Properties of a *Lactobacillus fermenti* bacteriocin

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SUMMARY

The properties of a bacteriocin derived from *Lactobacillus fermenti* strain 466 were investigated. The bacteriocin was present in low titre in supernatant fluids from overnight broth cultures and was not inducible by ultraviolet radiation. It was purified and concentrated to a titre of 1/1000 by dialysis, chromatography on Sephadex G100 and calcium phosphate gel columns. The bacteriocin is heat stable, and sensitive to trypsin and pepsin but not to lysozyme. No migration was demonstrated in electrophoretic fields in agar gel. Electron microscopy of the bacteriocin did not show any phage components. The bacteriocin is a macromolecular lipocarbohydrate protein which consists of 16 amino acids, four sugars, hexosamine and phosphorus. The biological activity of this complex is dependent on its structural integrity.

INTRODUCTION

Bacteriocins as defined by Reeves (1965) are produced by homofermentative and heterofermentative species of the genus *Lactobacillus* (de Klerk & Coetzee, 1961). The incidence of bacteriocinogeny in *Lactobacillus fermenti* is 15.5 % (de Klerk, 1967). All the *L. fermenti* bacteriocins have an identical host range and attempts to isolate resistant mutants have been unsuccessful. This has precluded the classification of these bacteriocins by cross-resistance tests (Fredericq, 1948). Colicins K (Goebel & Barry, 1958), V (Hutton & Goebel, 1961, 1962), F (E₂) (Reeves, 1963), A (Barry, Everhart, Abbot & Graham, 1965), I (Keene, 1966) and the unclassified colicin SG 710 (Nüske, Hösel, Venner & Zinner, 1957) are complexes of lipid, carbohydrate and protein. Megacin 216 (Holland, 1961) and a pyocin (Homma & Suzuki, 1964) are proteins. The specific chemical nature of all other bacteriocins is unknown (Hinsdill & Goebel, 1964; Reeves, 1965). The purpose of the present investigation was the purification and chemical analysis of one of the apparently homogeneous group of *L. fermenti* bacteriocins.

METHODS

Media. The liquid medium used was the MRS broth of de Man, Rogosa & Sharpe (1960) freed from high molecular weight constituents by dialysis (tubing size 18/32, Visking Co., Chicago, Ill., U.S.A.) under negative pressure. Solid medium was MRS broth diffusate + 2 % (w/v) agar.

Bacterial strains. The bacteriocinogenic *Lactobacillus fermenti* strain 466, the indicator *L. fermenti* strain FI and other indicator strains were described by de Klerk & Coetzee (1961). Cultures were incubated as previously described (de Klerk, 1967).

Preparation of bacteriocin. Crude preparations of bacteriocin were obtained by overnight growth of the bacteriocinogenic strain at 37° in broth diffusate medium. The culture was centrifuged, the supernatant fluid sterilized with chloroform and concentrated in a rotary evaporator at 40°. The concentrate was dialysed against de-ionized water to remove nutrients and small molecular weight bacterial products in the diffusate. The volume of the dialysis residue was further decreased by evaporation and then dialysed against 0.1 M-KCl + 0.05 M-tris + HCl buffer (pH 7.5).

Purification of bacteriocin. Crude bacteriocin was purified by gel filtration on Sephadex G100. Samples (2 ml.) were applied to columns (50 cm. × 2.5 cm.) and eluted with 0.1 M-KCl + 0.05 M-tris + HCl buffer (pH 7.5). Column effluents were collected in 5 ml. fractions and assayed for activity and the extinction at 280 m μ determined. High titre fractions were pooled and centrifuged at 200,000 g for 15 min. to remove bacterial debris. The supernatant fluid concentrated, dialysed against buffer and the dialysis residue filtered through Sephadex or chromatographed on calcium phosphate gel columns.

Adsorption chromatography of bacteriocin on calcium phosphate gel. Precipitated calcium phosphate was prepared according to Tiselius, Hjertén & Levin (1956), suspended in 1 mM-sodium phosphate buffer (pH 6.8) and applied to a chromatography tube (0.8 cm. × 12 cm.). A sample (2 ml. equiv., 10 mg. dry wt) of partly purified bacteriocin was dialysed against 1 mM-sodium phosphate (pH 6.8) and applied to the column. The bacteriocin was eluted with 1 mM-sodium phosphate buffer (pH 6.8) and step-wise elution with 10, 50 and 200 mM-sodium phosphate buffer solutions (pH 6.8; 50 ml. of each concentration) was continued. Fractions (5 ml.) were collected and assayed for activity and the extinction at 280 m μ determined.

Ultraviolet induction of bacteriocin. *Lactobacillus fermenti* strain 466 organisms from a 20 ml. overnight broth culture were deposited by centrifugation and re-suspended in an equal volume of sterile distilled water in a Petri dish. The suspension was irradiated with a 30 W. Hanovia Sterilamp (wavelength 2537 Å) from a height of 25 cm. At intervals of 5, 10, 15, 25, 40 sec., samples of 4 ml. were pipetted into 20 ml. warmed broth and incubated overnight in the dark. The supernatant fluids of these cultures were sterilized with chloroform and assayed for bacteriocin activity.

Assay of the bacteriocin. This was done by spotting drops of two-fold serial dilutions of the material in MRS broth on to agar plates seeded with the indicator strain and incubated overnight at 37°. The highest inhibitory dilution was the titre.

Analysis of bacteriocin

Purified bacteriocin was dialysed against repeated changes of distilled water and evaporated to constant dry weight under reduced pressure over P₂O₅. The bacteriocin (84.1 mg.) was then dissolved in distilled water (25 ml.).

Samples (0.2 ml.) of bacteriocin preparation were treated with 3 N-NaOH (0.1 ml.) and the protein content measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine albumin (Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.) as standard.

Total nitrogen was determined by a micro-Kjeldahl technique (Ballantine, 1957).

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were determined by the methods of Burton (1956) and Dische & Borenfreund (1957), respectively.

Bacteriocin (6.728 mg.) was hydrolysed in a sealed tube for 24 hr with 6 N-HCl

(5 ml.) at 105°. The acid was evaporated *in vacuo* over solid P₂O₅ and NaOH, and the amino acid composition determined with a Beckman automatic amino acid analyser, Model 120 B.

Bacteriocin (3 ml.) was treated with 12 N-perchloric acid (0.36 ml.) and left at 0° for 16 hr. The precipitated protein was removed by centrifugation and total phosphate in the supernatant fluid estimated by the method of Gomori (1942).

Estimation of lipids. The total lipid content of bacteriocin solution (10 ml.) was determined according to Salton (1953). The procedure consisted of hydrolysis with 6 N-HCl for 8 hr at 100°, followed by ether extraction of the hydrolysate.

Hexosamine estimation. Samples (2 ml.) of bacteriocin solution were hydrolysed in sealed tubes with 4 N-HCl for 15 hr at 100°. The acid was evaporated *in vacuo* over solid P₂O₅ and NaOH, and the liberated hexosamine separated from interfering substances (sugars and lipids) by cation exchange chromatography on columns (1 cm. × 10 cm.) of Amberlite IR 120 (H⁺-form) resin. The hexosamine was eluted with 2 N-HCl and estimated according to Boas (1953), with glucosamine hydrochloride (British Drug Houses Ltd.; BDH) as standard.

Liberation of carbohydrates. The bacteriocin solution (2.0 ml.) was hydrolysed for 2 hr at 100° in a final concentration of 2 N-H₂SO₄ and the hydrolysate applied to a column (1 cm. × 10 cm.) of Amberlite IR 120 (H⁺-form) cation exchange resin (Weibull & Bergström, 1958). Neutral sugars were eluted with water and concentrated under reduced pressure below 40° to a small volume. The sugar solution was transferred quantitatively to a 5 ml. standard flask and made to volume.

Paper chromatography of carbohydrates. One-dimensional descending chromatography was done on Whatman no. 1 filter paper with 2,4,6-trimethylpyridine as solvent (Partridge, 1948). D(+)-Galactose, D(+)-glucose, D(+)-mannose and L(+)-rhamnose (British Drug Houses Ltd.) were used as references. After developing the chromatograms for 40 hr, the solvent was removed by evaporation and the chromatograms sprayed with *p*-anisidine phosphate in ethanol (Mukherjee & Srivastava, 1952).

Quantitative estimation of carbohydrate components. A sample (0.5 ml.) of the sugar extract was applied as a band (10 cm. wide) in the centre and 8 cm. from the top of Whatman no. 1 filter paper (30 cm. × 57 cm.) together with two guide spots, 5 cm. from each edge, to which 50 µl. of sugar extract were applied. This chromatogram and a blank were developed for 40 hr in 2,4,6-trimethylpyridine and dried for 16 hr at room temperature. The positions of the separated sugars on the guide strips were revealed by the reagent and the corresponding areas excised from the experimental and blank chromatogram. Sugars and blanks were quantitatively eluted from each strip with distilled water into measuring cylinders (5 ml.) according to Dent (1947). Elution was stopped when 2 ml. of solvent had been collected and samples (1 ml.) were assayed for sugar concentration by the anthrone method of Scott & Melvin (1953).

Dissociation of protein and carbohydrate fractions. The phenol method of Goebel & Barry (1958) and hydrolytic techniques (Knox & Hall, 1965) were used in attempts to dissociate protein and carbohydrate components of the bacteriocin respectively.

Sedimentation of bacteriocin. This was done at 59780 rev./min. at 20° in the AN-D rotor of a Spinco model E ultra-centrifuge. A solution of about 1% purified

bacteriocin was used in a 12 mm. standard cell and photographs were taken at 8 min. intervals, with schlieren optics.

Action of enzymes on bacteriocin

Lysozyme. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of lysozyme (BDH; 100 µg./ml.) in 0.1 M-ammonium acetate for 5 hr.

Pepsin. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of crystalline pepsin (BDH; 1 mg./ml.) in 0.02 N-HCl for 24 hr. After digestion the mixture was adjusted to pH 7.0 with 0.1 N-NaOH.

Trypsin. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of crystalline trypsin (BDH; 0.5 mg./ml.) in 200 mM-sodium phosphate buffer (pH 7.5) for 3 hr. Control experiments were done without enzymes and bacteriocin titres determined.

Other investigations

Action of urea on bacteriocin. Bacteriocin was dissolved in a final concentration of 6 M-urea. Controls consisted of bacteriocin in water and a 6 M-urea solution. After 16 hr at 37° the solutions were assayed.

Chromatography on ion-exchange resins. This was done on Amberlite IR 410 (Cl⁻-form) anion and Amberlite IR 120 (H⁺-form) cation exchange resins.

Agar electrophoresis. Agar electrophoresis of purified bacteriocin at pH 5.2 and pH 8.5 was done by the method of Maré, Coetzee & de Klerk (1964).

Electron microscopy. Purified bacteriocin dissolved in distilled water was negatively stained with neutral potassium phosphotungstate (Brenner & Horne, 1959). Samples were mounted on carbon support films by a spreading technique (Bradley, 1962) and examined with a Philips EM 200 electron microscope.

RESULTS

Isolation of pure bacteriocin

Supernatant fluids of overnight cultures of *Lactobacillus fermenti* strain 466 in the MRS diffusate medium had inhibitory titres of 1/4. This medium did not contain molecules with a weight exceeding 10,000 (Leggett Bailey, 1962) and facilitated the separation of the bacteriocin which was not diffusible through the Visking tubing (de Klerk & Coetzee, 1961). Bacteriocin production was not enhanced by ultraviolet irradiation. One litre of crude bacteriocin was purified and concentrated to 2 ml. with a titre of 1/1000, this represented a recovery of 40–60 %. The primary separation of fractions on Sephadex (Fig. 1) and further purification on calcium phosphate gel (Fig. 2) are shown. In both graphs two peaks are present. The first had an absorption maximum of 277 mµ and contained all the activity. The second peak represented bacterial protein and broth constituents, and was virtually absent after repassage through either column. Ultracentrifugation of the purified bacteriocin revealed a single peak with an $S_{20,w}$ value of 2.8.

Chemical composition

The chemical composition of the bacteriocin is shown in Table 1. It consisted mainly of carbohydrate, protein and lipid, with lesser amounts of hexosamine and phosphorus, and was free from nucleic acids. The carbohydrate fraction (Table 2) contained four sugars; the mannose content being 56.7 %. The protein fraction con-

tained 16 amino acids (Table 3). Alanine, glycine, serine and the acidic amino acids were present in the highest concentrations.

Properties of the bacteriocin

Purified bacteriocin was a fluffy, white amorphous powder freely soluble in water. Aqueous solutions kept at room temperature for several days showed no loss of activity and neutral solutions withstood heating at 96° for 30 min. Activity was completely destroyed by trypsin and was decreased to half when treated with pepsin for 24 hr. The bacteriocin was unaffected by lysozyme. Phenol did not split a protein with bacteriocin activity from the complex; this corresponds to the findings of Barry *et al.* (1965) with colicin A. Attempts to separate the carbohydrate moiety by hydrolysis with hot 0.1 N-sulphuric acid or warm 10 % (w/v) trichloroacetic acid resulted in total

Fig. 1

Fig. 1. Chromatography of crude bacteriocin of *Lactobacillus fermenti* 466 on Sephadex G-100. A 2 ml. sample (titre: 1/1000) was applied to the column and eluted with 0.1 M-KCl + 0.05 M-tris + HCl buffer (pH 7.5). Fractions (5 ml.) were collected and assayed for: ●—●, extinction; and ■—■, inhibitory activity.

Fig. 2

Fig. 2. Chromatography of bacteriocin of *Lactobacillus fermenti* 466 on calcium phosphate gel. Partially purified bacteriocin was concentrated and dialysed against 1 mM-sodium phosphate buffer (pH 6.8). A 2 ml. sample (titre: 1/1000) was applied to the column and stepwise elution done with sodium phosphate buffer (pH 6.8) at concentrations of 1, 10, 50 and 200 mM. Fractions (5 ml.) were collected and assayed for: ●—●, extinction; and ■—■, inhibitory activity. (a) 1 mM, (b) 10 mM, (c) 50 mM, (d) 200 mM-sodium phosphate.

Table 1. Chemical composition of bacteriocin of *Lactobacillus fermenti* strain 466

	(%)		(%)
Nitrogen	4.96	Carbohydrate	53.20
Protein	23.80	Hexosamine	0.80
Lipid	20.80	Phosphorus	0.30

Table 2. Carbohydrate composition of bacteriocin of *Lactobacillus fermenti* strain 466

	(%)		(%)
Galactose	19.4	Mannose	56.7
Glucose	16.7	Rhamnose	7.2

inactivation. Extraction of a bacteriocin solution with chloroform or ether removed no lipid material and did not affect activity. The bacteriocin activity was also unaffected by treatment with urea. Agar electrophoresis at different pH values showed no migration of the bacteriocin when several different *Lactobacillus fermenti* strains were used as indicators. Anion and cation exchange resins did not retain the bacteriocin. Electron microscopic examination showed only amorphous material.

Table 3. *Amino acid composition of the protein fraction of bacteriocin of Lactobacillus fermenti strain 466*

Amino acid	Residues/1000 total amino acid residues	Amino acid	Residues/1000 total amino acid residues
Lysine	57.3	Glycine	111.2
Histidine	18.9	Alanine	134.3
Arginine	29.0	Valine	56.0
Aspartate	100.1	Methionine	4.5
Threonine*	87.6	Isoleucine	37.6
Serine*	101.0	Leucine	64.0
Glutamate	92.3	Tyrosine	30.7
Proline	47.2	Phenylalanine	28.3

* Corrected for loss during hydrolysis.

DISCUSSION

Lactobacillus fermenti strain 466 produces a single bacteriocin, which is non-inducible by ultraviolet radiation. The bacteriocin differs in the latter respect from megacin 216 (Holland, 1961), colicin F (Reeves, 1963) and pyocin (Homma & Suzuki, 1964). It is a lipocarbohydrate protein similar in general composition to colicins (Hinsdill & Goebel, 1964). Attempts to isolate an active protein fraction from the complex were unsuccessful although the phenol method ruptures covalent bonds (Holland, 1961) and an active protein was split from the colicin K complex in this way (Goebel & Barry, 1958). Mild techniques of hydrolysis which split sugar phosphate bonds to release a carbohydrate fraction (Knox & Hall, 1965) destroyed the biological activity of the bacteriocin. These findings support the conclusion that activity depends on the integrity of the lipocarbohydrate protein complex. The chemical composition of only one of the bacteriocins produced by Gram-positive organisms, megacin 216 (Holland, 1961), is known; this is a protein with traces of phosphorus and carbohydrate. The enterocin of *Streptococcus zymogenes* (Brock & Davie, 1963) is possibly a protein (Hamon, 1964) with a lipid component. The 16 amino acids of the *Lactobacillus fermenti* strain 466 bacteriocin correspond to those of megacin 216 (Holland, 1961), but the concentration of acidic amino acids in the latter bacteriocin is higher. The lactobacillus bacteriocin has a trace of methionine whilst cysteine/cystine are absent. This suggests that the three-dimensional structure of the macromolecule does not depend on the presence of disulphide linkages. The activity of this bacteriocin is probably independent of hydrogen bonds since it is not affected by high concentrations of urea.

The absence of migration in an electric field at different pH values was unexpected since the dicarboxylic amino acids, aspartate and glutamate, predominate. The bacteriocin diffused through 0.8 % (w/v) agar (de Klerk, 1967) and its electrophoretic immobility did not result from inability to penetrate the agar lattice. The failure of

ion-exchange resins to retain the bacteriocin confirms its lack of net ionic charge: its ionic groups may be masked by lipid.

Homma & Suzuki (1964) isolated a cell-wall protein with bacteriocin activity from *Pseudomonas aeruginosa*. Cell walls of *Lactobacillus fermenti* contain galactose, glucose, glucosamine and aspartate, glutamate, alanine and lysine (Ikawa & Snell, 1960). The bacteriocin of *L. fermenti* strain 466 differs in composition from its cell wall and is much more complex.

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