Antibacterial Compound from a Soybean Product Fermented by *Rhizopus oligosporus* (33930)

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For centuries, surprisingly large quantities of fermented soybean products have been consumed by the people of Asia. From their experiences, they consider fermented products as nourishing and easily digestible foods. The nutritional value of those fermented foods, however, has never been adequately investigated. Not until the 1960's were a number of studies made on an Indonesian soybean product, tempeh, which is prepared by fermenting soybeans with a species of *Rhizopus*. These studies (1–4) failed to confirm that the nutritional value and digestibility of soybean protein were improved by fermentation. Van Veen and Schaefer (5) observed the beneficial effects of tempeh on patients with dysentery in the prison camps of World War II. After scanning the dietary patterns of the Chinese in 1920, Adolph and Kiang (6) speculated on the possible relationship between the protein diet of soybean products and resistance to infection. The possibility of antibiotics being produced in oriental food fermentations always has intrigued us. In searching the literature, however, we could find no report on antimicrobial activity of any fermented foods, or of any antibiotics being produced by those molds commonly used in oriental food fermentations.

In the course of investigating the proteolytic enzyme systems of *Rhizopus oligosporus* Saito, a mold used for tempeh fermentation, we found that the mold produces a compound that inhibits the growth of bacteria associated with cheese making. The antibacterial compound was discovered when cheese was being made with the rennin-like enzyme produced by this mold. Although curd formation was excellent, the cheese failed to become acid because of failure of the lactic acid bacteria to grow. The present report describes the presence of an antimicrobial activity in tempeh and some properties of the material.

Methods and Materials. Cultures. Rhizopus oligosporus NRRL 2710 and NRRL 3271 were maintained on slants of potato–dextrose agar at 4°. Before each experiment, the organism was transferred to a fresh slant, which then was incubated at 28° for 7 days. A spore suspension for inoculation was prepared by adding sterilized distilled water to each slant.

Preparation of tempeh extracts. Tempeh fermentations were carried out in petri dishes as previously described (7). Soybean grits were washed, boiled, drain-dried, and inoculated with a spore suspension of *R. oligosporus*. Sixty g of inoculated soybean grits was packed in a sterilized petri dish and incubated at 31° for 12, 24, or 36 hr. Control samples of uninoculated soybean grits were treated similarly. After incubation, 100 ml of distilled water was used to extract the contents of each petri dish. The extracts were centrifuged at 18,000*g* for 15 min. The supernatant fluids then were sterilized through a Millipore[2] filter (0.45 μ).

Submerged fermentations. Submerged fermentations were started by aseptically transferring a spore suspension from agar slants to flasks each containing 100 ml of 5% skimmed milk or 2% soybean meal. The flasks and media had been previously sterilized by autoclaving at 121° for 20 min. Inoculated flasks were then incubated on a reciprocating shaker at 28° for 4 days.

Isolation of antimicrobial material. Because greater activity was demonstrated in fermentations carried out with 5% skimmed milk, isolation studies were made with

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filtrates from milk fermentations. Culture filtrates of *R. oligosporus* NRRL 3271 grown in 5% skimmed milk were first passed through a Seitz filter to remove spores and then concentrated tenfold under vacuum. The material precipitating from the concentrated culture filtrate between 30 and 75% saturation with ammonium sulfate was collected by centrifugation, dissolved in a minimum amount of buffer, and applied to a Sephadex G-100 column (2.5 × 40 cm, 0.1 *M* phosphate buffer at pH 5.8). Two distinct fractions were noted: One, the enzyme fraction had proteolytic activity; the other, was the substance having bacteria-inhibiting activity. To remove the proteinases contaminated by the overlapping of the two fractions, the inhibitory fraction was chromatographed on a diethylaminoethyl (DEAE)-cellulose column equilibrated with 0.1 *M* phosphate buffer, pH 5.8. The antibacterial compound had little or no retention by the column. On the other hand, the proteinases were bound to the cellulose. Consequently, the inhibitor was free of proteinases. It then was dialyzed against water and freeze-dried.

Assay of antibacterial activity. Tube dilution assays were used to assess antibacterial activity. The routine assay medium contained peptone (0.5%), yeast extract (2.0%), dextrose (1.0%), and monopotassium phosphate (0.2%) and was sterilized at 121° for 20 min. Testing samples were sterilized through Millipore filters (0.45 μ) and made into various concentrations by serial dilution. The assay medium containing no test material served as a control. The test organism was *Streptococcus cremoris* (NRRL B-634) which was maintained in peptone–yeast extract agar. This species, found in raw milk and milk products, is used in butter and cheese making. To prepare inoculum, the organism was transferred to 5 ml of routine assay medium and incubated for 20 hr at 31°. One-tenth ml of inoculum then was added to each assay tube. After incubating 20 hr at 31°, the bacterial growth was determined by turbidimetry and readings were made at 550 mμ against a blank of uninoculated medium set at zero absorbance (100% transmittance). The percentage of growth inhibition was used to express antibacterial activity, and was calculated from the following formula:

$$\frac{\text{Absorbance of control tube} - \text{Absorbance of test tube}}{\text{Absorbance of control tube}} \times 100.$$

Results. Antibacterial activity produced by *R. oligosporus* during tempeh fermentation is plotted in Figs. 1 and 2. Figure 1 indicates the growth of *S. cremoris* in media containing various amounts of extract prepared from 36-hr fermented tempeh as compared to that of control soybean extracts. The addition of control soybean extracts or small amounts of tempeh extracts to media did not change growth of the organism significantly. Its growth was greatly suppressed, however, when media contained more than 0.2 ml of tempeh extracts/6 ml of medium. This suppression suggests that there are growth inhibitors in the tempeh extract. Figure 2 shows that the amount of the growth inhibitor in tempeh increased as the fermentation time increased. After 12 hr of incubation, the mold growth was visible in soybean samples inoculated with *R. oligosporus*; but extracts prepared from these samples did not inhibit growth. Indeed, it stimulated growth over the control culture. In this repect the antibacterial substance produced by *R. oligosporus* behaved like many antibiotics which at low levels frequently stimulate growth. Conceivably, growth stimulation could occur because

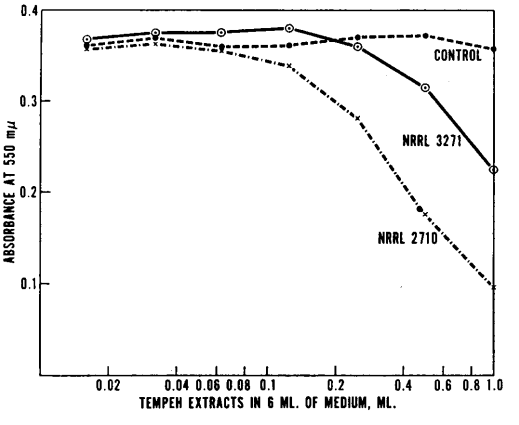

FIG. 1. Growth response of *Streptococcus cremoris* to various amounts of extracts prepared from tempeh after 36-hr fermentation by *R. oligosporus* NRRL 2710 and *R. oligosporus* NRRL 3271.

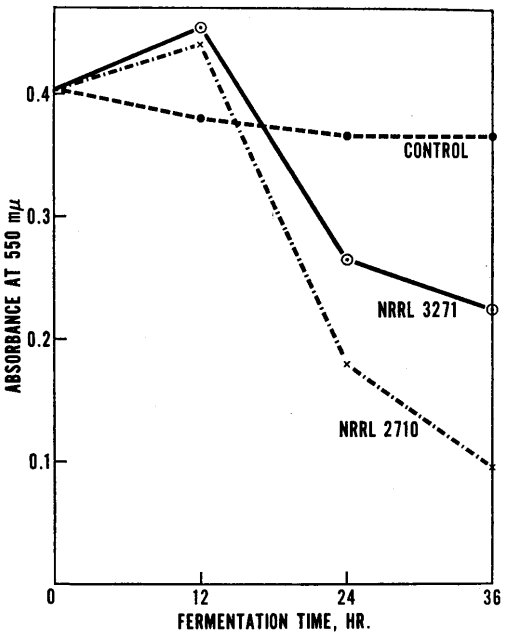

FIG. 2. Antibacterial activity of tempeh extracts prepared from 12- to 36-hr-fermented products by *R. oligosporous* NRRL 2710 and *R. oligosporus* NRRL 3271; test organism: *S. cremoris*.

of other factors. As the incubation time increased to 24 hr, luxurious mold growth was observed and extracts prepared from 24-hr fermented samples displayed a distinct growth-inhibitory activity. Mold growth and antibacterial activity continued to increase as incubation time was extended to 36 hr. The effect of extending the incubation time beyond 36 hr was not studied because soybean tempeh normally is complete after 20–24 hr at 31°. It is, therefore, evident that fermentation of soybeans by *R. oligosporus* produces antibacterial compound(s).

Tempeh extracts, culture filtrates of *R. oligosporus* grown in skimmed milk or soybean meal, and isolated products from culture broth then were tested against seven microorganisms, which included two gram-positive bacteria, two gram-negative bacteria, one acid-fast bacterium, one mold, and one yeast, by the routine paper disc assay to determine their antimicrobial spectra. Only two gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, were sensitive to these preparations.

The sensitivity of 25 microorganisms associated with cheese making and intestinal microflora of man is listed in Table I. Both the isolated preparation from culture broth and the crude tempeh extract were especially active against some of the gram-positive bacteria. Among the gram-negative bacteria tested, *Klebsiella pneumoniae* was the only one sensitive to the antibacterial compound produced by *R. oligosporus*. A second strain of this species was only slightly inhibited.

The antibacterial activity of crude tempeh extracts was rapidly destroyed at room temperature. The isolated product, on the other hand, was fairly stable. Fifty percent activity remained after boiling it for 1 hr. The isolated product also was fairly stable between pH 2–7. Gel electrophoretic patterns of isolated products showed four to five different components. The compound, therefore, has not been isolated in an unequivocally pure state. Its chemical and physical properties will be studied when further purification of the compound can be accomplished.

Discussion. Many fungi produce antibiotics. Antibiotics produced by Phycomycetes, however, are rare. Perhaps this situation is only a reflection of less study made on this class of molds. Almost all the known metabolite products of Phycomycetes are simple, low-molecular weight compounds (8). This knowledge may lead one to wonder whether Phycomycetes are capable of synthesizing the complex structures characteristic of many antibiotics. The finding of antibiotic materials produced by *R. oligosporus,* therefore, is unexpected.

Mucor and *Rihizopus* are two commonly found genera of Phycomcetes Species of these two genera have been widely used in oriental food fermentaions. The production of antibacterial agents by these species certainly would help to explain the nutritional value of fermented foods as claimed by natives, and the beneficial effects of tempeh on patients with dysentery as observed by Van Veen and Schaefer (5).

Antimicrobial tests indicated that the compound produced by *R. oligosporus* does not exhibit a broad spectrum activity, but it is

TABLE I. Antibacterial Activity of the Compound Produced by *R. oligosporus*.

Test organism	NRRL no.	Gram stain reaction	Inhibition (%)	
			Isolated product (50 μg/ml)	Tempeh extract (0.3 ml/ml)
Streptococcus lactis	B-633	+	70	100
Streptococcus cremoris	B-634	+	100	60
Streptococcus faecalis	B-1295	+	0	0
Leuconostoc dextranicum	B-1145	+	100	100
Leuconostoc mesenteroides	B-523	+	100	100
Leuconostoc citrovorum	B-1724	+	0	0
Lactobacillus casei	B-441	+	0	0
Lactobacillus plantarum	B-813	+	0	0
Salmonella pullorum	B-737	—	0	0
Salmonella paratyphi	B-118	—	0	0
Proteus vulgaris	B-123	—	0	0
Proteus mirabilis	B-3402	—	0	0
Escherichia coli	B-210	—	0	0
Escherichia freundii	B-2643	—	0	0
Staphylococcus aureus	B-313	+	97[a]	52
Bordetella bronchiseptica	B-140	—	0	0
Bacillus subtilis	B-765	+	100	100
Alcaligenes faecalis	B-170	—	0	0
Pseudomonas aeruginosa	B-23	—	0	0
Klebsiella pneumoniae	B-117	—	80	100
Clostridium botulinum	B-1218	+	33	91
Clostridium perfringens	B-3526	+	100	—
Clostridium sporogenes	B-1219	+	100	100
Clostridium butyricum	B-1024	+	100	100
Brucella abortus	B-1234	—	0	0

[a] Isolated product (250 μg/ml of growth medium).

very active against some gram-positive bacteria. The compound may not be an important antibacterial drug, but it is well established that antibiotics, in addition to minimizing infections, elicit growth-stimulating effects in animals. All these results, however, emphasize that antibiotics have a particularly striking growth-stimulating effect in diets that are deficient in any one of several vitamins (9) or proteins (10), or some growth factors (11) still unknown. Although there is no agreement on the mechanism of different types of growth-promoting effect, it is fairly well recognized that antibiotics stimulate a change in the intestinal microflora or in the intestinal wall of the animal, or both. *Clostridium sporogenes* and *C. perfringens* are both typical intestinal tract inhabitants. The antibacterial substance of *R. oligosporus* significantly inhibited the growth of each of the four *Clostridium* species tested.

Oriental people constantly are exposed to overwhelming sources of infection and their diets are frequently inadequate, yet they possess a wonderful resistance to disease. Our finding that an antibacterial agent is produced by *R. oligosporus* possibly offers a clearer understanding of the true value of tempeh in the diet of Indonesians, and perhaps of fermented foods in the diets of Orientals.

Summary. Strains of *R. oligosporus* produce an antibacterial compound especially active against some gram-positive microorganisms. The material can be extracted with water from soybeans fermented by *R. oligosporus*. It can also be recovered from culture broth by ammonium sulfate precipitation. The compound is fairly stable in its semipurified state and may consist of four or five components. The antibacterial activity produced by a mold commonly used in an orien-

tal food fermentation is considered significant for those people whose diets are often nutritionally inadequate. Conceivably ingestion of this material may confer disease resistance.

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Cytotoxicity of Phenothiazines on Chang Liver Cells as Measured by Enzyme Leakage (33931)

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Previous studies in this laboratory have utilized *in vitro* systems to demonstrate cytotoxicity of known and suspected hepatotoxic agents (1–3). Exposure to CCl_4 of suspensions of cells grown in tissue cultures led to rapid loss of intracellular enzymes (1). Chlorpromazine (CPZ), 10-(dimethylaminopropyl)-2-(chlorphenothiazine hydrochloride), an agent known to produce jaundice or hepatic dysfunction in humans (4), also led to leakage of intracellular enzymes from rabbit liver slices (3), while promazine (PZ) 10-(dimethylaminopropyl)-(phenothiazine hydrochloride) an agent which rarely produces hepatic injury in man (4) did not lead to this effect (3). In a further effort to develop a suitable *in vitro* model for the testing of hepatotoxicity, human liver cells (Chang) from tissue culture lines were exposed to PZ and CPZ at different concentrations. Leakage of lactate dehydrogenase, malate dehydrogenase, and asparatate aminotransferase into the medium was utilized as an index of injury to the liver cells.

Material and Methods. Chang human liver cells from tissue culture were obtained from Flow Laboratories, Rockville, Maryland. The cells were processed within 24 hr of arrival during which time they were kept at 4° refrigeration. Immediately before the experiment, the cells were centrifuged for 10 min at 1000 rpm, the original medium was discarded, and the cells were suspended in Hanks' base media 119 (IX), free of antibiotics and tested free of enzyme activity. For each experiment 1 ml of suspended cells containing approximately 10^6 cells/ml was mixed with 1 ml of the same medium used for suspension (controls) or 1 ml of the medium containing CPZ[3] or PZ[3] in different concentrations. The

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[3] Chlorpromazine hydrochloride (CPZ) and promazine hydrochloride (PZ), as the pure powders were each made available through the courtesy of the respective manufacturers: Smith, Kline and French, Philadelphia; and Wyeth Laboratories, Radnor, Pennsylvania.