ACIDOLIN: AN ANTIBIOTIC PRODUCED BY *LACTOBACILLUS ACIDOPHILUS**

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Acidolin was isolated from skimmilk cultured for 48 hours with *Lactobacillus acidophilus* (CHR. HANSEN's Laboratory strain 2181). It was extracted from the skimmilk with methanol and acetone and was further concentrated and purified by Sephadex G-25 gel filtration, high voltage electrophoresis, and thin-layer chromatography on silica gel. Ultraviolet, infrared, nuclear magnetic resonance, and mass spectra results are presented for the antibiotic. Acidolin has a low molecular weight (~200), is acidic in nature, possesses a yellow-brown color, and is highly hygroscopic and thermostable. Acidolin exhibits antimicrobial activity against enteropathogenic organisms and sporeformers and only limited activity against lactic-acid bacteria. It is non-toxic to tissue culture cells (H-Ep-2) and is more active against vaccinia than polio virus.

The production of antibiotics by lactobacilli has been reported by several workers: KODAMA[2] isolated lactolin in a crude powder form from a broth culture of *Lactobacillus plantarum*; VINCENT *et al.*[4] obtained lactocidin from solid agar medium seeded with *Lactobacillus acidophilus*; and SHAHANT *et al.*[3] reported partial purification of acidophilin from medium cultured with *Lactobacillus acidophilus*.

The present study describes the isolation, purification, characterization, and antimicrobial activity of an antibiotic designated as acidolin and produced in skimmilk by *Lactobacillus acidophilus* 2181.

Isolation

Acidolin in crude form was obtained from skimmilk (11% total solids) inoculated with *L. acidophilus* 2181 (kindly supplied by CHR. HANSEN's Laboratory, Inc., Milwaukee, Wisconsin) and incubated at 37°C for 48 hours. The extraction procedure, a modification of that reported by KODAMA[2], was as follows: 100 g of the cultured skimmilk, concentrated by lyophilization, was treated with one liter of methanol and then centrifuged for 10 minutes at 4,080 R. C. F. The supernatant was saved and extraction and centrifugation of the precipitate were repeated.

The supernatants were combined and reduced to a small volume with a rotary vacuum evaporator operated below 50°C. To the residue was added one liter of acetone and the system was centrifuged for 15 minutes at 16,300 R.C.F. The precipitate was again treated with acetone and centrifuged. The combined supernatants were filtered through Whatman 1 MM filter paper and concentrated by rotatory vacuum evaporation. The yellow viscous liquid remaining was sterilized by passage through a 0.2 μ Millipore filter. This fraction constituted

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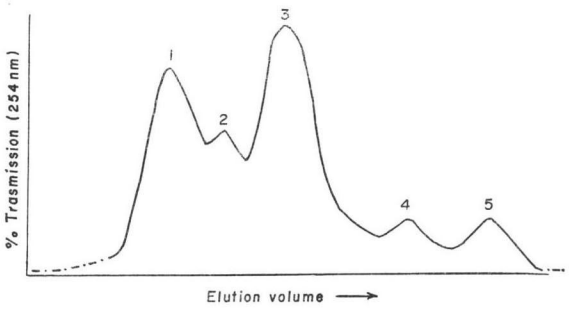
crude acidolin.

Assay

Antimicrobial activity was determined by the disc assay method[1]. The material was applied to a sterile filter disc (12.7 mm) which has been placed on the surface of solidified nutrient agar seeded with a 6~8-hour culture of an enteropathogenic strain (OSU 168) of *Staphylococcus aureus*.

The plate containing the disc was stored at 4°C for one hour to permit diffusion of the assay material and then was incubated at 37°C for 14~16 hours. The clear zone surrounding the disc was measured. A standard curve for crude acidolin was prepared on semi-log paper. The data show a linear relationship between the size of the inhibitory zone and the log of the concentration of crude acidolin. On an arbitrary scale, the 32.5 mm inhibitory zone was assigned the value of 100 inhibitory units. Based upon results obtained, the inhibitory units could also be calculated from the following linear regression equation: $Y=9.55X-190.78$, where Y=units of inhibition and X=the diameter in mm of the zone of inhibition.

Fig. 1. Elution pattern of crude acidolin passed through a Sephadex G-25 column. Elution buffer was acidified 0.15 N NaCl, pH 2.0.



Purification

Gel filtration.

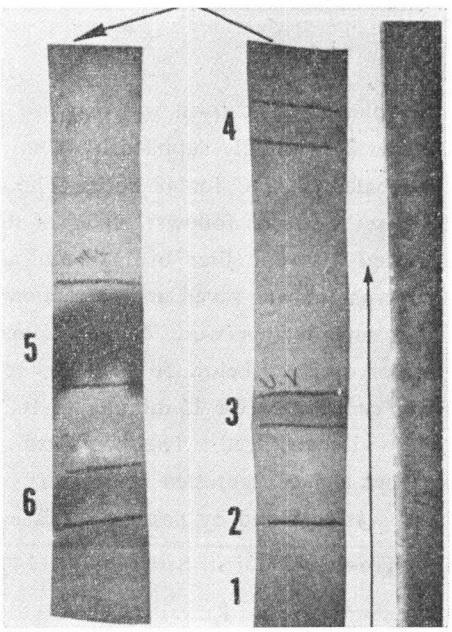
The crude acidolin was further purified by Sephadex G-25 gel filtration chromatography using 0.15 N NaCl buffer adjusted to pH 2.0 with 12 N HCl. The column size was 45×3 cm with a flow rate of 0.4~0.6 ml/min. The effluent was monitored at 254 nm by an Uvicord II, UV Spectrophotometer with attached recorder (LKB, Uppsala, Sweden).

In the elution pattern shown in Fig. 1, acidolin was concentrated in the fractions represented by the descending portion of Peak 1 and all of Peak 2. When these fractions were lyophilized, a yellow-brown residue remained; and upon assay, its antimicrobial activity was four times that of the crude acidolin at the same dilution.

High voltage electrophoresis.

Crude acidolin, 0.5~0.7 ml, was streaked on Whatman 3 MM filter paper. High voltage

Fig. 2. High voltage electrophoresis of acidolin. Arrow indicates direction of mobility. Areas 1 and 2, solvent controls; 3 and 4, ultraviolet absorbing; and 5 and 6, ninhydrin positive.



electrophoresis (HVE) was conducted in formic acid-acetic acid (60:240, v/v) buffer, pH 1.9, diluted to 3,000 ml with distilled water. Voltage was applied at 4,000 volts for 30 minutes at 20°C in a Savant High Voltage Electrophoresis apparatus (Hicksville, N.Y.). The paper was dried for 30 minutes at 100°C. The chromatogram was examined under ultraviolet (UV) light at 254 nm and upon spraying with ninhydrin solution.

Four compounds were detected by UV or ninhydrin reagent. The compounds were extracted with acetone. The extract was concentrated by rotatory vacuum evaporation and assayed for antimicrobial activity by the disc method. In the case of ninhydrin-sprayed papers, detected compounds were extracted from a duplicate unsprayed chromatogram.

For the chromatogram shown in Fig. 2, 1 and 2 were solvent controls, 3 and 4 absorbed at 254 nm, and 5 and 6 were ninhydrin-positive compounds. When assayed by the disc method, the inhibitory activity was located in UV-absorbing compound No. 3. Other areas on the chromatogram were devoid of activity.

Thin-layer chromatography.

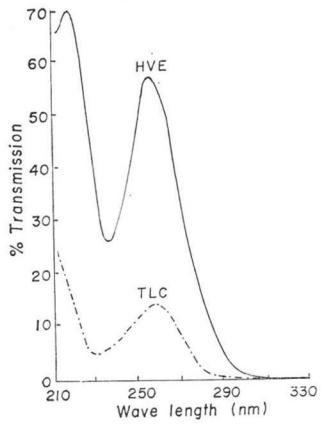
Acidolin isolated by Sephadex G-25 gel filtration or HVE contained detectable amounts of lactic acid. Therefore, thin-layer chromatography (TLC) on silica gel in a chloroform-methanol (90:10, v/v) solvent system was used to obtain acidolin free of lactic acid. The thin-layer plates were examined under UV light. Four spots absorbing at 254 um were detected. These were extracted separately with acetone, concentrated, and the residue was assayed for antibiotic activity. Only one compound with an R_f value of 0.52 demonstrated inhibitory activity.

Physical-Chemical Properties

Acidolin was highly acidic in reaction, soluble in water, methanol, and acetone, and only slightly soluble in chloroform. It has not been obtained in a crystal state.

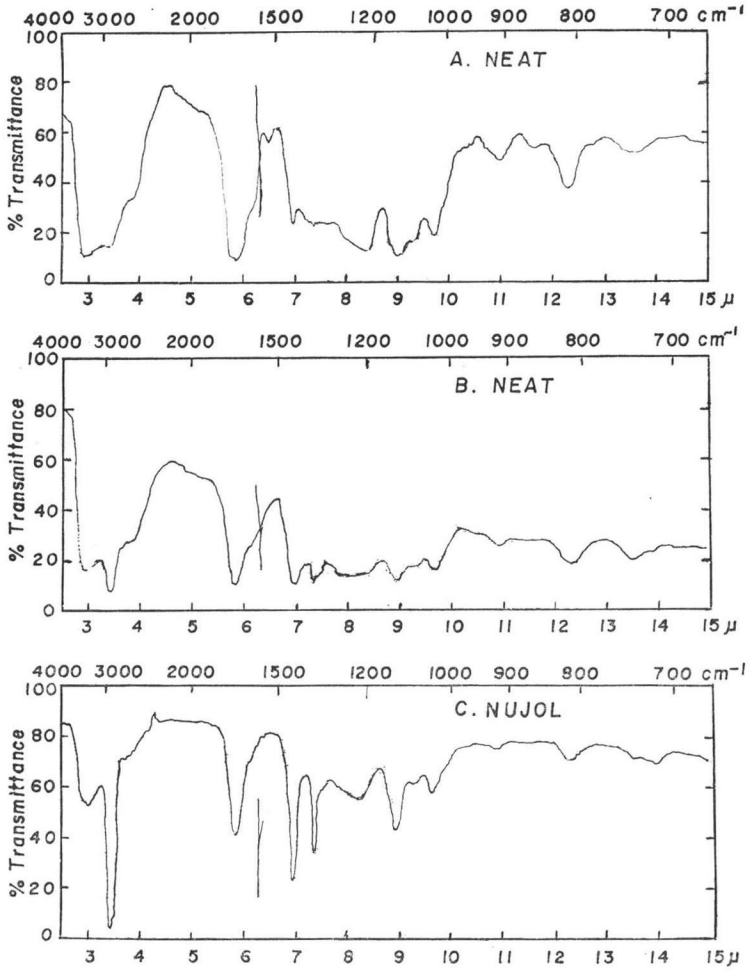
The UV spectra (Fig. 3) of acidolin purified by HVE or TLC revealed a maximum peak at 255 nm. The infrared spectra in neat or Nujol forms are shown in Fig. 4. The spectra show peaks at 3.5, 5.8, 6.9, 7.3, 8.4, 9.0, 11.0, 12.2, and 13.4 μ. The broad peak at 3.5 μ together with the absorption at 5.8 μ strongly suggests a carboxylic acid function. Peaks at 8.4 and 9.0 μ indicate C-O bends and the weak absorption at 12.2 and 13.4 μ indicates the possibility of an aromatic group.

Fig. 3. Ultraviolet absorption spectra of acidolin separated by high voltage electrophoresis (HVE) or thin-layer chromatography (TLC).



The nuclear magnetic resonance spectrum (Fig. 5) taken in a solution of acetone-*d*6 (Varian A-60) indicated the following: τ 4.21 (4H, singlet); τ 5.75 (1H, quartet, J=7.0 Hz); τ 8.57 and 8.70 (7H, singlets); and τ 9.12 (2H, multiplets). A small resonance at τ 8.02 and a triplet at τ 8.7 were partially obscured by larger, overlapping peaks. The singlet peak at τ 4.21 bore a proton similar to a cyclic functional group. However, this peak disappeared upon the addition of D_2O indicating that the peak was due to H_2O protons. The peak at τ 5.75 exhibited protons

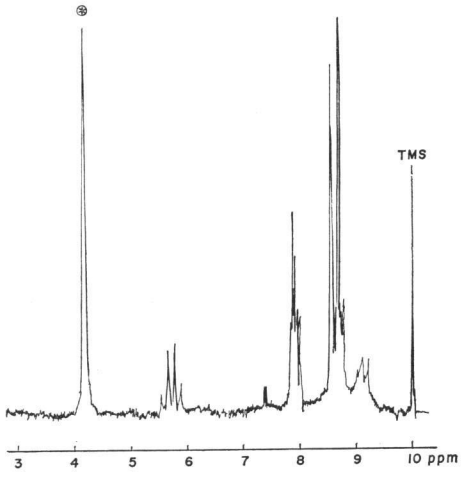
Fig. 4. Infrared spectra of acidolin isolated by gel filtration chromatography (A) or high voltage electrophoresis (B and C).



of CH_2CH_2O.

Acidolin separated by TLC was dissolved in acetone and injected into a gas chromatograph-mass spectrometer. The gas chromatogram showed a major peak at 10.44 minutes and this was analyzed in a Dupont 21-490 Mass spectrometer at 70 eV; 230°C, ion source temperature; and 1.5 kV accelerating voltage. The mass spectra (Fig. 6) show the parent peak at *m/e* 198 suggesting this to be molecular weight of acidolin. The base peak was at *m/e* 183 with other peaks at *m/e* 165 (18%) ($P\text{-}CH_3\text{-}H_2O$), 152 (10%), 141 (4%) ($155\text{-}CH_2$), 129 (5%), 120 (8%), 115 (6%) [$129\text{-}CH_2$], 105 (6%), 103 (9%), 91 (16%) [$C_7H_7^+$], 77 (15%) [$C_6H_5^+$], 52 (4%) [$C_4H_4^+$], and at *m/e* 28 (25%). These data indicate the possibility of a methyl ester of an acid. The peaks at *m/e* 91, 77, and 52 suggest a cyclic function similar to a benzylic group. A carbon chain larger or equal to two is probably ($R\text{-}CH_2CH_2\text{-}R'$) because of the presence of two (CH_2) fragmentations. This possibility was confirmed by the quartet peak observed on the NMR spectra. With a molecular weight of 198, it is assumed that no nitrogen is present unless N= 2, 4, 6, and so forth. However, micro-KJELDAHL analysis ruled out this possibility.

Fig. 5. Nuclear magnetic resonance spectrum of acidolin isolated by high voltage electrophoresis. TMS is for tetramethylsilane and $\tau = 10 - \delta$. Peak at 4.2 ppm disappeared upon addition of D_2O.



Disc assay against *S. aureus* OSU 168 indicated that the antimicrobial activity of concentrated or diluted acidolin was not affected by autoclaving at 121°C for 15 minutes. Similarly, storage of acidolin for 30 days at 4°C did not diminish its antibiotic activity.

With respect to one or more of the following: solubility, molecular weight, composition, stability, and/or UV and IR spectra, acidolin differed from lactocidin[4] or acidophilin[3]. Acidolin was not lactic acid, hydrogen peroxide, or a low molecular peptide. It is a newly identified antibiotic associated with *L. acidophilus* HANSEN's strain 2181.

Elucidation of the chemical structure of acidolin is now in progress and will be reported shortly.

Fig. 6. Mass spectrum of the major peak compound obtained from gas/liquid chromatography of acidolin.

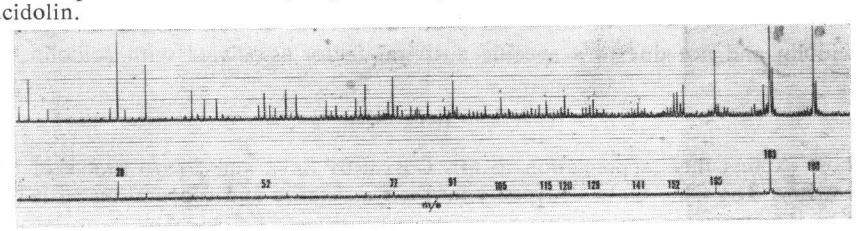


Table 1. Antimicrobial activity by the disc assay method of crude and purified acidolin.

Organism	Zones of inhibition (mm)	
	Crude extract (1/10 dilution)	Purified fraction (1/200 dilution)
Staphylococcus aureus OSU 168	35	23
Salmonella typhimurium OSU 471	28	17
Escherichia coli ATCC 25298	34	20
Escherichia coli B	31	17
Escherichia coli OSU 66	30	17
Bacillus cereus 7	32	20
Bacillus coagulan ATCC 7050	33	*
Streptococcus lactis C2	28	18
Streptococcus cremoris C1	15	0
Streptococcus cremoris R1	17	15
Lactobacillus acidophilus BAC	30	18
Lactobacillus acidophilus 2181	16	0

* Not determined.

Biological Activity

The antimicrobial activity of acidolin was determined by the disc assay method and results are shown in Table 1. Acidolin was effective against gram-positive and negative organisms including both enteropathogens and sporeformers. It was not effective against some of the lactic acid bacteria including the organism with which it was associated, *L. acidophilus* 2181.

Before undertaking the study of the antiviral activity of acidolin, the cytopathic effect (CPE) of different concentrations of acidolin on tissue culture cells was determined. A 24-hour healthy monolayer of H-Ep-2 cells was exposed for one hour to different concentrations of both the crude acidolin and the Sephadex-purified preparation. Five ml

Table 2. Effect of acidolin on synthesis of polio and vaccinia viruses.

Acidolin concentration	CPE*		pH
	Polio	Vaccinia	
0 (control)	+5	+5	7.30
1/640	+5	+5	6.80
1/320	+4	+4	6.50
1/160	+2	−	4.50
1/80	−	−	3.60
1/40	−	−	3.20
1/20	−	−	2.90

* CPE (Cytopathic Effect)
+5 Complete disintegration of the cells.
− Healthy confluent monolayer of cells.

of tissue culture fluid (Joklik, modified) containing from 0 to 5% crude or purified acidolin was added to each monolayer and the samples were incubated at 36°C for 72 hours. The CPE on the cells was determined microscopically. No toxic effect was noted with either preparation. The tissue culture fluid, however, turned yellow as a result of the low pH value.

To determine the effect of acidolin on synthesis of polio (Type 1, Ls-a strain) and vaccinia (IHD strain) viruses, serial dilutions of acidolin in the range of 1/20 to 1/640 were used. Results in Table 2 show that synthesis of the polio virus was inhibited at a dilution of 1/80, whereas, inhibition of the vaccinia virus occurred at a dilution of 1/160. Apparently, the vaccinia virus, a DNA virus, was more susceptible to acidolin than the polio virus, an RNA virus. However, inhibition of both viruses coincided with lowering of the pH by acidolin. At high pH (6.5~6.8), no inhibition of the viruses was observed indicating the possibility that inhibition may be due to the lowering of the pH of the cell culture system by the acidic nature of acidolin and not due to a specific antiviral factor associated with acidolin.

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Reference

1) HAUSLER, Jr., W.J. (Editor): Standard Methods of the Examination of Dairy Products. 13th ed. American Public Health Association, Washington, D.C. pp. 133~135, 1972
2) KODAMA, R.: Studies on lactic acid bacteria. II. Lactolin, a new antibiotic substance produced by lactic acid bacteria. J. Antibiotics 5: 72~74, 1952
3) SHAHANI, K.M.; J.R. VAKIL & R.C. CHANDAN: Antibiotic acidophilin and process of preparing the same. U.S. Patent No. 3, 689, 640. September 5, 1972
4) VINCENT, J.G.; R.C. VEOMETT & R.F. RILEY: Antibacterial activity associated with *Lactobacillus acidophilus*. J. Bact 78: 477~484, 1959