Rennin Enzyme of *Endothia parasitica*

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A microbiological screening program was instituted to search for an animal rennet substitute. Among 381 bacteria and 540 fungi tested, only one organism, *Endothia parasitica*, yielded a suitable enzyme substitute. The fungal rennin enzyme was crystallized and some of its properties were studied. It was found to be water-soluble, nondialyzable, precipitable with $(NH_4)_2SO_4$ and organic solvents (e.g., acetone and isopropanol), and destroyed by heating for 5 min at 60 C. It was determined to be most stable in water at pH 4.5 and to have an isoelectric point of pH 5.5. On acid hydrolysis, it yielded: alanine, ammonia, arginine, aspartic acid, cysteic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, threonine, tyrosine, and valine. No tryptophan was detected after alkaline hydrolysis. Its molecular weight was estimated to be in the range of 34,000 to 39,000. The milk-clotting activities of the fungal and animal rennins proved to be essentially identical in milk containing various concentrations of $CaCl_2$. Both rennins manifested comparable clotting activities in milk at pH 6.0 to 7.0.

Rennet is a term applied to any crude enzyme preparation of animal, plant, or microbial origin which curdles milk. Pure milk-curdling enzyme per se is designated rennin. The rennet normally employed in the production of cheese is derived from the abomasum of unweaned calves. This source of supply varies seasonally and is becoming progressively more scanty as a result of: (i) increasing cheese production, (ii) decreasing slaughter of calves, and (iii) decreasing exports of calf rennets to the U.S.A. by countries which retain the enzyme for home use. The need for a suitable rennet substitute is rapidly becoming acute.

Reviews of the literature by Dewane (12) and Veringa (Dairy Sci. Abstr. 23:197, 1961) reveal that substitutes for calf rennets have been sought among animal, plant, and microbial sources since the beginnings of this century. A survey of the literature published since the reviews by Dewane and Veringa discloses that the search for calf rennet substitutes is being actively pursued. Studies with rennets from three animal species have been described (14, 17, 25). There have also been reports of studies with plant rennets derived from the same genera cited in the aforementioned reviews (5, 11, 17, 22, 25), as well as from various other plant genera (5, 6, 19, 29).

Since 1960, there has been an upsurge in the survey of microorganisms for a suitable rennet. Nearly two score of organisms have been reported to yield promising rennet activity. These include: *Alcaligenes* sp., *Lactobacillus helveticus*, and *Streptococcus faecalis-lactis* [sic] (3); three species of *Bacillus*, including *B. cereus* (3, 25, 35, 36, 40); *Ascochyta visa*, *Colletotrichum atramentarium*, *Monascus anka*, and *Sclerotium oryzae-sativa* (K. Arima and S. Iwasaki, U.S. Patent 3,151,039, 1964); six species of *Aspergillus* (15, 26, 42); *Basidiobolus ranarum*, two species of *Conidiobolus*, and two species of *Entomophthora* (A. R. Whitehill and F. B. Ablondi, U.S. Patent 2,936,265, 1960); *Byssochlamys fulva* and *Fusarium moniliforme* (21); three species of *Mucor*, including *M. pusillus* (4, 20, 41; Arima and Iwasaki, Japanese, U.S., and Canadian Patents, 1963 and 1964); *Penicillium chrysogenum* (9, 21); and 10 species of *Rhizopus* (Arima and Iwasaki, Japanese, U.S., and Canadian Patents, 1963 and 1964; H. L. Wang, D. I. Ruttle, and C. W. Hesseltine, Abstr. 154th Meeting Am. Chem. Soc., Chicago, 1967). In addition, Srinivasan et al. (37) investigated about 230 molds and 43 bacteria, none of which was identified. Schulz et al. (31) cited a study carried out with 500 microorganisms (predominantly aerobic sporeformers).

The rennets elaborated by these microorganisms have proved inadequate in cheese production for a number of reasons. For example, they generate off-flavors, yield too much acid, render poor texture and body, exhibit excessive proteolysis, etc. Two cultures are reported to

synthesize rennets which produce organoleptically acceptable cheese. The rennet of *B. cereus* is claimed to produce a Cheddar cheese free from bitter flavor, though the cheese is described as being somewhat hard and acid (35). Also, *M. pusillus* is reported to produce a rennet which yields a limited number of cheeses (butter, Edam, and Tilsit) without bitter off-flavors (31).

The increasing number of attempts since the beginning of this century to discover a satisfactory substitute for calf rennet reflects the increasing urgency of the problem. A microbiologically derived substitute would be of particular value in view of its essentially unrestricted availability. An added dividend of a microbial rennet would be its acceptability for use by those having scruples about consuming cheeses prepared with animal rennet (8). For these reasons, we initiated a research program to discover a calf rennet substitute of microbial origin. As a result of this program, an organism, *Endothia parasitica*, was found which produces a rennin eminently suitable to replace calf rennet (Sardinas, U.S. Patent 3,275,453, 1966; Abstr. 154th Meeting Am. Chem. Soc., Chicago, 1967). The enzyme was crystallized and some of its properties were studied.

MATERIALS AND METHODS

Organisms. All organisms tested were obtained from the culture collection maintained by Chas. Pfizer & Co., Inc., Groton, Conn. One of the organisms, *E. parasitica*, has been deposited with the American Type Culture Collection, Washington, D.C., and is designated ATCC 14729.

Methods of culture. In the screening procedure, the organisms were grown in medium A [soy bean meal, 3%; cerelose, 1%; Matrix skim milk (Galloway-West Co., Fond du Lac., Wis.), 1%; $NaNO_3$, 0.3%; KH_2PO_4, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.025%; pH 6.8]. A total of 15 ml of this medium was placed into tubes (25 × 150 mm) and autoclaved for 20 min at 121 C. Seeded tubes were incubated at 28 C (thermophiles at 55 C) for 2 to 3 days on a rotary shaker. After growth, a sample of broth was taken to test for milk-clotting activity. After removal of the test sample, 2 ml of toluene was added to the tubes (excepting thermophiles), and the tubes were shaken for an additional 24-hr period. A second sample was then taken and similarly evaluated.

For the production of larger quantities of milk-clotting enzyme, 4-liter Pyrex fermentors (pots) were used. A description of these was given by Shull and Kita (34). The inoculum medium used was identical to medium VI described by Tendler and Burkholder (39). A total of 100 ml of the inoculum medium in a 300-ml Erlenmeyer flask was autoclaved for 20 min at 121 C. The culture under study was transferred to the flask from a fresh slant and incubated at 28 C for 2 days on a rotary shaker. The entire contents of the flask were used as inoculum for one pot. The pots contained 2 liters of medium A sterilized by autoclaving for 45 min at 121 C. After inoculation, the pots were incubated in a constant-temperature water bath at 28 C with aeration set at 0.5 volume per volume per min. Agitation of the medium was accomplished by stirring at the rate of 1,750 rev/min. Incubation generally proceeded for 48 hr.

Milk-clotting assay. The procedure used was a modification of the technique described by Ernstrom (16). In practice, 5 ml of the assay milk [12% skim milk (Difco) and 0.16% $CaCl_2$ in distilled water] was placed into a tube (16 × 150 mm), the contents were brought to 32 C in a constant-temperature water bath, and 0.5 ml of sample was added. Curd formation was observed by manually rotating the test tube from time to time so as to form a thin film on its inner surface. The end point was taken as the instant discrete particles were discernible. Activity was designated in rennin units per milliliter or per gram.

In the screening program, toluene was layered on the assay milk after the addition of broth, and any curd which formed was observed for at least 24 hr for peptonization and gas formation.

Recovery and crystallization of fungal rennin (FR). Broth filtrate of an *E. parasitica* fermentation was adjusted to pH 4.5 and concentrated under vacuum at 35 C to one-fifteenth of its initial volume. Anhydrous $(NH_4)_2SO_4$ was added to a concentration of 40% while stirring. The precipitated enzyme was collected by filtration and dialyzed. The non dialyzable substances were freeze-dried, reconstituted to a 15% solution, and cooled to 0 C; then 2 volumes of acetone at −15 C was added. The reprecipitated enzyme was collected after decantation of the acetone-water solution, reconstituted to a 15% solution, treated with 5% activated carbon, and filtered; the filtrate was freeze-dried. The solids were reconstituted finally to a 15% solution and cooled to 0 C. Cold (0 C) isopropanol was slowly added with stirring until the solution became opalescent. The suspension was refrigerated until the crystalline enzyme was completely deposited. Other purification techniques also were employed (Sardinas, U.S. Patent 3,275,453, 1966).

Ultraviolet-absorption spectrum of FR. The ultraviolet-absorption spectrum of crystalline FR was obtained with the Cary model 11 recording spectral photometer (Applied Physics Corp., Pasadena, Calif.).

pH stability of FR. A solution of crystalline FR at a concentration of 500 units/ml was subdivided into a number of portions. These were adjusted to pH 2 to 10, overlaid with toluene, maintained at 25 C for 24 hr, and then assayed for milk-clotting activity.

Thermostability of FR. Solutions of crystalline FR at a concentration of 500 units/ml and pH 4.5 were incubated at 10, 20, 37, 45, and 60 C. Samples were taken for potency determinations at various times.

Electrophoresis of FR. The materials and techniques used were substantially those described by Werum et al. (44). A total of 400 units of crystalline FR was added to each of several paper strips (dimensions, 3 × 30 cm). In one experiment, a series

of buffers described by the aforementioned investigators was employed. These had pH values of 4.0, 4.7, 6.0, 7.2, 8.0, and 9.3. In a second experiment, 0.025 M sodium acetate was used at pH 4.8, 5.0, 5.2, 5.4, 5.6, 5.8, and 6.0. In each case, a constant voltage of 150 v was applied for 5 hr.

Proteolytic activity of rennins. The proteolytic activity ("PV") of crystalline animal rennin (15 $\times$ 10^6 rennin units/g) and crystalline FR was determined by the procedure of J. Shovers and S. A. Morell (*unpublished data*).

Amino acid analysis of FR. An acid hydrolysate of crystalline FR was prepared (6 N HCl at 110 C for 10 hr) and analyzed for its amino acid composition by the method of Piez and Morris (28). To check for tryptophan, an alkaline hydrolysis was carried out in accordance with the procedure described by Sanger and Tuppy (30). The paper chromatography system of Partridge (27) with the use of *n*-butanol-acetic acid-water (4:1:5, v/v) was employed for detection of tryptophan. Ninhydrin spray reagent, 0.5% in acetone (w/v), was used for visualization of the amino acids.

Molecular weight determination of FR. The molecular weight of crystalline FR was estimated by the gel filtration technique detailed by Ward and Arnott (43). Bio-gel P-100 (Bio-Rad Laboratories, Richmond, Calif.) was used. The column was calibrated with: α-chymotrypsin [beef pancreas, estimated molecular weight, 22,500 (1), Mann Research Laboratories, Inc., New York, N.Y.], crystalline animal rennin [estimated molecular weight, 34,000 (13), Chas. Pfizer & Co., Inc., Milwaukee, Wis.], and hemoglobin [human, twice recrystallized, estimated molecular weight, 68,000 (38), Mann Research Laboratories]. The chromatography column (1.6 $\times$ 54 cm) contained 5.5 g of P-100 suspended in 0.05 M sodium acetate at pH 6.5. The rate of flow was adjusted to 12 ml/hr. Void volume (Vo) was 25 ml. The effluent volume (Ve) was measured at the protein peak. Fluid volume from the column to the detector (2.25 ml) was subtracted from measurements of Ve. Ultraviolet absorption of the effluent was measured at 280 mμ.

Effect of CaCl₂ concentration in milk on rennin activities. A standard suspension of skim milk for rennet assay was prepared without added CaCl₂. To portions of this milk, anhydrous CaCl₂ was added at concentrations of 2, 4, 6, 8, 10, 20, 40, 60, 80, and 100 $\times$ 10^{-3} M. Stock solutions of both crystalline fungal and animal rennins (150 units/ml each) were separately assayed, in triplicate, in each milk.

Effect of milk pH on rennin activities. Skim milk suspension for rennet assay was prepared in the usual manner, and portions were adjusted to pH 7.0, 6.75, 6.5, 6.3 (normal assay pH), 6.25, 6.0, 5.75, 5.5, 5.25, and 5.0. Stock solutions of crystalline fungal and animal rennins (150 units/ml each) were assayed separately in each milk.

Test for antibiotic production by E. parasitica. Freeze-dried solids of fermented broth filtrate and samples of fungal rennin at various stages of purification were reconstituted in water to concentrations of 5 and 15%. These were tested for antibiotic activity against *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Rhizoctonia solani,* and *Saccharomyces cerevisiae* by the paper-disc agar plate technique (23).

RESULTS

Culture screening. A total of 921 organisms from our culture collection was screened for rennet activity. This included 196 Ascomycetes, 104 Basidiomycetes, 114 Fungi Imperfecti, 126 Phycomycetes, and 381 bacteria (147 of which were thermophiles). Over half of these organisms curdled the assay milk. However, most curds were eventually peptonized. In some cases, there was obvious evidence of gas evolution, color formation, or generation of malodors. Only
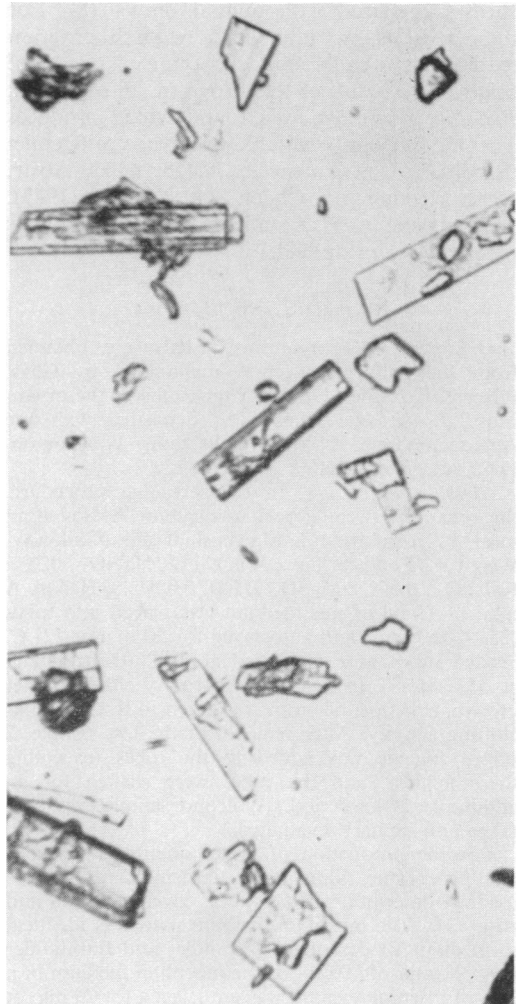

FIG. 1. *Photomicrograph of fungal rennin crystals.* $\times$ *340.*

one culture, *Endothia parasitica*, coagulated the milk without any evidence of peptonization or other undesirable effects.

Seven other strains of *E. parasitica*, from various culture collections, were tested, and all were found to curdle milk in the same manner as the original strain. A number of species of *Endothia*, viz., *E. fluens*, *E. fluens* series *xanthostoma*, *E. radicalis*, *E. tropicalis*, and *E. viridistroma*, were studied and found not to possess any significant rennet activity.

Fermentation of FR. The rennin elaborated by *E. parasitica* was produced in pot fermentations in sufficient quantities to supply material for isolation and purification studies. By use of the technique outlined above, crystals of fungal rennin were obtained (Fig. 1). The rennin activity was determined to be 1.6×10^6 units/g.

During fermentation, and at various stages of purification, samples were tested for antibiotic activity. No antimicrobial activity was manifested by any of the samples. The lack of antimicrobial activity can be taken as evidence for the absence of the two phytotoxins produced by certain strains of *E. parasitica*, viz., skyrin and diaporthin. Both of these phytotoxins are reported to possess antimicrobial activity (18). Skyrin is described as being intracellular and inhibitory to *S. aureus* and *B. subtilis*, but not to *R. solani*. On the other hand, diaporthin is extracellular and inhibitory to *R. solani*, but not to *S. aureus* and *B. subtilis*. Paper chromatographic analysis, which can reportedly detect diaporthin at levels as low as 0.01 μg (10), confirmed the absence of diaporthin.
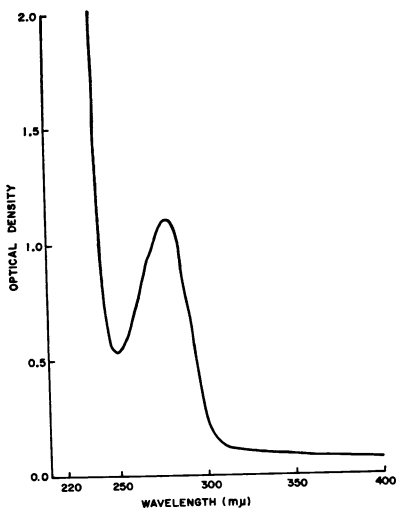
General properties of FR. FR was found to be water-soluble, nondialyzable, and precipitable with $(NH_4)_2SO_4$ and organic solvents (e.g., acetone and isopropanol). The ultraviolet-absorption spectrum of an aqueous solution of the crystals proved to be typical of a protein (Fig. 2). The maximal stability of FR was found to be at *p*H 4.5 (Fig. 3).

Thermostability of FR. Fungal rennin was found to be a relatively thermolabile protein. It was destroyed completely by heating at 60 C for 5 min. It is interesting to note that there is a 40% loss of potency at 20 C within 10 hr, but little loss for days thereafter. Perhaps the denatured protein affords some protection to the remaining undenatured enzyme (Fig. 4).

Isoelectric point of FR. In a preliminary electrophoretic study of crystalline FR, a broad range of *p*H and buffers was used (Fig. 5). To determine the isoelectric point (IEP), one buffer system (0.025 M sodium acetate), employing a narrower *p*H range, was chosen. The plot of migration of FR under these conditions indicates an IEP of *p*H 5.5 (Fig. 6).
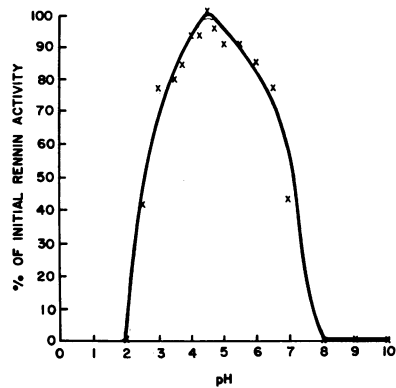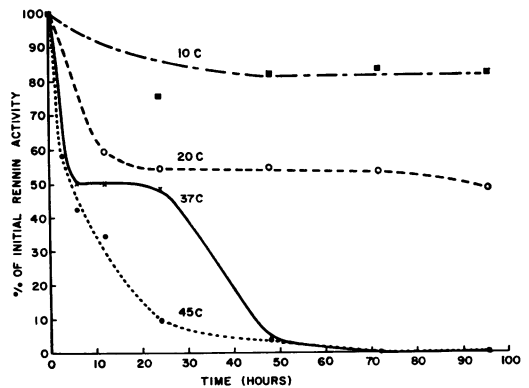
FIG. 3. *pH stability of fungal rennin.*

FIG. 2. *Ultraviolet absorption spectrum of fungal rennin.*

FIG. 4. *Thermostability of fungal rennin.*

Proteolytic activity of rennins. The proteolytic activity of crystalline FR was determined to be 4,930 PV units/g at *p*H 7.0 and 14,200 PV units/g at *p*H 5.5. For crystalline animal rennin, the proteolytic activity was 6,200 PV units/g at *p*H 7.0 and 26,000 PV units/g at *p*H 5.5.

Amino acid profile of FR. Acid hydrolysis of crystalline FR yielded ammonia and the following amino acids: alanine, arginine, aspartic acid, cysteic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, threonine, tyrosine, and valine. Paper chromatographic analysis of an alkaline hydrolysate failed to detect tryptophan.

Molecular weight of FR. The molecular weight of crystalline FR was estimated by gel filtration column chromatography to be about 34,000 (Fig. 7). The elution peaks of crystalline fungal
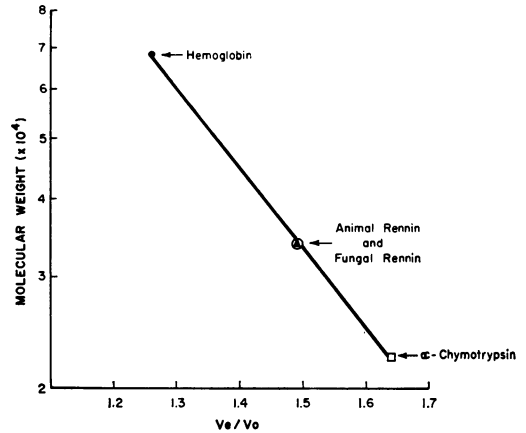
FIG. 7. *Molecular weight determination of fungal rennin by gel filtration.*
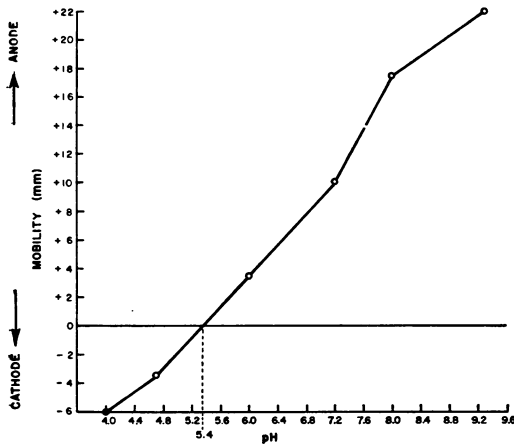
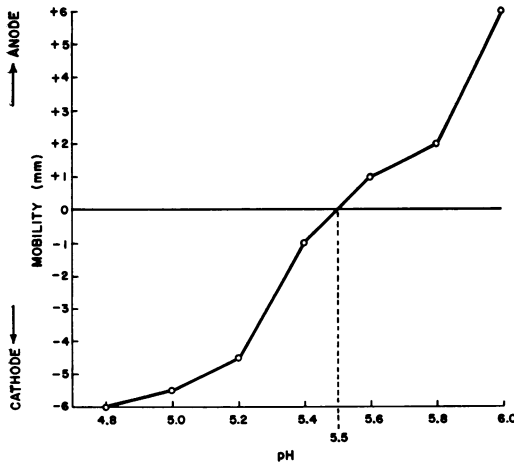FIG. 5. *Electrophoretic mobility of fungal rennin in a variety of pH buffers.*
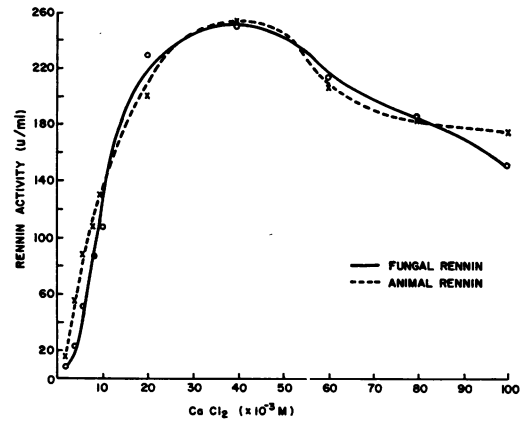
FIG. 8. *Fungal and animal rennin responses to various concentrations of CaCl₂ in milk.*

and animal rennins coincided exactly. A rerun of both enzymes in the same solution resulted in a single, sharp peak. However, a minimal molecular weight of 19,573 was calculated on the basis of an arginine content of 0.89 g per 100 g of protein. With two residues of arginine, the molecular weight is computed to be 39,146.

Effect of CaCl₂ concentration in milk on rennin activities. Figure 8 graphically illustrates the parallel response of fungal and animal rennins to various concentrations of added CaCl₂ to milk. Maximal potency for each rennin peaked at 40×10^{-3} M CaCl₂.

Effect of milk pH on rennin activities. Figure 9 indicates that the milk-clotting activities of fungal and animal rennins were substantially comparable between *p*H 6.0 and 7.0. However, below *p*H 6.0, animal rennin exhibited greater potentiation of its milk-clotting activity.
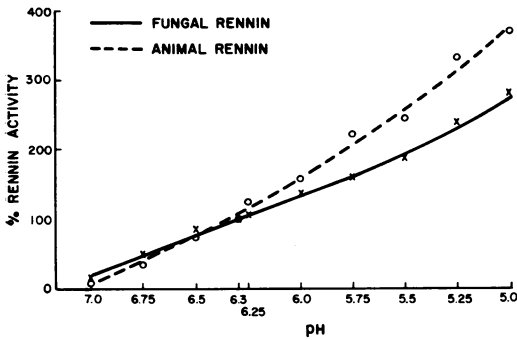
FIG. 6. *Isoelectric point determination of fungal rennin in 0.025 M sodium acetate.*

FIG. 9. *Effect of milk pH on the curdling activities of fungal and animal rennins.*

DISCUSSION

In the search for an animal rennet substitute, only one organism among the 900 studied possessed an enzyme capable of curdling milk without peptonization or production of other effects deemed undesirable in cheese manufacture. The enzyme is considered to be unique to *E. parasitica*, since all of the strains tested possessed it, in contrast to its absence in all of the other species of *Endothia* studied.

The enzyme was crystallized and some of its properties were studied. A comparison of the properties of fungal and animal rennins reveals similarities as well as dissimilarities. The molecular weight of animal rennin is reported to be in the range of 30,000 to 40,000 (2, 13, 32). The best estimate, based on the gel filtration column chromatographic technique, seems to be about 34,000 (13). Interestingly, the molecular weight of fungal rennin also was determined by gel filtration to be about 34,000. However, a molecular weight of 39,146 was calculated on the basis of its arginine content.

Fungal rennin was found to contain the same amino acids as animal rennin (32) except for the absence of lysine, methionine, and tryptophan. Mickelsen and Ernstrom (24) reported the maximal stability of animal rennin to lie broadly between pH 5.0 and 6.0. Fungal rennin, on the other hand, retained over 90% of its activity between pH 4.0 and 5.5, with a maximal stability at pH 4.5. The IEP of animal rennin is reported to be in the neighborhood of pH 4.5 (32), whereas fungal rennin has an IEP of pH 5.5. Both enzymes are mildly proteolytic, exhibit parallel potencies to various concentrations of calcium in milk, and manifest comparable curdling activities between pH 6.0 and 7.0. These latter properties are of especial importance in the evaluation of a rennet enzyme, since mild proteolysis is essential to the production of good

cheese, particularly aged cheese, and the calcium content and pH of milk vary seasonally and geographically.

When animal rennin is added to milk which is incubated below 15 C, no curd is formed, though some alteration of the milk occurs. If the temperature of this milk is then increased to 37 C, the milk will quickly clot (7). Fungal rennin acts in an identical manner (J. Shovers, *personal communication*).

In the final analysis, the crucial test for any rennet is the quality of the cheese made with it. Extensive production of a wide variety of cheese (e.g., Cheddar, Swiss, Colby, Italian varieties, etc.) manufactured with partially purified preparations of fungal rennin (Sure-Curd, trademark of Chas. Pfizer & Co., Inc.) were judged to be equal, or superior, to control cheeses made with animal rennet (33).

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