Antagonistic properties of lactic acid bacteria

Purification and characterization of a bacteriocin produced by *Lactococcus lactis* ssp. *lactis* SIK -83

Licentiate thesis

Elín Gudmundsdóttir

Department of Food Science, Chalmers University of Technology and
SIK, the Swedish institute for food research
1993
Antagonistic properties of lactic acid bacteria

Purification and characterization of a bacteriocin produced by Lactococcus lactis ssp. lactis SIK -83

Licentiate thesis

Elín Gudmundsdóttir

Department of Food Science,
Chalmers University of Technology
and
SIK, the Swedish institute for food research
1993

ISBN 91-7290-151-9
# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SUMMARY</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>PART I. LITERATURE SURVEY</strong></td>
<td>2</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>2</td>
</tr>
<tr>
<td><strong>LACTIC ACID BACTERIA</strong></td>
<td>4</td>
</tr>
<tr>
<td>Fermented food</td>
<td>4</td>
</tr>
<tr>
<td>Nomenclature</td>
<td>7</td>
</tr>
<tr>
<td>Metabolism</td>
<td>8</td>
</tr>
<tr>
<td><strong>ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA</strong></td>
<td>12</td>
</tr>
<tr>
<td>Acids</td>
<td>12</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>12</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>14</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>14</td>
</tr>
<tr>
<td>Reuterin</td>
<td>15</td>
</tr>
<tr>
<td>Bacteriocins</td>
<td>15</td>
</tr>
<tr>
<td>NISIN</td>
<td>21</td>
</tr>
<tr>
<td><strong>PART II. EXPERIMENTAL WORK</strong></td>
<td>28</td>
</tr>
<tr>
<td>BACKGROUND TO THIS STUDY</td>
<td>28</td>
</tr>
<tr>
<td>OBJECTIVE</td>
<td>29</td>
</tr>
<tr>
<td>DETECTION OF BACTERIOCIN ACTIVITY</td>
<td>29</td>
</tr>
<tr>
<td>FIRST ATTEMPTS AT PURIFICATION</td>
<td>31</td>
</tr>
<tr>
<td><strong>PURIFICATION - PRETRIALS</strong></td>
<td>32</td>
</tr>
<tr>
<td>Bacteriocin production</td>
<td>32</td>
</tr>
<tr>
<td>Bacteriocin assay</td>
<td>33</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation in the presence and absence of Tween 80</td>
<td>33</td>
</tr>
<tr>
<td>Column chromatography</td>
<td>35</td>
</tr>
<tr>
<td>Plasmid profile</td>
<td>37</td>
</tr>
</tbody>
</table>
SUMMARY

A bacteriocin produced by *Lactococcus lactis* ssp. *lactis* SIK-83 was purified and characterized. A purification procedure was developed for the bacteriocin when the bacteria were cultivated in M-17 broth supplemented with glucose. The extracellularly produced bacteriocin was isolated by ammonium sulphate precipitation followed by cation exchange, Phenyl Superose hydrophobic- and Pro-RPC reversed phase column chromatography. The SIK-83 bacteriocin was purified 10.926 fold. The purified bacteriocin was characterized with regard to amino acid composition while the sequence was determined from the gene for bacteriocin production obtained by PCR amplification. Amino acid composition analysis revealed the absence of aromatic amino acids and the presence of lanthionines. The SIK-83 bacteriocin is thus a lanthibiotic. The amino acid sequence showed that the bacteriocin is a variety of a nisin polypeptide where histidine in nisin A (residue 27) had been exchanged for asparagine. This bacteriocin is designated nisin Z.

The production of bacteriocins can be chromosomal or plasmid mediated. In this study plasmid profile analysis showed that plasmids are not involved in bacteriocin production. This explains the stability of production which was observed and is of great importance with regard to potential application.

*Key Words: Lactic acid bacteria, bacteriocins, lanthibiotics, nisin, purification*
PART I. LITERATURE SURVEY

INTRODUCTION

Lactic acid fermentation has been traditionally used to prevent spoilage as well as to improve the taste, aroma and texture of foods such as milk, vegetables and meat. Historically, food fermentations have been based on empirical knowledge. The natural lactic acid bacteria flora present on the raw material was used for fermentation, and conditions were manipulated to obtain improved results. Today, a variety of commercially fermented food is available on the market including youghurt, sour milk, fermented sausage, cheese, fermented vegetables, bread, wine and beer.

It was only during the last century that man realized that bacteria are involved in fermentation. With that knowledge studies of lactic acid bacteria commenced. Such studies have resulted in the introduction of inoculants or starter cultures into fermentation, an important step in industrializing the process.

Fermentation by lactic acid bacteria is characterized by the accumulation of lactic acid and other organic acids with an accompanying increase in acidity. This is of great importance in keeping spoilage bacteria at bay. There are, however, other metabolites and products of lactic acid bacteria which play a role in the antimicrobial character of fermentation, i.e. hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins. It is difficult to assess the role of each of these factors in total the antimicrobial activity observed in fermentations. Each of the factors mentioned above will be discussed here, with the main emphasis on bacteriocins.

Bacteriocins are by definition proteins or proteinous compounds produced by bacteria. They inhibit the growth of closely related species by a bactericidal mode of action (Tagg et al., 1976). The ability to produce bacteriocins is a widespread phenomenon in the bacterial world. Colicins produced by species of Escherichia coli and other enterobacteria were the first bacteriocins described. The first colicin was found by André Gratia in 1925. Since then much research has revolved round the colicins and several review articles are available (Reeves. 1965; Reeves, 1972; Mayr-Hartling, 1972). The first two bacteriocins
of lactic acid bacteria described were diplococcin, which is produced by strains of *Streptococcus cremoris*, and nisin, which is produced by strains of *Lactococcus lactis* (Whitebread, 1933; Roger and Whittier, 1928; Mattich and Hirsch, 1947).

Interest in bacteriogenic lactic acid bacteria and other gram-positive bacteria - such as *Staphylococcus* and *Bacillus* - has grown considerably in recent years. Many bacteriocins have been isolated, and their antimicrobial spectrum and genetic determinants studied. Not many of them, however, have been purified and chemically characterized. From the information available to date, bacteriocins of lactic acid bacteria seem to fall into three classes: large polypeptides >30,000 Da., small polypeptides with 30-50 amino acid residues and small lanthionine-containing polypeptides with 20 - 40 amino acid residues.

Special attention has been paid to bacteriocins of lactic acid bacteria used in food fermentation and preservation. The need to understand the role of bacteriocins in the competitiveness of bacteriocinogenic bacteria in fermenting ecosystems is of commercial interest, for example with regard to starter cultures. Another recent aspect of the growing interest in bacteriocins of lactic acid bacteria is their ability to inhibit the growth of the pathogenic bacteria *Listeria monocytogenes*, which have been correlated with recent outbreaks of listeriosis. Yet another field of interest is the possible preservative role of bacteriocinogenic lactic acid bacteria in inhibiting growth of pathogenic bacteria such as *Listeria* and *Clostridium*, in vacuum-packed food and food packed under modified atmosphere.

The genetic code for production of bacteriocins, as well as for immunity of the producer bacteria to its own bacteriocin, is often carried on plasmids. This ability can therefore be spontaneously lost, even from a whole population of bacteria. In some bacteria the plasmids carrying the genetic code for bacteriocin production and immunity have been integrated into the chromosomes resulting in a more stable production. The number of studies of the genetics of lactic acid bacteria has increased considerably in recent years. Increased knowledge in this field may lead to genetic methods being used to improve bacteriocin production, to combine different bacteriocins in order to expand their inhibitory activity, and to cooperatively target a variety of undesirable pathogenic and spoilage organisms in food.
LACTIC ACID BACTERIA

Fermented food
The origin of fermentation is lost in ancient, unrecorded history. It is likely that fermentation of grapes with yeasts came first and grape-wines were known in the Caucasus at least 8000 years ago. It is believed that the Sumerians used fermented milk drinks about the same time (Ragnarsson, 1984). Vegetable fermentation is believed to have its origin in the Orient, probably in China during the third century B. C. (Pederson, 1979).

Fermentation has historically been an empirical process which was passed on from generation to generation. Improvements based on observation were made from time to time. People have learnt from experience to manipulate conditions of fermentation in order to gain better products. Such manipulations include shredding, chopping and mincing raw material, tight packing to exclude air and enhance the anaerobicity of the system, and marinating raw material in salt or sugar brines. People also learnt to control the fermentation process by adding a batch of a previous successful fermentation to a subsequent fermentation. This was further developed by using starter cultures.

Lactic acid and other metabolites produced in fermentation are effective in inhibiting the growth of other bacteria that could spoil the food, making it unfit for consumption. Thus fermentation provided people with safer food with a prolonged shelf-life. In modern society man consumes fermented foods on a daily basis. It also resulted in a wider variety of foods from available raw-material (figure 1). Little energy is lost in the conversion of carbohydrates to organic compounds, so fermented food is almost as good an energy source as the raw material. There are potential health benefits from some species of lactic acid bacteria. Some bacteria and yeast produce vitamins, particularly the B vitamins, and thus add to the nutritional value of the food. Furthermore, the acid environment of fermented food provided good conditions for the preservation of vitamin C. There is also evidence that bioavailability of some minerals, i.e. iron, is improved in fermented food (Andersson et al., 1990). Apart from the improved nutritional value of some fermented food there are reports of improved digestion of lactose, control of intestinal infections, control of levels of serum cholesterol and anticarcinogenic activity and tumour suppression (Fernandes et al., 1987; Gilliland, 1990).
Interest has also focused on the use of lactic acid bacteria as probiotics to improve the growth and performance of domestic livestock (Sissons, 1989). With a ban pending on the use of antibiotics for such purposes, certain lactic acid bacteria may offer an alternative (Gilliland, 1990).

Successful food fermentation is the result of a succession of microorganisms taking part in the process. One particular species of microorganism initiates the fermentation and creates favourable conditions for another species to take over. Fermentation of sauerkraut is a good example. It is initiated by Leuconostoc mesenteroides. When conditions are favourable, strains of Lactobacillus brevis and Pediococcus cerevisiae will outgrow the Leuconostoc, and in turn be succeeded by Lactobacillus plantarum. This sequence is important for a successful fermentation. External conditions, such as number and kind of organisms present, salt concentration, cleanliness of the cabbage and vessels used, and temperature, will influence the process (Pederson, 1979).
Fig. 1 Variety of fermented foods.
Nomenclature

Definition of lactic acid bacteria was first given by Orla-Jensen (Orla-Jensen, 1919). Lactic acid bacteria are a heterogeneous group of bacteria without taxonomic boundaries, but all are gram-positive, catalase-negative, non-sporulating, non-pathogenic bacteria which have the ability to produce lactic acid from hexoses.

Lactic acid bacteria are divided into four genera: *Lactobacilli, Streptococci, Pediococci* and *Leuconostoc. Bifidobacteria*, specific to the intestinal tract, are in certain cases included in the group of lactic acid bacteria, and *Carnobacteria* form a relatively new genus, which includes some of the former Lactobacilli (Collins *et al.*, 1987; Schillinger and Holzapfel, 1990).

In recent years, two new groups have been formed from the genus *Streptococci* : the former lactic *Streptococci* (Lancefield group N), which are now designated *Lactococci*, and the former faecal *Streptococci* (*Streptococcus faecalis* and *Streptococcus faecium*), which are now called *Enterococci* (Hardie, 1986; Schleifer *et al.*, 1985; Schleifer and Kilpper-Bälz, 1984).

In the past, grouping of lactic acid bacteria has been based largely on gram-reaction, morphology, ability to ferment various carbohydrates and a general lack of the ability to produce catalase. More recently, DNA base composition, DNA homology, cell wall peptidoglycan type and immunological specificity of enzymes have come into use. By one of the newer methods, DNA base composition, expressed as moles% guanine+cytosine (G+C), organisms fall into two groups: those with a G+C DNA content of less than about 50 mol% are members of the *Clostridia-Bacillus* subdivision and those with G+C DNA content of over 50 mol% belong to the Actinomycetes subdivision. The genera *Lactobacillus, Pediococcus, Leuconostoc, Lactococcus, Enterococcus and Streptococcus* are members of the *Clostridia-Bacillus* group (Stackebrand and Teuber, 1988) (Figure 2).
Metabolism

Fermentation is a metabolic process in which carbohydrates and related compounds are oxidized and ATP is released in the absence of an external electron acceptor (anaerobic). The final electron acceptors are organic compounds.

Lactic acid bacteria have been divided into groups based upon the end product of carbohydrate (hexose) metabolism. Those which produce lactic acid as the major or sole product of glucose metabolism have been called obligative homofermentative (Streptococci, Pediococci and some Lactobacilli), while those which produce equal molar amounts of lactic acid, ethanol and carbon dioxide from hexoses are called obligative heterofermentative (Leuconostoc, some Lactobacilli and Bifidobacteria). There are three major pathways of hexose fermentation. These pathways have in common that only hexose phosphates with gluco-configuration are fermented. The difference between them lies in the way in which the carbon skeleton is split.

The homofermentatives ferment glucose by glycolysis, which is characterized by splitting the carbon skeleton into two triose-phosphate moieties by aldolase. The triose-phosphates are further converted to lactate. Heterofermentation in Leuconostoc and some Lactobacilli commences with oxidation of glucose-6-phosphate to gluconate-6-phosphate. This is followed by hexose decarboxylation and splitting of the resulting pentose-5-phosphate into a glyceraldehyde-3-phosphate and acetyl-phosphate. Glyceraldehyde-3-
phosphate is further metabolized to lactate, while, depending on hydrogen acceptors available, acetyl-phosphate is either metabolized to acetic acid or reduced to ethanol. The end products are thus 50% lactic acid, 25% carbon dioxide and 25% acetic acid or ethanol (Figure 3). If an additional electron acceptor is available (i.e. O₂ or fructose) ethanol is not formed, but O₂ is oxidized to H₂O₂ or H₂O, and fructose is reduced to mannitol. The homofermentative pathway yields two moles ATP per mole glucose while the pentose pathway only yields one mole ATP per mole glucose. In Bifidobacteria heterofermentation is initiated by splitting fructose-6-phosphate by phosphoketolase into a C-4 and C-2 moiety. The C-2 moiety is converted to acetate, while heptose-7-phosphate is formed from the C-4 moiety and a triose moiety, derived from splitting of an additional molecule of fructose-6-phosphate by transketolase. The heptose-7-phosphate is split into pentose-5-phosphate, finally resulting in acetate and lactate in the molar ratio of 3:2 (Kandler, 1983).

Figure 3. Difference between homolactic (A) and heteroaloactic (B) fermentation of glucose.
Pentoses are usually fermented by all heterofermentative bacteria through the phosphoketolase pathway. Some homofermentative bacteria possess an inducible phosphoketolase, and can ferment pentoses. These bacteria are referred to as facultative heterofermentative (Kandler and Weiss, 1986). End-products of homofermentative lactic acid bacteria may be shifted in some strains when limited amounts of carbohydrates are present. Thomas et al. (1979) found, for example that homofermentation by Streptococcus (Lactococcus) lactis was shifted to heterofermentation when glucose was limited, and lactate formation was shifted to formate, acetate and ethanol.

Heterofermentative and facultative heterofermentative lactic acid bacteria can utilize an assortment of organic compounds for fermentation. Reduction of fructose to mannitol was mentioned above. McFeeters and Chen (1986) demonstrated that Lactobacillus plantarum can utilize mannitol anaerobically and utilize three types of compounds as electron acceptors: citric acid cycle intermediates, a-keto acids and intermediates found in the heterofermentative pathway. Although they lack cytochromes and catalase, most heterofermentative species contain flavoprotein oxidases and peroxidases and can utilize oxygen as an alternative electron acceptor resulting in the formation of hydrogen peroxide. Lactate can be oxidized to pyruvate and hydrogen peroxide by means of L-lactate oxidase or NAD-independent D-lactate dehydrogenase. Aerobic conversion of pyruvate into acetate and carbon dioxide with a yield of 1 mol ATP per mol pyruvate has been observed in Streptococcus faecalis and Lactobacillus plantarum (Kandler, 1983). Lactic acid bacteria are, as mentioned above, generally classified as catalase- and cytochrome-oxidative-phosphorylation negative, as they are incapable of synthesizing hemeporphyrins, which are essential components of cytochromes. According to Condon (1983), such a classification should not go unqualified as not all catalases are heme-proteins. Non-heme catalases, so-called pseudocatalases, have been observed in species of Streptococci, Leuconostoc, Pediococci and Lactobacilli. A few lactic acid bacteria are even able to form heme-catalases if grown in the presence of hematin. Cytochromes have also been observed in some strains grown in the presence of hematin (Condon, 1983).

Several metabolites of lactic acid bacteria are of practical interest. Production of diacetyl from citrate is, for example, essential in lactic acid bacteria used in the production of butter and cheddar cheese to obtain the typical taste of these products. Fermentation of malic acid to lactic acid, malo-lactic fermentation, is of great importance in controlling
the acidity and sensory characteristics of wines. Lactic acid bacteria produce a number of polysaccharides which are defined by their location related to the cell. Among polysaccharides, located outside the cell are dextrins, which are of great industrial and medical importance. Dextrins are, for example, used in filtration gels, Sephadex, and as binding substances in medical products.
ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA

Acids
Accumulation of organic acids is, as mentioned above, a characteristic of lactic acid fermentation. The main acids are usually lactic acid and acetic acid. The following three factors have been found important with regard to the preservation activity of acids: (i) reduction in pH, (ii) the extent of the dissociation of the acid and (iii) the specific effect of the acid molecule (Ingram et al., 1956). At constant pH, antimicrobial activity of acids consisting of more than four carbons generally increases with chain length (Woolford 1975). Acids with a chain length of above ten carbons are, however, not particularly effective because of their low solubility in water.

Accumulation of acids reduces the pH of the surroundings. A low pH can affect microorganisms in two ways: by prolonging their lag phase and by impairing their heat stability. The effect of undissociated acids is, however, probably the main cause of inhibition of microorganisms by organic acids. The antimicrobial activity of undissociated acids is attributed to their lipophilic character, which allows them to penetrate the microbial cell wall. Inside the cell, where the pH is higher, the acids dissociate and release protons and conjugate bases. This will destroy the membrane proton-motive force and thus impair ATP synthesis and transport depending on it (Freese et al., 1973; Adams and Hall, 1988). Antimicrobial activity of short carbon-chain acids will thus depend on their degree of dissociation (pKa) and the pH of the external medium, which in turn depends on its buffering capacity. Most short-chain organic acids have a pKa of between 3 and 5 and are therefore most effective at low pH values.

In a well buffered system with a moderately low pH (4-6) acetic acid with pKa 4.75 is more effective than lactic acid (pKa 3.86) because a greater portion of acetic acid is in an undissociated form. Adams and Hall (1989) found an apparently synergistic interaction between the two acids in weakly buffered media; this was ascribed to the potentiation of acetic acid in the more acidic environment created by the lactic acid.

Hydrogen peroxide
Many lactic acid bacteria can perform oxidation of various organic compounds by means
of flavin-containing enzymes, oxidases, peroxidases or NAD-independent dehydrogenases (Kandler, 1983).

All aerobic metabolism of microorganisms involves biochemical reactions which are a consequence of interactions between the microorganisms with oxygen, finally resulting in metabolites such as superoxide ion or hydrogen peroxide, which may be further converted to hydroxyl radicals and singlet oxygen. Hydrogen peroxide is, under normal aerobic conditions, the only one of the possible oxygen metabolites that is known to accumulate to any significant extent (Condon, 1983).

Several lactic acid bacteria have been reported to form hydrogen peroxide. Dahiya and Speck (1969) reported inhibition of *Staphylococcus aureus* by hydrogen peroxide formed by *Lactobacillus lactis* and *Lactobacillus bulgaricus*. Price and Lee (1970) reported that lactobacilli produced sufficient amounts of hydrogen peroxide to inhibit *Pseudomonas* species. Gilliland and Speck (1975) have reported that hydrogen peroxide production by lactobacilli was involved in causing inhibition of psychrotropic bacteria, and that hydrogen peroxide produced by *Lactobacillus acidophilus* was also involved in inhibiting growth of intestinal and food-borne pathogenic bacteria. Collins and Aramaki (1980) studied the hydrogen peroxide formation of strains of *Lactobacillus acidophilus* in milk and found that an impractically large number of the bacteria would be required for sufficient accumulation of hydrogen peroxide to be a factor in extending the shelf-life of milk.

There are indications that lactic acid bacteria respond to a sublethal concentrations of hydrogen peroxide by inducing a protective system which helps them survive normally lethal levels of the peroxide. Two groups of *N-streptococci* exposed to sublethal level (0.5mm) of hydrogen peroxide survived a challenge with a lethal concentration (5.5) at a substantially greater rate than cultures which were not first exposed to sublethal levels (Condon, 1987).

Inhibition by hydrogen peroxide has been explained by its strong oxidizing effect on the bacterial cell, and to the destruction of basic molecular structure of cell proteins (Lindgren and Dobrogosz, 1990).
Carbon dioxide

Carbon dioxide accumulates in fermented plant material by two means; endogenous respiration of the plant material and as a by-product of heterofermentation. Low concentrations of carbon dioxide can stimulate the growth of some organisms, while high concentrations will inhibit the growth of others. There are two ways in which carbon dioxide influences food preservation: it plays a role in creating an anaerobic environment by replacing molecular oxygen, and it has direct antimicrobial activity.

There have been two main theories to explain the antimicrobial activity of carbon dioxide: that carbon dioxide inhibits enzyme reactions necessary for growth (King and Nagel, 1975), and that the cell membrane is its main target. The latter supposes that carbon dioxide interacts with lipids of the cell membrane and decreases the permeability of ions (Sear and Eisenberg, 1961).

Eklund (1984) reported that the uptake of amino acids in membrane vesicles of *Escherichia coli* and *Bacillus subtilis* was not seriously inhibited by carbon dioxide at concentrations up to 80%. Growth of these bacteria, as well as *Bacillus cereus* and *Pseudomonas aeruginosa*, was inhibited to a greater extent than amino acid uptake. These results do not support the view that carbon dioxide influences membrane permeability.

Diacetyl

Strains within certain species from all genera of lactic acid bacteria can produce diacetyl from citrate and pyruvate. Diacetyl is best known as the component responsible for the aroma and flavour of butter.

Jay (1982) showed that 0.004M (350 ppm) of diacetyl will inhibit most organisms. It has greater antimicrobial activity against gram-negative bacteria and fungi than against gram-positive bacteria. Lactic acid bacteria and anaerobic bacteria are most resistant.

Diacetyl has a strong aroma and is a volatile component so that its suitability as a food additive is limited. Jay (1982) suggested that, because of its antimicrobial properties and volatility, diacetyl could be used as a microbial dip for utensils and surfaces.
Reuterin

Reuterin is a low-molecular weight, non-proteinous antimicrobial substance produced by *Lactobacillus reuteri*, which inhabits the gastrointestinal tract of humans and animals. Reuterin has been purified and identified as an equimolar mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde. It has a broad spectrum of antimicrobial activity against certain gram-positive- and gram-negative bacteria as well as yeasts, fungi and protozoa (Lindgren and Dobrogosz, 1990). Among pathogenic organisms inhibited by reuterin are species of *Salmonella*, *Shigella*, *Clostridium*, *Staphylococcus*, *Listeria*, *Candida* and *Trypanosoma*.

Bacteriocins

Bacteriocins are antimicrobial substances produced by a large assortment of bacterial species. The first such antimicrobial substance was described by Gratia in 1925. This substance was produced by a strain of *Escherichia coli* and showed inhibitory activity only against related species of *Escherichia coli*. After the discovery of more such substances produced by species of *Escherichia coli*, they were collectively called colicins. Later, when similar substances from other genera of bacteria had been observed, the name bacteriocins was suggested (Jacob et al., 1953).

Through the years there have been many reports of apparent bacteriocin activity. In many cases, this apparent bacteriocin activity has been due to other agents, including bacterial phages, lysozymes and other cellulolytic enzymes. Bacteriocins must therefore fulfil the following criteria used to define these substances:

1. A narrow inhibitory spectrum of activity centred around the homologous species.
2. The presence of an essential protein moiety.
3. A bactericidal mode of action.
4. Attachment to specific receptors.
5. Plasmid borne genetic determinants of bacteriocin production and of host cell bacteriocin immunity.
6. Production by lethal biosynthesis (i.e. commitment of the bacterium to produce a bacteriocin that will ultimately lead to cell death) (Tagg et al. 1976).
These criteria are generally applicable to the colicins while bacteriocins of gram-positive bacteria are more diverse. It is generally agreed that the first three points should be fulfilled for a substance to be called a bacteriocin. Some bacteriocins of lactic acid bacteria and other gram-positive bacteria, however, exhibit a broader spectrum of antimicrobial activity than stipulated in criterion 1.

The first report of an antimicrobial substance of lactic acid bacteria came when Roger (1928) reported antagonistic activity of *Streptococcus lactis* against *Lactobacillus bulgaricus*. Whitehead (1933) reported two strains of lactic *streptococci* isolated from milk, which produced substances with a marked inhibitory action on the growth of other streptococci. The substances from the two strains were apparently the same. By treating the substances with trypsin and pepsin he also determined that they were proteinous (polypeptides). The antagonistic agent observed by Roger (1928) was later studied by Mattich and Hirsch (1944; 1947), who found it to be a polypeptide. It was given the name nisin. The substance studied by Whitehead (1933) was further studied by Oxford (1944) and called diplococcin. It was confirmed that diplococcin was protein-like, of small molecular weight and contained no sulphur or phosphorus.

Diplococcin exhibited a narrow antimicrobial activity and was not effective against sporeformers. It powerfully inhibited closely related species, while other gram-positive cocci were inhibited to a lesser extent (Oxford, 1944).

When Davey and Richardson (1981) later examined 150 strains of *Streptococcus cremoris*, 11 were found to produce diplococcin. They purified the diplococcin produced by *Streptococcus cremoris* 346, and found it very unstable at room temperature and that a rapid loss of activity occurred upon heating it to 100°C. It was found sensitive to trypsin, pronase and a-chymotrypsin. The molecular weight of purified diplococcin was estimated as 5300 Da., and it did not contain lanthionine or b-methyl-lanthionine.

Davey (1984) found that the ability to produce diplococcin could be transferred by conjugation from *Streptococcus cremoris* 346 to two plasmid-free *Streptococcus cremoris* recipients. Diplococcin production coincided with the acquisition of a 54 Mda. plasmid in both recipients. Spontaneous loss of this plasmid restored the Dip- phenotype.
*Streptococcus cremoris* is a very important multi-strain starter culture in cheese-making. The diplococcin-producing strains in the multi-strain culture predominate over other lactic lactococci, which is a disadvantage. This was overcome by treating the cultures at elevated temperatures and thereby obtaining strains which had lost the bacteriocin-coding plasmid but keep all other properties intact (Davey and Pearce, 1980).

Nisin has a broader antimicrobial activity than diplococcin. It will inhibit growth of most gram-positive bacteria and inhibit outgrowth of spores. Because of the extensive studies carried out on nisin and because of its importance as a commercial food preservative a special section will be devoted to nisin (page 21).

Since the first reports on nisin and diplococcin were published bacteriocinogeny has been demonstrated within all genera of lactic acid bacteria. Table 1 shows many of the bacteriocins and bacteriocin-like substances which have been reported. Some of these substances have been purified and characterized, their molecular size, amino acid composition and sequence have been determined and genetic determinants localized. In other cases, the antimicrobial spectrum has been reported and a proteinous nature demonstrated. In some cases, the substances have not yet been sufficiently characterized to determine whether they are bacteriocins or something else.

Several bacteriocins or bacteriocin-like substances have been isolated from species of the genus *Lactobacillus*. Of these, seven have been well characterized. Five of these seven are heat-stable, one moderately heat-stable and one heat-labile. The molecular size ranges from 2500 Da. to 35000 Da. One, produced by *Lactobacillus fermenti*, is a protein containing lipocarbohydrates, another which is produced by *Lactobacillus helveticus* is a glycoprotein, while the others are simple proteins. In some cases, genetic determinants for bacteriocin production reside on plasmids, while in others, evidence for chromosomal determinants has been demonstrated. The antimicrobial activity spectrum varies from revolving around closely related species to activity against all genera of lactic acid bacteria (Barefoot and Klaenhammer, 1983; 1984; Muriana and Klaenhammer, 1987; 1991; DeKlerk and Smith, 1967; Upreti and Hindsill, 1973; 1975; Joerger and Klaenhammer, 1986; 1990; Daeschel et al., 1990; Mørtvedt and Nes, 1990; Mørtvedt et al., 1991). Some of these bacteriocins - or the producer-bacteria - have been tested and found effective against strains of Listeria monocytogenes, *Staphylococcus aureus*, spores
of *Clostridium botulinum* and the gram-negative bacteria *Aeromonas hydrophila* (Harris *et al.*, 1989; Okereke and Montville, 1991; Lewus *et al.*, 1991). Other bacteriocins or bacteriocin-like substances of Lactobacilli, as yet less well characterized, have been reported. In most cases, a proteinous nature has been demonstrated. The antimicrobial spectrum and heat stability also vary widely among these substances (for references see Table 1).

*Pediococci* are important in the fermentation of vegetables, and have also been used as starter cultures in the fermentation of meat and dairy products. It is therefore of importance to study bacteriocinogenesis among this genus. A few bacteriocins of pediococci have been reported. Only one has been purified and the molecular size has been determined as approximately 2700 Da. (Bhunia *et al.*, 1987; 1988). Another one has been partially purified (Gonzales and Kunka, 1983). Their antimicrobial spectrum varies from including numerous gram-positive bacteria, among them strains of *Listeria* monocytogenes, *Staphylococcus aureus* and spores of *Clostridium botulinum*, to being centred around closely related species (Pucci *et al.*, 1988; Harris *et al.*, 1989; Spelhaug and Harlander, 1989). The genetic determinants for bacteriocin production have been linked with plasmids in all cases (Gonzales and Kunka, 1983; Ray *et al.*, 1989; Hoover *et al.*, 1988; Daeschel and Klaenhammer, 1985; Graham and McKay, 1985).

There are some reports of bacteriocins produced by the genera *Leuconostoc* and *Carnobacterium*. So far, none of these have been well characterized apart from establishing a proteinous nature and in some cases, a broad antimicrobial spectrum against gram-positive bacteria (for references see Table 1).

Bacteriocins of *Lactococci* seem to fall into three categories: nisin or nisin-like bacteriocins, non-nisin-like bacteriocins and diplococcin or diplococcin-like bacteriocins. *Lactococci* are important as starter cultures in the dairy industry. More studies of the genetic and biochemical properties of their bacteriocins are therefore needed. Diplococcin was briefly discussed above (pages 15-16); nisin will be discussed below.
### Table 1. Bacteriocins and bacteriocin-like substances reported in the literature.

<table>
<thead>
<tr>
<th>PRODUCING ORGANISM</th>
<th>NAME</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus ssp. C-136</td>
<td>NN</td>
<td>Harris et al., 1989</td>
</tr>
<tr>
<td>and UAL36</td>
<td>NN</td>
<td>Ahn and Stiles, 1990b</td>
</tr>
<tr>
<td>Lactobacillus sp. 100-37</td>
<td>NN</td>
<td>McGormick and Savage, 1983</td>
</tr>
<tr>
<td>Lactobacillus acedophilus AC1</td>
<td>NN</td>
<td>Mehta et al., 1983</td>
</tr>
<tr>
<td>Lactobacillus acetophilus NCFM</td>
<td>NN</td>
<td>Ferreira and Gilliland, 1988</td>
</tr>
<tr>
<td>Lactobacillus acetophilus N2</td>
<td>Lactacin B</td>
<td>Barefoot and Klaenhammer, 1983; 1984</td>
</tr>
<tr>
<td>Lactobacillus acetophilus 88</td>
<td>Lactacin F</td>
<td>Muriana and Klaenhammer, 1987; 1991</td>
</tr>
<tr>
<td>Lactobacillus acidophilus LAPT 1060</td>
<td>Acidophilucin A</td>
<td>Toba et al., 1991a</td>
</tr>
<tr>
<td>Lactobacillus bavarius MN, and JX</td>
<td>NN</td>
<td>Lewus et al., 1991</td>
</tr>
<tr>
<td>Lactobacillus brevis B37</td>
<td>Brevicin 37</td>
<td>Rammelsberg and Radler 1990</td>
</tr>
<tr>
<td>Lactobacillus casei 880</td>
<td>Caseicin 80</td>
<td>Rammelsberg and Radler, 1990</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii JCM 1106 and JCM 1107</td>
<td>Lacticin A</td>
<td>Toba et al., 1991b</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii JCM 1248</td>
<td>Lacticin B</td>
<td>Toba et al., 1991b</td>
</tr>
<tr>
<td>Lactobacillus fermenti 466</td>
<td>NN</td>
<td>DeKlerk and Smith, 1967</td>
</tr>
<tr>
<td>Lactobacillus helveticus LP27</td>
<td>Lactacin 27</td>
<td>Upreti and Hindsill, 1973; 1975</td>
</tr>
<tr>
<td>Lactobacillus helveticus 481</td>
<td>Helveticin J</td>
<td>Joerg and Klaenhammer, 1986; 1991</td>
</tr>
<tr>
<td>Lactobacillus plantarum</td>
<td>NN</td>
<td>Schröder et al., 1980</td>
</tr>
<tr>
<td>Lactobacillus plantarum NDCO1193</td>
<td>Plantaricin B</td>
<td>West and Warner, 1988</td>
</tr>
<tr>
<td>Lactobacillus plantarum C-11</td>
<td>Plantaricin A</td>
<td>Daeschel et al., 1990</td>
</tr>
<tr>
<td>Lactobacillus plantarum LKE</td>
<td>NN</td>
<td>From, 1991</td>
</tr>
<tr>
<td>Lactobacillus plantarum BN</td>
<td>NN</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus plantarum UAL 59</td>
<td>NN</td>
<td>Ahn and Stiles, 1990b</td>
</tr>
<tr>
<td>Lactobacillus plantarum Lb 75 and Lb 529</td>
<td>NN</td>
<td>Schillinger and Lücke, 1989</td>
</tr>
<tr>
<td>Lactobacillus sake Lb706</td>
<td>Sakasin A</td>
<td>Schillinger and Lücke, 1989</td>
</tr>
<tr>
<td>Lactobacillus sake 45</td>
<td>Lactacin S</td>
<td>Mørdvedt and Nes, 1990</td>
</tr>
<tr>
<td>Lactobacillus viridescens PX and QX</td>
<td>NN</td>
<td>Lewus et al., 1991</td>
</tr>
<tr>
<td>Lactobacillus ssp. UAL11 and C-136</td>
<td>NN</td>
<td>Harris et al., 1989</td>
</tr>
<tr>
<td>Pediococcus acidilactici PAC-10</td>
<td>Pediocin PA-1</td>
<td>Gonzales and Kunka, 1983</td>
</tr>
<tr>
<td>PRODUCING ORGANISM</td>
<td>NAME</td>
<td>REFERENCES</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>---------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Pediococcus acidilactici</td>
<td>Pediocin A7H</td>
<td>Ray et al., 1989, Elswas et al., 1991</td>
</tr>
<tr>
<td>Pediococcus acidilactici PO2, B5627</td>
<td>NN</td>
<td>Hoover et al., 1988</td>
</tr>
<tr>
<td>and PC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pediococcus acidilactici E, F, and M</td>
<td>Pediocines</td>
<td>Ray et al., 1989</td>
</tr>
<tr>
<td>Pediococcus acidilactici</td>
<td>NN</td>
<td>Nielsen et al., 1990</td>
</tr>
<tr>
<td>Pediococcus pentosaceus MC-03</td>
<td>NN</td>
<td>Hoover et al., 1988</td>
</tr>
<tr>
<td>Pediococcus pentasaceus L7230</td>
<td>Pediocin A</td>
<td>Fleming et al., 1975</td>
</tr>
<tr>
<td>and FBB-61</td>
<td></td>
<td>Daeschel and Klaenhammer, 1985</td>
</tr>
<tr>
<td>Pediococcus cerevisiae FBB63</td>
<td>NN</td>
<td>Graham and McKay, 1985</td>
</tr>
<tr>
<td>Lactococcus lactis ssp. cremoris</td>
<td>Type I-VIII</td>
<td>Gais et al., 1983</td>
</tr>
<tr>
<td>.....&quot;.....&quot;.....&quot;....&quot;....&quot;actis</td>
<td>(not</td>
<td></td>
</tr>
<tr>
<td>&quot;.....&quot;.....&quot;....&quot;actis SIK-83</td>
<td>lactostrepsins</td>
<td></td>
</tr>
<tr>
<td>.....&quot;.....&quot;.....&quot;....&quot;actis LMG2130</td>
<td>Lactococcin A</td>
<td>Andersson, 1986, Andersson et al., 1988</td>
</tr>
<tr>
<td>Lactococcus lactis ssp. cremoris</td>
<td>Lactostrepsins</td>
<td>Hol et al., 1991</td>
</tr>
<tr>
<td>.....&quot;.....&quot;.....&quot;....&quot;actis</td>
<td>(there among</td>
<td>Kozak et al., 1978</td>
</tr>
<tr>
<td>.....&quot;.....&quot;.....&quot;....&quot;actis</td>
<td>Las5)</td>
<td>Bardowski et al., 1979</td>
</tr>
<tr>
<td>Lactococcus lactis ssp. lactis</td>
<td>Nisin</td>
<td>Mattich and Hirsch, 1947</td>
</tr>
<tr>
<td>Lactococcus lactis CNRZ 481</td>
<td>Lacticin 481</td>
<td>Plard et al., 1990</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
<td>NN</td>
<td>Carminati et al., 1989</td>
</tr>
<tr>
<td>Lactococcus lactis ssp. cremoris 346</td>
<td>Diplococcin</td>
<td>Oxford, 1944</td>
</tr>
<tr>
<td>Lactococcus lactis ssp. diacetylactis WM4</td>
<td>NN</td>
<td>Scherwitz et al., 1987</td>
</tr>
<tr>
<td>Lactococcus lactis ssp. diacetilactis S50</td>
<td>S50</td>
<td>Kojic et al., 1991</td>
</tr>
<tr>
<td>Leuconostoc PO18</td>
<td>NN</td>
<td>Orberg and Sandine, 1984</td>
</tr>
<tr>
<td>Leuconostoc UAL 14</td>
<td>NN</td>
<td>Harris et al., 1989</td>
</tr>
<tr>
<td>Leuconostoc gelidum IN139</td>
<td>NN</td>
<td>Harding and Shaw, 1990</td>
</tr>
<tr>
<td>Leuconostoc gelidum UAL 187</td>
<td>NN</td>
<td>Hastings and Stiles, 1991</td>
</tr>
<tr>
<td>Leuconostoc sp. VX and OX</td>
<td>NN</td>
<td>Lewus et al., 1991</td>
</tr>
<tr>
<td>Leuconostoc sp. UAL14 and UAL104</td>
<td>NN</td>
<td>Ahn and Stiles, 1990b</td>
</tr>
<tr>
<td>Carnobacterium piscicola UAL 26</td>
<td></td>
<td>Ahn and Stiles, 1990b</td>
</tr>
<tr>
<td>and UAL 89</td>
<td></td>
<td>Schillinger and Holzapfel, 1998</td>
</tr>
<tr>
<td>Carnobacterium sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carnobacterium piscicola GN</td>
<td>NN</td>
<td>Lewus et al., 1991</td>
</tr>
<tr>
<td>and DX</td>
<td></td>
<td>Ahn, C. and M. E. Stiles, 1990a</td>
</tr>
</tbody>
</table>

NN = Not named
NISIN

Nisin was, as stated above, found some decades ago. Because of its industrial application it is one of the most important bacteriocins. The first evaluation of nisin as a food preservative was made in Swiss cheese, where it effectively controlled blowing caused by growth of Clostridium (Hirsch et al., 1951). Further studies in food systems led to the commercial production of nisin by Aplin and Barret in the United Kingdom under the name of Nisaplin. The World Health Organization (WHO) accepted nisin in 1969 and its use is now allowed in 48 countries around the world.

The use of nisin is allowed mostly in cheese and processed cheese, pasteurized milk and long-life milk, clotted cream and canned vegetables. Its use has been tested in other food systems such as meat and sausages where it has not been very successful because of its interaction with meat particles and membrane phospholipids.

Furthermore its use has been tested in alcoholic beverages such as wines and beer, where it has been found effective in keeping unwanted lactic acid bacteria at bay, and in controlling malo-lactic fermentation (Ogden, 1986; Ogden and Tubb, 1985; Ogden and Waites, 1986; Ogden et al., 1988; Radler, 1990a; 1990b; Daeschel et al., 1991).

It was realized early on in the studies of nisin that there were several different polypeptides involved (Hirsch, 1951). By separating a batch of nisin by counter-current distribution, Berridge et al. (1952) identified five polypeptides with different partition coefficients. Amino acid analysis by paper chromatography showed a striking similarity between nisin A, B and C, but nisin D was slightly different. It did not seem to contain valine and methionine, present in the other three. It also seemed to have some of the sulphur-containing amino acids in an oxidized form. Nisin A and B, however, showed five times more antimicrobial activity against Streptococcus agalactiae than C and D. The complete structure of one of these peptides, nisin A, was elucidated by Gross and Morell (1971), and their proposal has been confirmed by recent NMR and FAB-MS studies (Slijper et al., 1989; Barber et al., 1988) and a recently published method on the synthesis of nisin (Fukase et al., 1988).
Nisin is a small peptide, consisting of 34 amino acid residues. The nisin molecule contains the \(\alpha,\beta\)-unsaturated amino acids dehydroalanine and dehydrobutyryl (\(\beta\)-methyldehydroalanine) (Gross and Morell, 1967; 1968), as well as the sulphur-containing lanthionine and \(\beta\)-methylthionine (Newton and Abraham, 1953; Gross and Morell, 1970). Amino acids are joined by sulphur atoms to form five internal cyclic structures, one ala-S-ala, which is lanthionine, and four ABA-S-ala, which are \(\beta\)-methylthionine (ABA being amino butyric acid) (Figure 4).

Chan et al. (1989) have isolated and characterized two natural degradation products from nisin found in commercial samples of nisin about three years after the expiry date. They found three identifiable components, nisin, nisin1-32, cleaved at residue 32, and (des-DAla5) nisin1-32, cleaved at residue 32 as well as at residue 5. They found that nisin1-32 was essentially as active as nisin against a number of gram-positive organisms, while the other, (des-DAla5) nisin1-32 was at least 500 times less active. By treating nisin in the same manner as Gross and Morell (1968), with hydrochloric acid in glacial acetic acid, they found that, under these conditions, the major degradation product is (des-DAla5) nisin1-32. Chan et al. (1989) suggest that the C-terminal residues DAla33 and Lys34 are not required for antimicrobial activity. They suggest that nisin A and nisin B, reported by Berridge et al., (1952) could be nisin and nisin1-32. They state further that it remains to be established whether the DAla5 residue per se is required for activity or whether the absence of activity of (des-DAla5) nisin1-32 is due to one ring structures being left open.

![Figure 4. The structure of nisin A.](image-url)
In spite of containing amino acids for which there is no genetic code, nisin is synthesized by the protein-synthesizing mechanism (Hurst, 1966; Ingram, 1969). Ingram put forward the hypothesis that a nisin precursor polypeptide chain, containing cysteine, serine and threonine, was ribosomally synthesized. Serine and threonine were dehydrated to dehydroalanine and dehydrobutyryl, respectively, which upon condensation with cysteine residues form lanthionine and b-methyllanthionine (Ingram, 1969; 1970). Such inactive precursor protein which was converted to nisin was observed by Hurst and Paterson (1971) who suggested that nisin was synthesized in at least two stages: synthesis of a precursor molecule by the accepted protein synthesis mechanism and posttranslational modifications with the involvement of enzyme(s) into the active nisin molecule. The gene which encodes for the precursor of nisin has been cloned and sequenced. The encoded precursor is 57 amino acid long, with a 23-residue leader region and a 34-residue structural region. The structural region contains serine, threonine and cysteine at the positions required to form nisin by a series of posttranslational modifications, involving dehydration of serine and threonine to dehydro forms and cross-linking with cysteine residues, as proposed by Ingram (Buchman et al. 1988).

By 1H-NMR studies of nisin, Slijper et al. (1989) found indications that nisin is not a random coil but is folded in a unique conformation with especially the N-terminal part well constrained. The side chain of proline (residue 9), which is part of the second ring, points in the direction of the first ring (formed residues 3 to7). The side chains of the methionines (residues 17 and 21) face each other.

It is interesting to note the structure of bacteriocins produced by other gram-positive bacteria, such as Bacillus subtilis and strains of Staphylococcus, which also contain lanthionines (Figure 5). Bacteriocins, or antibiotics, containing these amino-acids have commonly been called lanthibiotics, and it has been suggested that they are all derived from a common ancestor (Allgaier et al., 1985; Gross and Kiltz, 1973; Kellner et al., 1988; Schnell et al., 1988). These bacteria are phylogenetically related and belong, as mentioned earlier, to the Clostridium-Bacillus group.
Nisin exhibits antimicrobial activity against a limited range of microorganisms. It has, however, an antimicrobial effect against a wide range of gram-positive bacteria. It inhibits certain strains of *Staphylococcus* (Gowan *et al.*, 1954), *Streptococcus*, *Pediococcus*, *Micrococcus*, *Leuconostoc* and *Lactobacillus* (Ogden and Tubb, 1985; Radler, 1990a) as well as a majority of sporeforming species of *Clostridia* and *Bacillus* (Mattich and Hirsch, 1947). It has recently been shown to exert a significant inhibitory effect against strains of *Listeria monocytogenes* (Benkerroum and Sandine, 1988; Carminati *et al.*, 1989). Spelhaug and Harlander (1989) demonstrated slight inhibition by nisin produced by *Lactococcus lactis* 11454 against several gram-negative bacteria, including *Aeromonas hydrophila* E9071 and AH2, *Escherichia coli* 0157:H7 and *Virio parahaemolyticus* A865957. To determine whether this inhibitory effect was indeed due to nisin, they tested a non nisin-producing mutant of *Lactococcus lactis* 11454 against these organisms and found no zone of inhibition around the cured variant. They therefore used various proteases to determine whether the effect was due to nisin. The inhibitory effect was unaffected by trypsin, but eliminated by a-chymotrypsin and pronase. The authors suggested that *Lactococcus lactis* 11454 might produce two types of inhibitors: nisin and some other inhibitor which was pronase-sensitive and responsible for the effect on gram-negative organisms. Production of more than one bacteriocin by the same organism has been demonstrated previously (Holland and Roberts, 1964; Kozak *et al.*, 1978; van Belkum *et al.*, 1989).
It has long been known that some bacterial spores are sensitive to nisin. Activation of spores requires some sort of trigger, chemical or physical, and is reversible. Three stages of swelling have been recognized during the germination and outgrowth of spores: germination swelling, pre-emergent swelling and emergency and elongation (Hitchins et al., 1963). The germination swelling is irreversible. Nisin is believed to inhibit the germination process at the stage of pre-emergent swelling (Hitchins et al., 1963; Lipinska, 1977). Bacillus spores have been classified into two groups: the small-spored species, in which envelope rupture takes place by mechanical pressure (M-type) and large-spored species, in which outgrowth takes place by lysis mechanism (L-type) (Gould, 1964). The latter type is much more nisin-resistant. It was first thought that nisin increased the sensitivity of spores to heat, but it has now been demonstrated that heat-damaged spores are characterized by increased sensitivity to nisin (Heinemann et al., 1965). The M-type of spores is more sensitive to nisin than the L-type, but after heat damage the sensitivity of the L-type is considerably increased.

Hirsch and Grinstead (1954) reported that nisin is sporocidal for Clostridium butyricum but for other Clostridia that they tested. Scott and Taylor (1981) have demonstrated that nisin is effective in preventing the outgrowth of Clostridium botulinum types A, B, and E in bacterial media. Type E spores were most sensitive to nisin and type A least sensitive. The effectiveness of nisin in preventing outgrowth of Clostridium botulinum spores is increased by the following factors; lower pH, lower spore-loading, increased temperature and length of heat shocking.

The cytoplasmic membrane has been suggested as the primary target of nisin. An immediate cessation of biosynthesis has been observed on addition of nisin to sensitive cells (Sahl and Brandis, 1982). An efflux of low-molecular weight substances, such as amino acids and K+ ions, and an immediate collapse of the membrane potential of whole cell membranes after addition of nisin has been demonstrated. (Sahl and Brandis, 1983; Ruhr and Sahl, 1985). A similar effect has been observed for other lanthionibiotics (Kordel and Sahl, 1986). Further studies by Sahl et al. (1987) on the effect of nisin on bacterial membranes and artificial lipid bilayers indicated that nisin requires energized membranes to exert its disruptive effects. With intact cells, a threshold potential of -50 to -80 mv was required at pH 7.5, and at pH 5.5 a threshold potential of less than -50mv was sufficient. Single-channel recordings indicated that nisin formed pores, permeable to
hydrophilic solutes with a molecular weight of up to 500 Da. By macroscopic conductivity measurements, an indication was found that nisin acts in a voltage-dependent fashion. It has been suggested that dehydro residues play an important role in the mechanism of the antibiotic action of nisin by reacting with one or more nucleophils in a sensitive cellular target (Liu and Hansen, 1990).

Gross and Morell (1968) showed that nisin can be chemically degraded by the hydrolytic cleavage of the C-terminal residues dehydroalanine-lysine, resulting in the release of pyruvyllysine. They found that this made the nisin inactive. Nisin can also be inactivated by some digestive enzymes and by non-proteolytic enzymes from species of Bacillus cereus and Bacillus polymyxa (Jarvis, 1967). Jarvis and Farr (1971) partly purified a nisin-inactivating enzyme from Bacillus cereus and studied its substrate specificity. The enzyme inactivated nisin A, B, C, and E but not nisin D. They found indications that the C-terminal residues DAla33 and Lys34 were reduced to alanyllysine in enzymatically inactivated nisin. Their conclusion was that the enzyme, nisinase, was a dehydroalanin reductase.

Genetic determinants for nisin production and nisin resistance, which are linked with sucrose metabolism, have been demonstrated both plasmid borne (Kaletta and Entian, 1989) and chromosomal (Buchman et al., 1988; Dodd et al., 1990). As has been suggested for other bacteria (Klaenhammer, 1988), it is possible that in some nisin-producing bacteria plasmids carrying the genes for nisin-production and nisin-resistance may have been incorporated into the chromosome.

Many researchers have studied the possible toxicity of nisin. Both acute toxicity and long-term effects of feeding overdoses to many generations of rats and mice have been studied. No indications of toxicity have been observed (Hara, 1962; Frazer, 1962). As nisin is a polypeptide and, like the bacteriocins discussed above, sensitive to proteolytic enzymes found in the digestive tract, any residues in the food would be degraded by digestive enzymes.

Procedures for the purification of nisin have been published. In the commercial production, nisin is produced by fermentation of Lactococcus lactis in non-fat milk medium. The nisin is then concentrated, separated, spray-dried and milled into fine
particles and stabilized with sodium chloride.

In 1957 Cheeseman and Berridge published a procedure for preparing nisin. The method consisted of extracting nisin with n-propanol containing NaCl. This was followed by a number of fractionation precipitations and further purification of concentrated material by counter-current chromatography. Gross and Morell (1967) seem to have used a similar method in their work on the structure of nisin.

A method published by Baily and Hurst (1971) consisted of breaking cells mechanically followed by extraction with cold HCl. Cl-ions were exchanged for acetate on Amberlite resin column and the eluate precipitated with acetone at -19°C. This was followed by a pH gradient chromatography on CM-cellulose. Several peaks were obtained, three of which contained antimicrobial activity. Wilimowska-Pelc et al. (1976) described a method for purification of commercial nisin. They fractionated nisin on columns of BioGel P-10. This was followed by ion-exchange chromatography on CM-Sephadex G-25. The activity of purified material increased 40-fold. This material gave three bands in electrophoresis.
PART II. EXPERIMENTAL WORK

BACKGROUND TO THIS STUDY

The lactic acid bacteria SIK-83 was isolated from lactic acid fermented carrots. Initially, the bacteria was identified as *Lactobacillus plantarum* and the bacteriocin designated plantarincin SIK-83 (Andersson *et al.*, 1988). On further analysis it was found that the bacteria were not *Lactobacillus plantarum* but *Lactococcus lactis* ssp. *lactis*.

The bacteriocin of these bacteria exhibits an inhibitory spectrum against a number of gram-positive bacteria including strains of *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. It has been shown to prolong the log phase of *Staphylococcus aureus* and inhibit spheroplasts of the gram-negative bacteria *Erwinia carotovora* and *Pseudomonas aeruginosa*. The crude bacteriocin is heat-stable, can be boiled for 2 hours or autoclaved at 121°C for 15 minutes. However, when partly purified, it was inactivated by autoclaving. The inhibitory activity against *Staphylococcus aureus* was affected by treatment with trypsin and pepsin. It was found that *Staphylococcus aureus* could adapt to the bacteriocin. The killing kinetics of the bacteriocin was of first order. A linear correlation was found when killing rates were plotted against concentration of bacteriocin.

The bacteriocin bound to sensitive cells, but binding to resistant gram-positive cells or gram-negative cells was not observed. If cells to which bacteriocin bound were treated with protease, they could be rescued. Pretreating sensitive cells with heat or protease did not affect the binding of the bacteriocin, indicating that binding sides were not proteinous. Binding to sensitive cells was accompanied by leakage of material which absorbs ultraviolet light at 260 nm and followed by cell lysis (Andersson, 1986; Andersson *et al.*, 1988). In a footnote to their report in 1988 Andersson *et al.* state that the bacteriocin produced by *Lactococcus lactis* ssp. *lactis* SIK-83 might be similar to nisin.

Andersson (1986) reported that the activity of SIK-83 bacteriocin was affected by trypsin and pepsin. Nisin has been shown to be resistant to both these proteolytic enzymes (Jarvis and Maloney, 1969; Hurst, 1981; Spelhaug and Harlander, 1989). Furthermore, small discrepancies in antimicrobial activity have been observed between nisin and the SIK-83 bacteriocin. These discrepancies indicated that, although the bacteriocin could well be nisin it was not necessarily nisin A.
OBJECTIVE

It has been reported that the bacteriocin produced by *Lactococcus lactis* ssp. *lactis* SIK-83 might be similar to nisin (Andersson *et al.*, 1988, footnote). As discussed above nisin can be of different varieties; nisin A, B, C and D. The aim of this study was to establish the definite identity of the SIK-83 bacteriocin. In order to do this a purification procedure had to be developed.

DETECTION OF BACTERIOCIN ACTIVITY

Many methods of detection of bacteriocins have been described. The most usual techniques are all derived from those of Gratia and Fredericq (1946). They are based on the ability of bacteriocins to diffuse in solid and semisolid culture media. When the bacteriocin has diffused, the bacteria has to be killed with chloroform vapours before the media is inoculated with a suitable indicator strain.

There are many modifications of this basic method. Kékassy and Piguet (1970) described a method which did not require killing the producer organism with chloroform vapours. Instead, the agar was loosened from the edge of the petri dish, the dish inverted and the agar made to fall into the lid, so that the producer bacteria were at the bottom of the agar instead of on top. The indicator strain is then inoculated. This is a widely used method. Tagg and McGiven (1971) described a modification, in which holes are punched into the agar in a petri dish and the bottom sealed with a drop of melted agar. Then standardized amounts of bacteriocin preparations are added to the wells and the bacteriocin allowed to diffuse into the agar. The agar is then treated as described in the method of Kékassy and Piguet. A modification of this method is to seal the wells with soft agar after diffusion of the bacteriocin, and then to overlay the plates with soft agar containing the indicator bacteria (Andersson *et al.*, 1988).
Figure 6 shows the clear zones around holes filled with bacteriocin.

Throughout this study, qualitative bacteriocin activity was determined by an overlay assay. Holes were punched into agar plates and the bottom sealed with soft agar (0.7% agar). 100 ml of bacteriocin-containing culture supernatants were applied to the holes and the bacteriocin allowed to diffuse into the agar. The dry holes were filled with soft agar and overlayed with melted soft agar (45°C) containing 20 ml of fresh culture of the indicator bacteria. The plates were incubated overnight and examined for clear zones.

Bacteriocin activity was quantified in a microtiter plate assay system, as described by Mørtvedt and Nes (1990). Twofold dilutions of bacteriocin extracts (100 ml) in nutrient broth were prepared in microtiter plates. 150 ml of fresh indicator culture adjusted with nutrient broth to optical density of 0.1-0.4 at 600 nm were added and the plates incubated for three hours at 30°C. Inhibition of the growth of the indicator bacteria was measured spectrophotometrically at 600 nm by using a Titertek Multiscan. An arbitrary unit (Au) was defined as the amount of bacteriocin which inhibited the growth of the indicator bacteria by 50% (had 50% of the turbidity of the indicator culture without bacteriocin under standard assay conditions).
FIRST ATTEMPTS AT PURIFICATION

Initially, the SIK-83 strain was cultivated in MRS broth. First attempts at purification by ammonium sulphate precipitation and column chromatography with Sephadex G-100 were unsatisfactory. A very small bottom pellet was obtained on ammonium sulphate precipitation; the bacteriocin stuck to the walls of the vessels used and floated on the surface. As a consequence quite a lot of the precipitated material was lost during this step. The material which was retained was dissolved in phosphate or citrate buffer and applied to a Sephadex G-100 column. Two peaks were obtained but no antimicrobial activity was found in any of the eluted fractions. Sephadex G-25 was then tried, but the results were similar. It was speculated that the bacteriocin had been only partly eluted with contaminating proteins from the MRS medium but in amounts too small to be detected. Because of the hydrophobicity, the remains were thought to have bound to the Sephadex gel. In an attempt to overcome the hydrophobic behaviour of the bacteriocin and prevent losses of bacteriocin activity, Tween 80 was added to the eluting buffer. Activity was, however, not recovered. The bacteriocin was then incubated with Sephadex G-100 and G-25 gel in phosphate buffer. Within 15 minutes, all activity was lost and it was concluded that it had bound so tightly to the Sephadex gel that it was difficult or impossible to get it off.

An attempt was made to precipitate the bacteriocin with organic solvents. Acetone, butanol and chloroform were tested in turn. By lowering pH with HCl, acetone precipitation at -20°C resulted in crystallization. Little activity was, however, recovered. Precipitation with butanol also resulted in minimal recovery of bacteriocin activity. This may have been due to intermolecular aggregation. When chloroform was used bacteriocin activity was found in the precipitate. 5% chloroform was added to the cell-free MRS supernatant which contained the bacteriocin. The mixture was shaken and left at 4°C overnight. It was then centrifuged at 14000 rpm (GS34 rotor, Sorvall centrifuge) for 15 minutes. A slurry was obtained as a bottom pellet. This was washed with water saturated with chloroform after which the chloroform was then removed in a rotary evaporator (35°C). The bactericidal activity of chloroform-precipitated pellet was considerably greater than after ammonium sulphate precipitation or >32000 Au/ml. No activity was observed by adding chloroform or water-chloroform mixture to microtiter plates. The slurried pellet was dissolved in phosphate buffer (20mM, pH 6.0) and applied to Sephadex G-25 column. Two peaks were obtained, one of which contained the
bacteriocin activity. There was, however, very little absorption of ultraviolet light at 280 nm, at which wavelength the chromatography was monitored. Measurements at 210 nm gave a much greater response indicating either the absence of proteins or absence of aromatic amino acids.

By ultrafiltration of eluted material with CentriconTM micro-concentrators (Amicon), activity was found in the retentate when membranes with a molecular cut-off of 3000 Da. were used but not when membranes with a molecular cut-off of 10000 Da. were used. The molecular size was therefore estimated to be between 3000 and 10000 Da.

Due to lack of facilities for removing chloroform residues from large batch preparations of bacteriocin, this method was not further pursued.

It was speculated that the MRS-broth in which the bacteria were cultivated might contain some component(s) which aggregated with the bacteriocin, making the aggregate hydrophobic and non-active. An attempt was therefore made to cultivate the bacteria in a medium containing fewer components than the MRS-broth. For this purpose, M-17 medium was chosen. In spite of its simpler composition, this medium will support growth of lactic acid bacteria and bacteriocin production. The M-17 was supplemented with 0.5% glucose or 0.5% lactose respectively. The bacteria grew equally well in presence of both carbohydrates and produced bacteriocin freely in both. Glucose was chosen for further work. The M-17 medium supplemented with glucose will hereafter be referred to as GM-17. Ammonium sulphate precipitation was more successful when this medium was used than when the bacteria had been cultivated in MRS-broth.

Before the final purification procedure was decided some pretrials were carried out. In order to report the purification procedure clearly the pretrials, that lead to this particular procedure being chosen, will be described and discussed separately. Thereafter the final purification procedure will be described.

**PURIFICATION - PRETRIALS**

**Bacteriocin production**

One of the differences between MRS and GM-17 broths is that MRS broth contains
Tween 80. The effect of Tween 80 upon bacteriocin production and detection of bacteriocin activity was tested. SIK-83 was grown in GM-17 broth and aliquots were taken at an optical density of 0.55, 0.85 and >1.0 and bacteriocin activity was quantified by the critical dilution method. *Pediococcus pentosaceus* LA 61 was used as an indicator bacteria. Figure 7 shows that the presence of Tween 80 results in the detection of somewhat more bacteriocin activity in the exponential phase of bacterial growth. When the stationary phase is reached and bacteriocin activity is maximal, however, there is no detectable difference.

![Bar graph](image)

**Figure 7.** Bacteriocin production in GM-17 with and without Tween-80.

**Bacteriocin assay**

Qualitative and quantitative bacteriocin assay was carried out as earlier described.

**Ammonium sulphate precipitation in the presence and absence of Tween 80**

To test the most effective ammonium sulphate precipitation both in the presence and absence of Tween 80, two batches of the SIK-83 bacteria were grown overnight at 30°C in GM-17 broth with and without the addition of 0.1% Tween 80. After the cells were removed by centrifugation 20, 30 and 40% ammonium sulphate (wt/vol) was added to aliquots of both batches. The ammonium sulphate was dissolved and the mixtures were allowed to stand at 4°C for 1 hour and then centrifuged at 8000 rpm. (Beckman centrifuge, rotor J10) The pellets were dissolved in phosphate buffer (20 mM, pH 6.0) and bacteriocin activity quantified using *Lactococcus lactis* ssp. *lactis* IL 1403 and *Pentococcus pentosaceus* LA 61 respectively as indicator bacteria. Figures 8 and 9 show
that maximal bactericidal activity was obtained at precipitation with 30% ammonium sulphate, both in the presence and absence of Tween 80. Bactericidal activity was, however, considerably greater in the presence of Tween 80. Figures 8 and 9 also show the difference in sensitivity of the indicator bacteria.

Figure 8. Bacteriocin activity recovered from precipitation with different concentrations of ammonium sulphate. Indicator bacteria is *Pediococcus pentosaceus* LA 61.

Figure 9. Bacteriocin activity recovered from precipitation with different concentrations of ammonium sulphate. Indicator bacteria is *Lactococcus lactis* IL 1403.
Column chromatography
To test the best binding of concentrated bacteriocin from ammonium sulphate precipitation to a cation exchange column, three Econo columns (Biorad), 2.5x20 cm, were packed to a column height of 2.5 cm with S-Sepharose and equilibrated with 20 mM phosphate buffer at pH 6.0, 7.0 and 8.0, respectively. 3x70 ml of bacteriocin concentrated by ammonium sulphate precipitation were adjusted to pH 6.0, 7.0 and 8.0, respectively, and applied to the appropriate column. After the columns were washed with the appropriate buffer, bound material was eluted with 20 ml of 1M NaCl. The procedure was repeated with bacteriocin produced in the presence of 0.1% Tween 80. As Figure 10 shows, the best binding was obtained at pH 6.0, both in the presence and absence of Tween 80. The figure shows furthermore that the bacteriocin bound considerably better to the cation exchange column at all three pH values tested in the absence of Tween 80.

![Figure 10. Binding to S-Sepharose cation exchange column at different pH values in the presence and absence of Tween 80.](image)

Before the next column to be used in the purification procedure was choses, the degree of hydrophobicity of the bacteriocin was assessed by packing small columns with Phenyl Superose and Octyl Superose, respectively. The material obtained from the cation exchange was applied to these columns and eluted with water, 50% ethanol or 95% ethanol and tested for antimicrobial activity. The best results, as seen in Figure 11, were obtained by using Phenyl Superose and eluting the bound material with water. The second best results were obtained by using Octyl Superose eluting with 95% ethanol.
Figure 11. Binding of bacteriocin concentrated by cation exchange was tested by Phenyl-Superose and Octyl-Superose hydrophobic gel chromatography. Bound material was eluted with water, 50% ethanol and 95% ethanol respectively.

To test which reversed phase column would be most suitable for the final purification step, the material obtained from Phenyl Superose column was applied to Bond Elut C2, and C18 columns and bactericidal activity eluted with methanol, ethanol and 2-propanol, respectively. Eluted fractions were assessed for bacteriocin activity. It was found that the bacteriocin was retained better on the C2 than on the C18 column, and that the recovery was better when it was eluted with either ethanol or 2-propanol compared with methanol.

From the pretrials it was concluded that 30% (w/vol) ammonium sulphate was optimal for precipitation of this bacteriocin. The higher activity of bacteriocin in culture supernatants and after precipitation in the presence of Tween-80 was attributed to the capacity of the detergent to prevent inter- or intramolecular aggregation of bacteriocin molecules which would result in non-detectability of the bacteriocin in spite of its presence.

Figures 8 and 9 show that the indicator bacteria *Lactococcus lactis* IL 1403 were much more sensitive to the bacteriocin than *Pediococcus pentosaceus* LA 61. As the *Lactococcus lactis* strain IL 1403 also grew faster than the *Pediococci* under the conditions used, making it possible to limit the incubation time to 3 hours, it was decided to use the Lactococci IL 1403 as indicator bacteria in further experiments.
Figure 10 shows that very little bacteriocin activity was recovered from the cation exchange column when Tween-80 was present. Figure 11 shows, furthermore, that although less bacteriocin activity was detected after ammonium sulphate precipitation in the absence of Tween-80, it was restored after cation exchange column chromatography. This further indicates inter- or intra-molecular binding of bacteriocin molecules, which rendered them inactive in the absence of Tween-80. It also shows that Tween-80 destroys the ionic-nature of the bacteriocin molecules so that they do not bind to the ion-exchange column. On the basis of this information, it was decided to carry out large batch preparations without the detergent.

The pretrials also showed that, although this bacteriocin seemed hydrophobic in nature relatively mild conditions of hydrophobic chromatography gave the best results. It bound better to Phenyl Superose than to Octyl Superose and could be eluted from the Phenyl Superose column with water. It also bound better to carbon columns with a short chain length. Conditions in large batch preparations were chosen accordingly.

All column materials used in this study were supplied by Pharmacia.

**Plasmid profile**

Because of the stable bacteriocin production of *Lactococcus lactis* SIK-83, it was decided to determine the plasmid profile of the bacteria and compare it with the plasmid profile of a variant of the bacteria which had previously been cured of bacteriocin production (Bac-).

Plasmid DNA from *Lactococcus lactis* SIK-83 Bac+ and Bac- was isolated by the alkaline lysis procedure (Maniatis et al., 1982) adding 1/20 vol. 20 mg/ml lysozyme and 1/20 vol. 5000 units/ml mutanolysis to the lysis buffer. DNA was analysed by agarose gel electrophoresis (0.8%). The gel was stained with ethium bromide and destained in distilled water. Figure 12 shows the plasmide profile of *Lactococcus lactis* SIK-83 Bac+ and Bac-. The profiles were identical for both bacteria and only one possible plasmid band appeared. This indicates that no plasmids are involved in bacteriocin production.
Figure 12. Plasmid profile of SIK-83 Bac- and Bac+
FINAL PURIFICATION AND CHARACTERIZATION OF SIK-83 BACTERIOCIN

EXPERIMENTAL PROCEDURE

Cultures and media
Two strains of *Lactococcus lactis*, the bacteriocin-producing strain, SIK-83, and *Lactococcus lactis* ssp. *lactis* IL1403, an indicator strain, were used in the study. The SIK-83 strain was maintained on MRS-agar and the IL 1403 on GM-17 agar. Both bacterial strains were reinoculated biweekly. The liquid medium M-17 broth (Difco) supplemented with 0.5% glucose (GM-17) was used. The SIK-83 was propagated in GM-17 broth overnight before being grown overnight in two litres of GM-17 broth.

Bacteriocin activity
Bacteriocin activity was quantified as earlier described.

Ammonium sulphate precipitation
Two litres of GM-17 were inoculated with SIK-83 and incubated overnight at 30°C. Cells were removed by centrifugation for 15 minutes at 6000 rpm (Beckman centrifuge, J10 rotor). Ammonium sulphate was added to a final concentration of 30% (wt/vol) and dissolved by stirring slowly. The mixture was kept at 4°C for 60 minutes. After centrifugation for 20 minutes at 8000 rpm. The supernatant was tested for bacteriocin activity and then discarded. The pellet was dissolved in 900 ml of 20mM phosphate buffer, pH 6.0, and the solution was tested for bacteriocin activity.

Cation exchange chromatography
The bacteriocin, 900 ml, concentrated by ammonium sulphate precipitation, was applied to a cation exchange column, packed with S-Sepharose. equilibrated with 20mM phosphate buffer at pH 6.0. Bound material was eluted with 30 ml of 1M NaCl and assayed for bacteriocin activity.

Hydrophobic and reversed-phase chromatography
10% ammonium sulphate (wt/vol) was added to the bacteriocin-containing fraction eluted from the cation exchange column and the fraction was applied to a prepacked
Phenyl Superose HR5/5 column (Pharmacia). The column was equilibrated with 20 mM phosphate buffer at pH 6.0 to which a final concentration of 10% ammonium sulphate had been added. The bacteriocin activity was eluted by gradually decreasing the salt concentration by exchanging the high salt buffer with water over a 30-minute period. The flow rate was 1 ml/min. Fractions of 2 ml were collected and tested for bacteriocin activity. The bacteriocin-containing fractions were pooled and applied to C1/C8 ProRPC 5/2 reversed phase column (Pharmacia) equilibrated with 10mM phosphate buffer at pH 5.6. The flow rate was 1ml/min, and bacteriocin activity was eluted with a linear gradient of 2-propanol. 1.5 ml fractions were collected and tested for bacteriocin activity. Phenyl Superose and reversed-phase chromatography were performed using FPLC system (Pharmacia).

**Protein measurements**

Optical density was measured at 214 and 280 nm. The method of Lowry et al. (1951) using BSA protein standard (Sigma) was also applied.

**Amino acid composition analysis**

Standard amino acid analysis and sequencing were carried out at Biochemical Institute at the University of Oslo.
Figure 13. Flowchart showing the purification process.
RESULTS AND DISCUSSION

Bacteriocin production
As shown in Figure 7, bacteriocin production was maximal at an optical density of >1, or after overnight growth, indicating that the bacteriocin was produced extracellularly during late log phase and stationary phase.

Ammonium sulphate precipitation
Only 52% of the bacteriocin activity found in culture supernatant was recovered after ammonium sulphate precipitation. As seen in Figure 7, considerably more activity was recovered when bacteria were grown in the presence of Tween 80. This could be explained by aggregation of bacteriocin molecules to contaminating proteins precipitated by ammonium sulphate, or to aggregation between bacteriocin molecules.

Cation exchange chromatography
Cation exchange chromatography on S-Sepharose with the column equilibrated at pH 6.0 resulted in a higher recovery of bacteriocin activity than was obtained after ammonium sulphate precipitation. This indicates that bacteriocin molecules were present in the pellet obtained by ammonium sulphate precipitation but that they were in an inactive state. A 2185-fold purification of the bacteriocin was obtained by cation exchange (Table 2).

Phenyl-Superose hydrophobic column chromatography
From the Phenyl Superose HR 5/2 column, a peak consisting of three 2-ml fractions was eluted between 40% and 50% water in the high salt (10% ammonium sulphate) phosphate buffer (20mM, pH 6.0) and water gradient. Each of the three fractions had a specific activity of around 11000 Au/ml and recovery of the fractions totalled 29%. When pooled they showed a specific activity of 11904 Au/ml, which is a recovery of 30%.
Figure 14. Hydrophobic column chromatography using a prepacked Phenyl Superose column connected to an FPLC system. A gradient of 20 mM phosphate buffer (pH 6.0) supplemented with 10% ammonium sulphate and water was used. Optical density was measured at 214 nm.

**Reversed-phase chromatography**

From the Pro-RPC HR5/2 reversed phase column, one peak, consisting of three 1.5 ml fractions, was eluted at 70% 2-propanol in a gradient system of 10 mM phosphate buffer: 2-propanol (Figure 15). The middle fraction of the three had a specific activity of 24038 Au/ml, while each of the two side fractions had a specific activity of only 4860 Au/ml. When 0.1% Tween-80 was added to the three fractions, respectively, the specific activity of the two side fractions was increased to 30000 Au/ml, or by a factor of 6, while the middle fraction had unchanged specific activity. The different specific activity of the three fractions and the fact that adding Tween-80 could restore activity to the less active fractions could indicate aggregation of bacteriocin molecules. The two side fractions did not, however, co-elute, which indicates a difference between them. The possibility that there is more than one bacteriocin present was considered and with that in mind the three fractions were vacuum centrifuged separately. The middle fraction was taken for amino acid analysis and the other two frozen and kept at -80°C.
Figure 15. Pro-RPC reversed-phase column chromatography of SIK-83 bacteriocin. 6 ml of SIK-83 bacteriocin (11940 Au/ml) obtained from Phenyl Superose hydrophobic column were applied to the Pro-RPC column, equilibrated with 10 mM phosphate buffer, pH 5.6. A 30 minute gradient of the same buffer and 2-propanol was used for elution. Absorption was measured at 214 nm. 1.5 ml fractions were collected and assayed for bacteriocin activity.

Protein measurements

After cation exchange, the absorption at 280 nm was minimal. Protein measurements by the method of Lowry and other methods based on dye binding to aromatic amino acids were unsuccessful, indicating that there are no aromatic amino acids in the bacteriocin. Optical density was therefore measured at 214 nm, and calculations of specific activity are based on those measurements. Absorption of the surrounding medium at each step was also determined and subtracted. Absorption of 7 different concentrations (between 50 mg/ml and 1 mg/ml) of commercial nisin dissolved in phosphate buffer (20mM, pH 5.6) was measured. Figure 16 shows that there is a linear relationship between concentration and absorption at 214 nm. As many compounds absorb ultra violet light at this wavelength, it was felt that it could not be stated with any certainty that the absorption was only due to protein. Specific activity is therefore expressed as arbitrary units divided by volume divided by optical density at 214 nm (AU/ml/A214). Because of the linear relationship between concentration of commercial nisin and absorption at 214 nm, expressing the specific activity as Au/mg protein would give the same relative results for recovery and the degree of purification.
Figure 16. Linear relationship between concentration of commercial nisin and absorption at 214 nm. The regression coefficient is 0.99.

Table 2. Purification of SIK-83 bacteriocin

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Volume ml</th>
<th>Optical density 214 nm</th>
<th>Total activity</th>
<th>Specific activity AU/ml/A214</th>
<th>Recovery %</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>2000</td>
<td>225.3</td>
<td>1000000</td>
<td>2.2</td>
<td>100</td>
<td>47</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>950</td>
<td>5.3</td>
<td>522500</td>
<td>104</td>
<td>52</td>
<td>2185</td>
</tr>
<tr>
<td>Cation exchange</td>
<td>30</td>
<td>5.2</td>
<td>750000</td>
<td>4808</td>
<td>75</td>
<td>5411</td>
</tr>
<tr>
<td>Phenyl superose column</td>
<td>6</td>
<td>4.2</td>
<td>300000</td>
<td>11905</td>
<td>30</td>
<td>10926</td>
</tr>
<tr>
<td>Pro-RPC reversed phase column</td>
<td>1.5</td>
<td>5.2</td>
<td>187500</td>
<td>24038</td>
<td>18.8</td>
<td></td>
</tr>
</tbody>
</table>

Amino acid analysis and gene sequencing

Results from amino acid composition analysis carried out on the middle fraction from the peak obtained from the Pro-RPC column, with a specific activity of 24038 Au/ml confirmed the absence of aromatic amino acids. Peaks consistent with lanthionines were observed. Amino acids were not detected upon direct N-terminal amino acid sequencing, indicating that the N-terminal was blocked.
Because of the probability that the bacteriocin was a variety of nisin it was decided to try to sequence the gene for bacteriocin production. This work was carried out together with the Laboratory for Microbial Genetics at Ås in Norway. Two primers were made for PCR amplification of chromosomal DNA from SIK-83. The primers were chosen from the published sequence of the precursor nisin A structural gene (Dodd et al., 1990). They were a 28-mer upstream and a 30-mer downstream of the gene, both including a Clal site. The PCR reaction was run for 35 cycles. The same primers were used for sequencing the amplified gene from SIK-83. A total of 225 bases were sequenced and translated using the Universal genetic code. The sequence obtained was almost identical to that of precursor nisin A, found in open reading frame 2 in the publication of Dodd et al. (1990). The only exception was that one base, C in nisin A, was exchanged for A in the SIK-83 bacteriocin. This point mutation results in a change of one amino acid; histidine (residue 27) in nisin A has been exchanged for asparagine in the SIK-83 bacteriocin. This variation of a nisin polypeptide is designated nisin Z (see Appendix).

FUTURE ASPECTS

In recent years, interest in bacteriocinogeny in lactic acid bacteria has grown considerably. This increased interest can, to a large extent, be explained by growing concern over health aspects of some food preservatives now used, and to the desire to find biologically produced alternatives.

There are two ways in which bacteriocins can be introduced into food systems: the producer bacteria can be inoculated into the food or the bacteriocin can be isolated and added as a chemical. Either way, knowledge of the chemical and physical properties of bacteriocins, their antimicrobial spectrum and genetic determinants is a basic requirement.

Lactic acid bacteria have been used for food fermentation for centuries. The process has been considered safe because of the lack of any evidence to the contrary.

Heineman et al. (1965) listed the requirements that compounds used as an aid to heat sterilization should fulfil. These include that their toxicology should be acceptable by recognized authorities; they should be sufficiently effective at relatively low
concentrations; they must not have any deleterious effect on the properties of the food in which they are used; they must be economically acceptable to industry; they should be stable during storage and should not have any medical use. Similar requirements are appropriate for biologically produced food preservatives.

The one bacteriocin, nisin, which has been internationally accepted as a food preservative fulfils all the above requirements. Nisin is mainly used in dairy products and canned foods. There are, however, new potential fields for its use opening up. The use of nisin in meats has not been successful because of its poor solubility at the pH of meats and because of its binding to phospholipid molecules. Furthermore, it is not effective against all spoilage organisms or pathogens associated with meat. It can, however, be used in conjunction with nitrates, thus decreasing the levels of nitrates necessary for preservative effect.

Studies of the use of other bacteriocins as food preservatives are still in their infancy. Such studies have, to a large extent, revolved around the use of bacteriocins for the preservation of meat. Refrigeration retards the outgrowth of spoilage bacteria in meat, but eventually psychrotropic spoilage bacteria will grow out and cause spoilage. With the increase in vacuum and modified atmosphere-packaging of meat, there is potential danger of the outgrowth of psychrotropic, anaerobic pathogens. Some bacteriocinogenic *Pediococci* have been shown to have a potential use in fresh and vacuum-packed meat (Nielsen *et al.*, 1990; Yousef *et al.*, 1991), but much research is needed before use of lactic acid bacteria as meat preservatives can be commercialized.

There is a growing interest in the ability of bacteriocins of lactic acid bacteria to inhibit the outgrowth of *Listeria monocytogenes*. Outbreaks of listeriosis have mainly been associated with raw milk or milk products, but vegetables and meats have also been involved. Many bacteriocins have been demonstrated to inhibit the growth of *Listeria* under laboratory conditions. Further studies in food systems are required.

When a starter culture is inoculated it is regarded as part of the raw material. The addition of bacteriocins as chemical substances, on the other hand, needs permission from the appropriate authorities, and before such permissions are forthcoming extensive tests have to be carried out. Such a process is both time-consuming and expensive.
Improving the bacteriocin production of a starter culture is therefore the more promising way. Knowledge of the genetics of lactic acid bacteria will provide the means. The use of genetic methods for the improvement and construction of bacteriocin-producing lactic acid bacteria has great potential. There are possibilities, by molecular cloning and manipulation of genes, of improving bacteriocin production and of expanding the inhibitory activity of industrial strains.

In conclusion it can be said that although there are difficulties to overcome, the use of lactic acid bacteria and their bacteriocins as food preservatives has great potential.
Appendix

From personal communication with Dr. Ingolf Nes I got the information that a group in Holland was working on an identical lanthibiotic. In November 1991 an article dealing with the identification of this bacteriocin was published in the European Journal of Biochemistry. Unfortunately I did not receive knowledge of this publication until after I had submitted the present study (in January 1992). In the Dutch study the gene for bacteriocin production is sequenced, leading to the identification of an identical bacteriocin to that produced by SIK-83 (John W. M. Mulders, Ingrid J. Boerigter, Harry S. Rollema, Roland J. Siezen and Willem M. de Vos, 1991. Identification and characterization of the lanthibiotic nisin Z, a natural nisin variant. Eur. J. Biochem. 201; 581-584).
Acknowledgements

I would like to thank my supervisor Dr. Rolf Andersson for giving me the opportunity to carry out this work and for his support along the way. I am also most grateful to Professor Caj Eriksson at Chalmers University of Technology for his invaluable help.

The work of Dr. Knut Sletten at the University of Oslo on the amino acid analysis of this bacteriocin is gratefully acknowledged. I also want to thank Dr. Jon Nissen Meyer at the Laboratory of Microbial Gene Technology at Ås for his help and advice with the purification procedure and Dr. Ingolf Nes and Dr. Leiv Sigvar Håvarstein at Ås for their help with the genetic work. Last but not least I want to express my gratitude to Dr. Ingolf Nes for receiving me at his laboratory where a great deal of this work was carried out as well as for his helpful advice and discussions.
REFERENCES:


