3.0 to 9.0. The effectiveness of bacteriocin depends on both its concentration and the bacteria producing it.

Thus, while SalB belongs to the class II LAB bacteriocins it is not homologous to bacteriocins of the anti-listeria family. The primary structure of the predicted translational product of the SalB structural gene indicates that SalB is synthesized as a precursor protein (1). In addition to acidification, SalB also has a strong anti-listerial activity. Therefore, both the bacterium and its products are good candidates for the restoration of a digestive system that is disorganized by pathogenic bacteria.

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