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review

Bacteriocins of Lactic Acid Bacteria – Properties, Range of Inhibitory Activity and Methods of Detection

Bakteriocini bakterija mliječne kiseline – svojstva, raspon inhibicijskog djelovanja i metode određivanja

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Summary

Lactic acid bacteria (LAB) represent a group of safe organisms beneficial to health and important food preservers. It has been found that some members display antagonistic effects against pathogenic and spoilage bacteria. LAB can produce a variety of substances with antibacterial activity such as metabolic end products, antibiotic-like substances and bactericidal proteins, termed bacteriocins. This review summarises the current knowledge on the properties of bacteriocins, their structure, spectrum of activity and mode of action. The methods used to study bacteriocins are discussed.

Sažetak

Bakterije mliječne kiseline predstavljaju zajednicu neškodljivih mikroorganizama čije djelovanje može biti korisno za zdravlje, a važni su za očuvanje hrane. Utvrđeno je da neki sojevi djeluju antagonistički prema patogenim bakterijama i bakterijama kvarenja. Bakterije mliječne kiseline proizvode brojne spojeve antibakterijskog djelovanja, kao što su krajnji produkti metabolizma, spojevi slični antibioticima i baktericidni proteini nazvani bakteriocini. U ovom su pregledu iznesene suvremene spoznaje o svojstvima bakteriocina, njihovoj strukturi, rasponu i načinu djelovanja. Također su prikazane metode za proučavanje bakteriocina.

Introduction

Lactic acid bacteria (LAB) are industrially important organisms because of their fermentative ability as well as their health and nutritional benefits. They give fermented foods distinctive flavours and textures while preventing spoilage, extending shelf life and inhibiting pathogenic organisms. Preservation of fermented foods by LAB is due to different substances like organic acids (lactic, acetic), hydrogen peroxide, diacetyl, secondary reaction products and bacteriocins which have potential to inhibit a variety of other microorganisms that are usually closely related to the producer bacterium *(1)*. By the desire of consumers for »natural« food products, these microorganisms have been recognised as a potential source of biopreservatives for foods. They essentially have a GRAS (generally recognized as safe) identity having been consumed in large numbers by people for thousands of years with no ill effects *(2)*. LAB are normal residents of the gastrointestinal tract of human beings and it is agreed that their presence in the gastrointestinal tract is essential for healthy life. Among other substances, bacteriocins are believed to be important in the ability of LAB to compete with other microflora of gastrointestinal tract *(3)*.

The original meaning of the term »bacteriocin« was greatly influenced by characteristics common to colicins which were the prototype bacteriocins. There is no universally accepted definition, but the description has expanded to recognise the differences between colicins and bacteriocins produced by Gram-positive bacteria *(4)*. Today, bactericidal peptides or proteins produced by bacteria are, for the most part, called bacteriocins and the term »colicin« now implies a bactericidal protein produced by varieties of *E. coli* and closely related *Enterobacteriaceae*. Klaenhammer *(5)* defined bacteriocins as »proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer microorganism«. Bacteriocins are heterogeneous compounds which vary in molecular weight, biochemical properties, activity spectra and mechanism of action. Investigations concerning bacteriocins have historically focused on four general areas *(5)*: identification and description of novel bacteriocins, characterisation of bacteriocin production and mode of action, characterisation of plasmids encoding bacteriocin production and immunity and classification of bacteria based on bacteriocin production or sensitivity.

Elements of composition and/or structure of bacteriocins

The structure of LAB bacteriocins has shown that there exist three groups of molecules: lantibiotics, low molecular weight peptides (< 50 amino acids) which contain lanthionine, low molecular weight peptides (< 15000 Da) without lanthionine and the last category of bacteriocins which includes non-lantibiotic, large molecules (> 15000 Da). Lantibiotics are a family of peptides containing α, β-unsaturated amino acids (dehydroalanine and dehydrobutyrine) and thioether amino acids (lanthionine and β-methyl-lanthionine). The most extensively characterised bacteriocin of LAB is nisin. It is produced by various strains of *Lactococcus lactis* subsp. *lactis* (*Lactococcus* = *Lac.*) and belongs to the lantibiotics. Lacticin 481 produced by *Lac.lactis* subsp. *lactis* CNRZ481 is another lantibiotic, smaller than nisin and whose N-terminal amino acid sequence does not show any resemblance to nisin. Other low molecular weight peptides which do not contain lanthionine are diplococcin produced by *Lac. lactis* subsp. *cremoris* 346, lactococcin A produced by *Lac. lactis* subsp. *diacetylactis* WM4, *Lac. lactis* subsp. *cremoris* LMG2130 and lactacin F produced by *Lactobacillus acidophilus* 11088 (*Lactobacillus* = = *L.*). The last category of bacteriocins includes bacteriocins produced by *L. helveticus* LP27 (lactocin 27, which is a glycoprotein), *L. helveticus* 481 (helveticin J), *L. fermenti* (fermenticin) and *L. casei* B80 (caseicin 80) *(5–8)*.

Activity spectra, biochemistry and stability of bacteriocins produced by LAB

Spectrum of activity

Klaenhammer *(5)* defined two classes of bacteriocins according to their spectrum of activity. One includes bacteriocins active against bacteria taxonomically close to the producer and the other is composed of bacteriocins with a relatively broad spectrum of activity against Gram-positive bacteria. Some of the most known bacteriocins produced by LAB and their spectrum of activity are summarised in Table 1.

The inhibitory power of the *Lactococcus* was probably first recognised in the 1930s when the inhibition of commercial cheese starter cultures by similar dairy bacteria was reported. The two best characterised proteins causing this phenomenon in lactococci are nisin and diplococcin. Nisin is unusual in that it can inhibit most Gram-positive bacteria including spores of *Clostridium* spp. and of *Bacillus* spp. These bacteria are responsible for most of deterioration of canned foods and the addition of nisin to these products can reduce the nitrite used as a preservative. Besides, nisin prevents late blowing of pressed cheese caused by *Clostridium tyrobutyricum* spores. It is also active against *Lysteria monocytogenes*. Nisin was the first recognised antimicrobial that showed promise for practical use in food preservation and remains the only bacteriocin produced by LAB that has realised commercial applications in food processing and fermentation. Commercial production of nisin started in the 1950s and is available under the trade name Nisaplin *(2)*.

Table 1. Spectrum of inhibitory activity of bacteriocins produced by LAB *(6)*

Tablica 1. Inhibicijsko djelovanje bakteriocina bakterija mliječne kiseline *(6)*

Bacteriocin	Producer organism	Spectrum of activity
Nisin	*Lac. lactis*	Gram-positive bacteria
Diplococcin	*Lac. cremoris*	*Lactococcus* sp.
Lactostrepcins	*Lac. lactis*	*Lactococcus* sp., β-haemolytic streptococci, *L. helveticus, Leuconostoc* sp., *Clostridium* sp.
Lacticin 481	*Lac. lactis*	*Lactococcus* sp. *L. helveticus, L.bulgaricus, Leuconostoc* sp., *S. thermophilus, Cl. tyrobutyricum*
Lactococcin A	*Lac. cremoris*	*Lactococcus* sp.
Lactocin 27	*L. helveticus*	*L. helveticus, L.acidophilus*
Helveticin J	*L. helveticus*	*L. helveticus, L.bulgaricus, L.casei*
Lactacin B	*L. acidophilus*	*L. leichmanii, L.bulgaricus, L. helveticus, L.casei*
Lactacin F	*L. acidophilus*	Idem + *L.fermentum*, + *E.faecalis*
Bac*	*L. acidophilus*	some strains of genera: *Lactobacillus, Lactococcus, Pediococcus, Listeria, Enterococcus, Staphylococcus, Bacillus, and Clostridium*
Plantaricin A	*L. plantarum*	*Lactobacillus* sp., *Pediococcus* sp. *E. faecalis*
Plantaricin B	*L. plantarum*	*L. plantarum, Leu.mesenteroides, L. damnosus*
Plantaricin S	*L. plantarum*	*Lactobacillus* sp., *Leuconostoc sp., Lactococcus* sp., *Pediococcus* sp.
Sakacin A	*L. sake*	*Lactobacillus* sp., *Lys. monocytogenes*
Lactocin S	*L. sake*	*Lactobacillus* sp., *Leu.mesenteroides, P. acidilactici, P. pentosaceus*
Caseicin 80	*L. casei*	*L. casei*
Brevicin 37	*L. brevis*	*Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp.
Pediocin A	*P. pentosaceus*	Lactic acid bacteria, *Clostridium* sp., *S. aureus, Lys. monocytogenes, B. cereus*
Pediocin PA-1	*P. acidilactici*	Lactic acid bacteria, + *Lys. monocytogenes*

E. = Enterococcus; S. = Streptococcus; Cl. = Clostridium; Leu. = Leuconostoc; P. = Pediococcus; Lys. = Lysteria.

* Bacteriocin is produced by strain *L. acidophilus* LF221, isolated from faeces of a newborn child *(21,22)*.

* Bakteriocin bakterije *L. acidophilus* LF221, izolirane iz fecesa dojenčadi (21,22).

Biochemical properties

Among biochemical properties tolerance to heat, to pH and sensitivity to enzymes are important. Bacteriocins differ greatly with respect to their sensitivity to inactivation by changes in pH and temperature. Many of the bacteriocins produced by LAB are only stable at acid and neutral pH and are inactivated at a pH above 8.0. This indicates that the substances are well adapted to the environment of the bacteria producing them. Heat resistance is a major characteristic of bacteriocins produced by LAB, but Toba et al. *(9)* reported about heat-labile bacteriocin produced by *Lactobacillus acidophilus* LAPT 1060. Heat resistance can vary considerably; bacteriocins are heat resistant at 60 °C and 100 °C for 30 min and to autoclaving at 121°C for 15 to 20 min, respectively. This heat resistance suggests that the activity of bacteriocins is based on molecular structures which are relatively small and uncomplicated. All bacteriocins are inactivated by at least

one protease. They are usually inactivated by proteolytic enzymes of pancreatic origin (trypsin and α-chymotrypsin), sometimes of gastric origin (pepsin). This high sensitivity of bacteriocins to metabolic proteolytic enzymes is very interesting with respect to food safety, because the ingestion of bacteriocins would not alter intestinal ecology. Some bacteriocins produced by *Lactobacillus* spp. contain a carbohydrate, lipid and/or phosphorous moiety. The presence of such a non-proteinaceous moiety can indicate the sensitivity of these bacteriocins to glycolytic (α-amilase), lipolytic (lipase) and phospholipolytic (phospholipase) enzymes *(10–20)*. Some examples are summarised in Table 2.

Biosynthesis and conditions of maximal production

Bacteriocin biosynthesis occurs during or at the end of exponential growth. Generally, they are extracellular products but a part of antimicrobial activity may be retained within the cell. The most important factors which influence the bacteriocin production are: medium, pH, temperature and physiological state of the cells. Several groups have studied the culture conditions of the producing strain which would lead to maximal bacteriocin production. Most authors have noted that bacteriocin production is correlated with the quantity of biomass produced. As a result of this, maximal bacteriocin production could be obtained by supplementing a given culture medium with growth-limiting factors, such as sugars, vitamins, nitrogen sources, by regulating bacterial cultures at a given pH or by choosing the best adapted culture medium. Recent studies have shown that in many cases more bacteriocin is produced under stress conditions when growth is weaker *(6,23–29)*.

Mode of action

One of the common characteristics attributed to bacteriocins is that inhibition is bactericidal, not bacteriostatic, with the possible exception of lactocin 27, leucocin A-UAL 187 and leuconocin S. Their action is rapid (within several minutes) and their lethal effect is higher on exponentially growing cells than on stationary phase cells *(6,8,30)*.

According to Tagg et al. *(4)* the action of bacteriocins on sensitive cells is a 2-step process. The first phase is the adsorption of bacteriocins on specific or nonspecific receptors on the cell envelopes of host bacteria. At this time, bacteriocins are sensitive to proteases. In many cases, sensitive cells, following exposure to the bacteriocin, can be rescued by treatment with proteolytic enzymes. The second irreversible phase involves pathological changes in the target cell, specific to each bacteriocin. Most published work has shown that bacteriocins of LAB are nonspecifically adsorbed to Gram-positive bacteria. Bhunia et al. *(31)* observed that a fraction containing lipoteichoic acid (LTA) could be responsible for the attachment of pediocin AcH. LTA is present in the walls of Gram-positive bacteria and are absent in walls of Gram-negative bacteria. Data obtained by Chikindas et al. *(32)* suggest that pediocin PA-1 functions in a voltage-independent manner but requires a specific protein in the target membrane.

The bacteriocin literature led us to the hypothesis that the primary target of bacteriocins from LAB is most probably the cytoplasmic membrane, since they initiate reactions which alter the membrane permeability disturbing membrane transport or dissipating the proton motive force (PMF) in sensitive cells *(32–37)*.

Table 2. Sensitivity to enzymes, thermotolerance and acidotolerance of bacteriocins produced by LAB *(6)*
Tablica 2. Osjetljivost bakteriocina bakterija mliječne kiseline na enzimsko djelovanje te otpornost na temperaturu i kiselost *(6)*

Bacteriocin	Inactivating enzymes	Thermotolerance Acidotolerance
Nisin	α-chymotrypsin, nisinase	+ (115 °C, pH = 2), – (neutral pH)
Diplococcin	α-chymotrypsin, trypsin, pronase	purified: – (1 h, 100 °C) unpurified: + (1 h, 100 °C, pH = 5), – (1 h 100 °C, alkaline pH)
Lactostrepcins	α-chymotrypsin, trypsin, pronase, phospholipase D	+ (100 °C, 10 min) – (neutral pH)
Lacticin 481	α-chymotrypsin, pronase, ficin, proteinase κ, rennet	+ (1 h, 100 °C, pH = 4,5 and 7,0) stable from pH = 2 to pH = 8
Lactocin 27	trypsin, pronase	+ (1 h, 100 °C)
Helveticin J	trypsin, pronase, ficin, pepsin, proteinase κ, subtilisin	– (30 min, 100 °C)
Lactacin B	proteinase κ	unpurified: + (1 h, 100 °C) purified: + (3 min, 100 °C)
Lactacin F	trypsin, ficin, proteinase κ	+ (15 min, 121 °C)
Bac (unpurified)*	trypsin, proteinase κ, pronase, α-amyloglicosidase, lipase	+ (5 min, 100 °C), – (20 min, 115 °C) stable from pH = 2 to pH = 9
Plantaricin B	α-chymotrypsin, trypsin, pronase, pepsin, lipase, α-amylase	+ (30 min, 100 °C)
Sakacin A	trypsin, pepsin	+ (20 min, 100 °C)
Caseicin 80	trypsin, α-chymotrypsin, pronase, proteinase κ, pepsin	– (10 min, 60 °C, pH = 2)
Brevicin 37	trypsin, pronase	+ (1 h, 121 °C, pH = 2 to 4)
Pediocin A	pronase	+ (1 h, 100 °C)
Pediocin PA-1	α-chymotrypsin, pepsin, papain	+ (10 min, 100 °C) – (15 min, 121 °C)

+ : stable – : unstable

* Bacteriocin is produced by strain *L. acidophilus* LF221, isolated from faeces of a newborn child *(21,22)*

* Bakteriocin bakterije *L. acidophilus* LF221, izolirane iz fecesa dojenčadi *(21,22)*

Methods of detection and determination of bacteriocin activity

Demonstration of antagonism from one strain of bacteria against another is very common. When investigating bacteriocins produced by LAB we must always be aware of the fact that bacteriocins are only one category of substances produced by LAB that are inhibitory to other bacteria. Besides bacteriocins, possible inhibitors produced by LAB include organic acids, enzymes, defective bacteriophages and other metabolic by-products as hydrogen peroxide and diacetyl. Before determination of bacteriocin activity questions that can be asked include the following *(30)*: Should the test be direct or deferred? What is the effect of the medium's pH, buffering capacity, nutrients content, redox potential, or protein binding capacity? How were the production culture and indicator strain handled? How long was the assay incubated? Is the bacteriocin inducible? How important is cell density? Is the bacteriocin freely diffusing? Is the bacteriocin heat sensitive or highly prone to degradation by common proteolytic enzymes? How is bacteriocin activity measured (by measuring of zones or optical density readings)?

Methods of detection

There are many techniques for detecting bacteriocin production. Most are based on the diffusion of bacteriocins through solid or semisolid culture media to inhibit growth of a sensitive organism.

The two basic methods that are commonly used are simultaneous (or direct) and the deferred antagonism procedures *(4,30,38,39)*. The simplest direct test and the one most widely used for the preliminary screening is the »spot-on-the-lawn« test. Here the test and indicator cultures are grown simultaneously. The lawn is generally seeded before inoculation of the test strains. The density of the indicator lawn is an important determinant of the sensitivity of the method. Variations of this procedure include the use of overlapping drops, wells cut into freshly seeded pour plate cultures and filled with agar containing the test organism and well-diffusion assay *(40)* where supernatants from bacteriocin-producing cultures are placed in wells cut into agar seeded with a sensitive organism. The latter method is often used as bioassay for the determination of bacteriocin titers.

In deferred antagonism, the test organism is grown on agar for a period of time. The bacteria are then killed by exposure to chloroform or heat, and an overlay of the indicator strain in melted agar is placed on the surface. A useful alternative of indirect methods include the flip-streak and the spot-on-the-lawn assay. In the flip-streak method, the bacteriocin-producing strain is streaked on a medium and after incubation a bacteriocin sensitive organism is streaked perpendicular to it on the reverse side of the agar (which must be flipped). For the spot-on-the-lawn method, the bacteriocin producer is spotted on an agar medium and a lawn of sensitive bacteria is poured over the resultant colony. The modification of the well diffusion assay is done by pouring melted and tempered soft agar seeded with an overnight indicator culture in a sterile Petri dish and allowing it to harden. It is then incubated until growth is evident. Then the wells are cut and filled with cell-free supernatant of bacteriocin-producing culture. It is also possible that the test culture is then spot or strike inoculated onto this plate. Incubation of plates continues and plates are examined for clearing around the wells or spots. The clear zones are »lysis zones« and not »inhibition zones« as of well diffusion and spot test assay commonly used. This method gives false-negative results when the bacteriocin tested is bactericidal without causing cell lysis. The appearance of clear zones of inhibition on the indicator lawn around the colonies or wells can be taken as a positive result. However, bacteriocins are not the only antimicrobial substances causing inhibition zones. Inhibitory activity can also be due to organic acids, hydrogen peroxide or bacteriophages. The improved deferred »sandwich« method of Hechard et al. *(42)* as well as the examination of the clearing zone on a spot deferred antagonism assay plate for the presence of lytic bacteriophages *(42)* may confirm inhibition due to the release of bacteriocin. The new technique described by Toba et al. *(43)* is an adaptation of the well diffusion assay on microdilution plates. It eliminates the need to cut out agar.

Deferred antagonism procedures often prove more sensitive than simultaneous antagonism and permit the independent variation of the time and conditions of incubation of the test and indicator cultures.

Determination of activity

The most generally applied method for the activity determination of bacteriocin is the Critical Dilution Method *(44)*. The basis of the method is simple and consists of: preparation of a series of dilutions of the sample (usually a two-fold series are used), deposition of uniform drops (quantity) from each dilution on the surface of a nutrient medium (or wells cut in the agar) seeded with a uniform standard inoculum of the indicator strain, after a standardised period of incubation, recording the last dilution causing the zone of inhibition.

The activity is defined as the reciprocal of the highest dilution affecting a zone of inhibition of the indicator lawn and is expressed as activity units (AU) per millilitre.

$$AU/mL = (1/(\mu L \text{ of bacteriocin} \times 1/2^n)) \times 1000$$

n = dilution, n = 0 for undiluted sample

A quantitative alternative for the agar diffusion method is turbidometry. The criteria used for the determination of inhibitory effects are based on the changes of turbidity measured as absorbance – optical density of the indicator culture in liquid medium. Usually the end optical density after a specified incubation period is measured in parallel in the cultures with and without the bacteriocin.

Unit of activity is defined as a reciprocal of the highest dilution of a sample resulting in a 50 % decrease of maximum absorbance after a definite time of incubation. With this method the calculation of activity unit of bacteriocin is more precise. This method also enables the determination of lower level of bacteriocin because the amount of the bacteriocin sample is not so limited as on a hard medium. We can vary the amount of the cells of indicator strain and the amount of the bacteriocin added.

We recently used this method, slightly modified, for the determination of activity of bacteriocin produced by *L. acidophilus* LF221 and got very good results. With the application of the suitable pH indicator after incubation the reading of results is easier and there is no need to measure the optical density *(23)*.

Conclusions

Bacteriocins from LAB are of great interest because of their potential for improving the overall quality and safety of foods. The direct addition of bacteriocins or the use of bacteriocin-producing LAB associated with foods may provide a novel means of preserving foods from the spoilage and/or pathogenic bacteria. Bacteriocins may also be present in established food systems (i.e. ripened cheeses, fermented milks, sausages) as by-products of starter fermentations. The addition of bacteriocin-producing isolates to various foods is an excellent alternative to direct addition of bacteriocins. Several studies reported the cloning of bacteriocin genes into nonbacteriocinogenic LAB. There are possibilities to improve selected starter cultures with this technique. Besides all this advantages among LAB the probiotics can be found. Obviously more work needs to be done to develop the potential of the bacteriocins from LAB for use in the marketplace.

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