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MICROCIN 7: PURIFICATION AND PROPERTIES

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Microcin 7 is an antibiotic peptide, produced and excreted to the culture medium by E. coli strains harboring the plasmid pRYC7. This peptide was extracted from the culture media by adsorbing it on octadecyl silica. It was purified by gel filtration on Sephadex G-25 and reverse phase high performance liquid chromatography. Its amino acid composition is the following: Ala (0.8), Arg (1.9), Asx (1.9), Gly (1.5), Met (0.8) and Thr (0.9). The purified peptide does not react with ninhydrin and it is resistant to carboxypeptidase degradation, indicating that the molecule may be a cyclic or end-blocked oligopeptide.

Microcins were described by our group in 1976 as dialysable antibiotics produced by enterobacteria (1), different from the known bacteriocins in that microcins are not inducible by DNA-damaging agents, but resembling them in that both production of and immunity to these antibacterials are coded for by plasmid DNA (2).

Estimations of their size and composition as well as examples of their mode of action have been published. In one case, an oligopeptide seemed to be involved in the depolarization of the membrane of sensitive cells (3) and in another case, a non-peptidic derivative of methionine was shown to be inhibiting methionine biosynthesis in the treated cells (4). The uncertainities about their structure are due to the fact that, to date, no microcin has ever been purified to homogeneity, mainly because they are present in the culture media in very low concentrations which, in the case of microcin 7, we have estimated it to be around 0.5 mg/l.

Microcin 7 is an antibiotic peptide produced by E. coli strains that carry a 43 kilobase plasmid named pRYC7, which also codes for the immunity to this antibacterial (M. Novoa, personal communication). We have extracted the active peptide present in 157 l of E. coli culture, purified it to chromatographic homogeneity and analyzed its amino acid composition.

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ABBREVIATIONS: RP-HPLC: Reverse phase high performance liquid chromatography.

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MATERIALS AND METHODS

Strains and culture conditions.

E. coli K12 strain GS7 (F^-, araD139, ΔlacU169, rpsL, relA, thi, malE::Tn5) (5) was obtained from Dr. F. Moreno, along with its derivative RYC25, which carries the microcinogenic plasmid pRYC7. Both strains were routinely grown at 37ºC with agitation in minimal medium M9 (6), supplemented with thiamine (10 mg/l).

The relationship between the growth of E. coli RYC25 and the appearance of microcin 7 activity in the medium was studied by incubating 1 l of M9 medium, inoculated with 8 x 10^9 viable cells, in 2 l Erlenmeyer flasks. The bacterial growth was followed by measuring the optical density of the cultures at 660 nm with a Spectronic 20 colorimeter (Bausch and Lomb). At each hour, the bacteria in two of the flasks were pelleted by centrifugation at 10,000 r.p.m. for 10 min in a GSA rotor (Sorvall) kept at 4ºC. The supernatant from each flask was passed through a prepacked octadecyl silica microcolumn (SepPak-C18, Waters), which was then washed with 3 ml of deionized water and 2 ml of 80% methanol. The methanolic extract was evaporated to dryness under reduced pressure, the residue was dissolved in water and its antibacterial activity was assayed as described below. The results for the two flasks were averaged.

Antibiotic activity assay.

Microcin 7 activity was estimated by measuring the diameters of the zones of growth inhibition, produced by 25 µl of suitable dilutions of a sample on a plate seeded with a sensitive strain. The samples and two tetracycline standards were deposited into wells cut out of plates containing 25 ml of M9 medium with 1.5% agar, previously overlayed with 3 ml of 0.7% agar seeded with approximately 10^8 viable cells of E. coli GS7 from an overnight culture. The diameters of the inhibition zones were measured after 15 hours of incubation at 37ºC and the results were interpolated on a regression line constructed with tetracycline. In these conditions the results of the assay are very reproducible and both microcin 7 and tetracycline give linear relationships between the logarithm of the antibiotic concentration and the diameter of the inhibition halo.

One activity unit (1 u) was defined as the antibiotic activity present in a preparation that produced, in the assay described above, an inhibition zone of the same diameter as the one produced by 0.3 µg of tetracycline. When amino acid analyses of microcin 7 became available, we determined that 1 u was the activity of 0.2 nmoles of the peptide (0.2 µg if a molecular weight around 1,000 is assumed, see Results).

Extraction of microcin 7.

E. coli RYC25 was grown in M9 medium in a Biolafitte fermenter (157 l in two batches), at a growth rate shown in Figure 1. The cells were col-

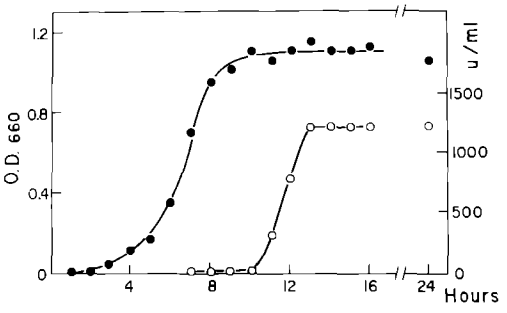

Figure 1. Correlation between microcin 7 production and cell growth. The growth of E. coli RYC25 in M9 medium (●) and the appearance of microcin 7 activity (○) in the culture supernatants were followed as described in the text.

lected by centrifugation 4 hours after reaching the stationary phase of growth and the supernatants were passed through two columns, 5 cm in diameter and connected in parallel, packed with 20 g of Bondapak-C18/Porasil B (Waters) each. The packing was done in methanol and the columns were washed with 10 volumes of deionized water before applying the supernatants.

The batches were processed one at a time, at flow rates of approximately 5 l/h and when all the medium had passed through the silica bed the columns were washed with 2 volumes of deionized water. Microcin 7 activity was eluted with 1 l of methanol, the methanolic solution was concentrated to 100 ml under reduced pressure and 200 ml of chloroform and 300 ml of water were added. The organic phase was extracted twice with deionized water and the aqueous fractions were pooled and lyophilized.

After each adsorption step, the octadecyl silica was regenerated by washing it with 500 ml of dimethyl sulfoxide, followed by 1 l of methanol, in a sintered glass funnel.

Purification of microcin 7.

The active preparations obtained as described above were dissolved in 50 mM KCl and filtered through a Whatman GF/C filter. The filtrate was chromatographied on Sephadex G-25 (fine) as described in the legend to Figure 2 and the active fractions from each run were pooled and concentrated on two SepPak-C18 cartridges coupled in series. The salt was washed out with 6 ml of deionized water and the microcin was eluted with 4 ml of methanol containing 0.1% of trifluoracetic acid. These preparations were evaporated to dryness under reduced pressure and the residue was taken up in the appropriate solvent for RP-HPLC.

HPLC chromatographies were performed with a model 204 Liquid Chromatograph (Waters), fitted with either a fixed wavelength Uvicord S absorbance detector (LKB) or a model 480 variable wavelength detector (Waters). The columns used were a 0.4 x 30 cm μBondapak-C18 (Waters) and a 0.4 x 25 cm Lichrosorb RP-18 (Merck) with a 5 μm irregular packing. All chromatographic runs were done at room temperature and the details are given in the legend to Figure 3.

Protease digestions.

Samples (100 u) of the active preparations obtained after Sephadex chromatography were digested with 8 units of trypsin (Sigma type XI, DPCC

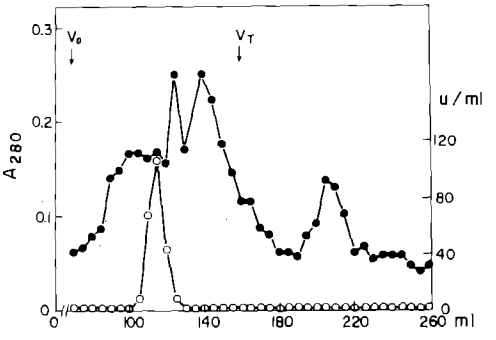

Figure 2. Sephadex G-25 chromatography of a crude extract of microcin 7. The delipidated and lyophilized material obtained as described in Methos was dissolved in 50 mM KCl at a concentration of 50 mg/ml. The column was equilibrated and eluted with the same solvent. Samples never exceeding 5% of the bed volume were applied and the elution was performed at flow rates between 2 and 4 ml/cm·h (1.6 x 80 cm column). Effluents were collected as 5 ml aliquots that were monitored for their absorbance at 280 nm (○) and for their antibiotic activity (●), as described in the text.

treated) or 0.01 units of subtilisin (Sigma protease type VII) in 100 µl of 0.4 M ammonium bicarbonate, pH=7.8, at 37ºC for 6 hours. The antibiotic activity of the digestion products was assayed as described above.

From 3 to 5 nmoles (15-25 u) of the homogeneous peptide were digested with carboxypeptidases under the following conditions: (A) 4 units of carboxypeptidase A (Merck) in 50 µl of 0.2 M N-methylmorpholine, pH=8.2. (B) 4 units of carboxypeptidase B (Merck) in 50 µl of the same buffer. (C) 9 units of carboxypeptidase Y (Sigma) in 25 µl of 0.1 M pyridine/acetic acid, pH=5.5. Digestions were performed at 37ºC for 15 hours and the products were lyophilized, taken up in sample buffer and applied to the amino acid analyzer.

Amino acid analysis.

The peptide was hydrolysed with 5.7 N HCl containing β-mercaptoethanol (0.02%), in sealed and evacuated glass tubes kept at 110ºC for 18 hours. The products were identified with a Beckman 121-MB autoanalyzer equipped with a Data 126 integrator. Tryptophan was qualitatively assayed with p-dimethylaminobenzaldehide (7).

RESULTS AND DISCUSSION

Purification of microcin 7.

The use of a mild and efficient extraction procedure to recover the microcin 7 present in the culture supernatants of E. coli RYC25, instead of working with concentrates of this type of media as previously described (1,3) has enabled us to purify for the first time a microcin to chromatographic homogeneity (Table I, Figure 3). The adsorption on octadecyl silica is simultaneously an extraction, concentration and purification step, because it yields the antibacterial concentrated 80-fold and free of the bulk of salts, which are the main solid constituents of the supernatant solutions.

During the purification of this compound by RP-HPLC, we have observed a strong influence of the aqueous component of the mobile phases on the recovery of the activity. In fact, microcin 7 is not eluted at all from the reverse-phase columns when water free of traces of ammonium salts (i.e.

Table I

Purification of microcin 7

	Total units (x10-5)	Sp. act. (u/mg dry wt.)	Recovery (%)
Supernatant	3.7	0.2	100
Bondapak-C18	3.7	123	100
Organic extraction	2.4	185	65
Sephadex G-25	2.2	1290	60
RP-HPLC[a]		5000	

The details of the purification procedure are described in the text.

[a]Only a fraction of the material obtained after gel chromatography was purified by RP-HPLC.

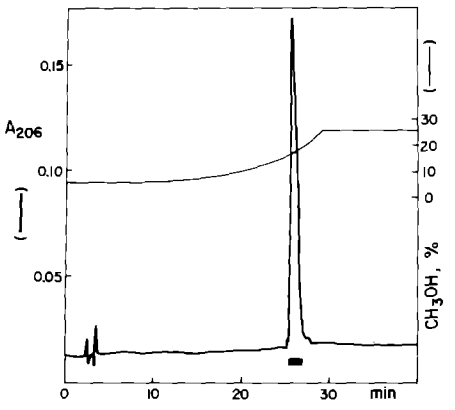

Figure 3. Final RP-HPLC step in the purification of microcin 7. A 100 µl sample containing 40 nmoles (200 u) of microcin 7 in 0.1% trifluoracetic acid was applied to a Lichrosorb RP-18 column. The elution was performed with a gradient of methanol in 0.1% trifluoracetic acid, at a flow rate of 1 ml/min. The solid bar indicates the position at which the antibiotic activity is eluted.

distilled over permanganate) is used without silanol-blocking additives. These results can be interpreted as evidence of a mixed mechanism of retention for microcin 7, in which both silanophilic and hydrophobic interactions play a role, the former predominating in the absence of silanol-blocking ions (like ammonium ions). This dual mechanism shows up particularly when peptides with basic groups are chromatographied in a RP-HPLC system (8,9), and this is in agreement with the observed presence of arginine in the composition of this microcin (Table II).

We believe the preparations obtained after several runs of HPLC to be homogeneous, because they give unique and symmetrical peaks when chromatographied in different RP-HPLC systems, using ultraviolet detection at 206

Table II

Amino acid composition of microcin 7

Amino acid	moles/mol Arg
Ala	0.8
Arg	1.0
Asx	1.9
Gly	1.5
Met	0.8
Thr	0.9

Conditions as described in the text. The assay for Trp gave negative results.

and 214 nm. The amino acid composition of samples purified independently was always identical.

Properties of microcin 7.

Microcin 7 is soluble in water, methanol, acetonitrile, ethanol and 1-propanol, but it is insoluble in 1-butanol, chloroform, acetone and diethyl ether. Its antibiotic activity is completely lost upon digestion with trypsin or subtilisin. The chromatographic behavior of microcin 7 in Sephadex gels (Figure 2) and HPLC columns and its amino acid composition are consistent with a small size and moderate polarity for this compound which, if we assume it to be an octapeptide with the composition shown in Table II, would have a molecular weight of 810.

This peptide does not react with ninhydrin nor is it degraded by carboxypeptidases A, B or Y, indicating that it lacks a free amino terminus and that it may not have a free L-amino acid at the carboxyl end either. This suggests that the molecule is cyclic or end-blocked. In this last case, the blocking groups cannot be very big, or at least not very hydrophobic, and certainly they cannot resemble the fatty acids found at the amino termini of certain proteins and peptide antibiotics, because that would require much higher concentrations of methanol or acetonitrile to elute the microcin from the reverse-phase columns (10,11,12).

This antibacterial is produced without the necessity to induce it with DNA-damaging agents, when the producer strain is well into the stationary phase of growth, and it seems to be perfectly stable in the culture medium (Figure 1). This contrasts with the known properties of most colicins and resembles the production kinetics of secondary metabolites.

We think that it would be of interest to clarify the relationship, if any, between this antibacterial, produced by a microorganism isolated from the human intestinal tract, and the known colicins and peptide antibiotics. As a first approach, work on the structure and mechanism of action of this microcin is in progress.

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