Table V. Foaming Capacity and Foaming Stability of the Oilseeds *Tetracarpidium conophorum*, *Cucumeropsis edulis*, and *Citrullus vulgaris* (Mean Values of Duplicate Samples)

	vol of foam after whipping, mL			
	foaming capacity		foaming stability	
sample	1/2 min	1 min	10 min	2 h
1, *Tetracarpidium conophorum* flour	66		14.9	14.6
2, *Cucumeropsis edulis* flour	144.8		97.2	93.8
3, *Citrullus vulgaris* variety 1 flour	141.7		90.3	82.8
4, *Citrullus vulgaris* variety 2 flour	146.2		93.0	90.0
5, soy flour[a]		160	131	14.6
6, soy concentrate (Isopro)[a]		400	28	93.8
7, soy isolate[a]		660	603	82.8
8, sunflower[a]		600	522	9.0

[a] Literature source Lin et al. (1974). Isopro denotes method of purification.

The foaming capacity and the foaming stability of the flours are shown in Table V. The foaming capacity of the flours are inferior to that of soy flour (shown in the table for comparison). The isolate of the studied oilseeds showed little or no foaming power. However, *Citrullus* and *Cucumeropsis* showed very good foam stability comparable to that of soy flour and isolate after 2 h.

Tetracarpidium showed very little foaming capacity and stability.

Summary. The solubility profile of the oilseeds showed that proteins of *T. conophorum* are highly soluble at a low pH, a factor that it may be of use in beverages.

Citrullus and *Cucumeropsis* have potential as human food because of the good functional properties—fat holding capacity, emulsion capacity, and water holding capacity. Their foaming capacity is very low, but the stability of the foam in *Citrullus* and *Cucumeropsis* is good after 2 h.

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Amylase Activities in Fungal Rennets and Whey Protein Concentrate Powder

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Amylase activities were characterized in fungal rennets and in whey protein concentrate powder. Starch hydrolysis was detected by the release of radioactive low molecular weight compounds from starch. Hydrolysis patterns indicative of α-amylase or glucoamylase attack on the starch substrate were observed. Among the fungal rennets tested, those derived from *Endothia parasitica* cultures contained amylolytic activities that were more heat sensitive than those derived from *Mucor* cultures. Time-dependent reductions in yield value were observed when such whey concentrate powders were added to starch-containing foods.

Fungal protease preparations are commonly used as substitutes for calf rennets in cheese making (Ernstrom and Wong, 1974). They are less expensive and readily available unlike the calf rennets, which are subject to the uncertainties of the agricultural industry. Fungal rennets may be derived by the fermentation of media containing wheat bran, corn meal, soybean meal, casein, and/or dried milk (Arima and Iwasaki, 1965; Sardinas, 1966; Charles et al., 1970). Simple purification steps may then be used to produce a partially purified preparation enriched in milk clotting activity. Three species, *Mucor pusillus* var. *Lindt*, *Mucor miehei*, and *Endothia parasitica* are the source of the fungal rennets used in the United States. These fungi also readily produce amylase activity when grown in culture (Barnett and Fergus, 1971; Chapman et al., 1975; Fergus, 1969) on many types of media (Adams and Deploey, 1976). The occurrence of amylase activities in commercial fungal protease preparations used as rennet substitutes in cheese making is, therefore, not surprising.

The possibility of amylase contamination in these crude protease preparations has received scant attention, although de Koning et al. (1969) were aware of the presence of amylase activities in bacterial milk clotting preparations. In fact, they suggested that the detection of these enzymatic activities in calf rennets would provide a useful test for their adulteration with the less expensive bacterial

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rennets. Apart from this aspect, the occurrence of amylase activity in rennets would normally be of no concern in cheese making since there is no substrate for this enzyme. However, whey is a major byproduct of the cheese industry, and its production is on the increase (Zall et al., 1982). New uses are being sought for whey, and these might include supplementing starch-containing foods with this highly nutritious product. Under these circumstances, the presence of residual amylase activities might restrict whey usage to nonstarchy foods. The purpose of the work reported here was to characterize and identify such amylase activities in rennets and whey and to determine their fate during subsequent processing into whey protein concentrate (WPC) powder. Particular attention was paid to their heat stability since this is the most logical means for removing residual enzymatic activities in whey. Radiolabeled ^{14}C-starch substrates provided the sensitivity needed for the detection of residual amylase activity (Malacinski, 1971) in whey and food products.

MATERIALS AND METHODS

Rennets and WPC. Emporase, derived from *Mucor pusillus* var. *Lindt*, was obtained from Dairyland Food Laboratories, Inc., Waukesha, WI. Marzyme, derived from *Mucor miehei*, was from Marschall Division, Miles Laboratories Inc., Madison, WI. Sure Curd, from *Endothia parasitica*, was a product of Pfizer, Inc., Milwaukee, WI. Samples of WPC were obtained from local suppliers.

Desalting. Aliquots of rennet (10 mL) were desalted on a 23 × 2.5 cm column of Bio-Gel P10 (Bio-Rad Laboratories, Richmond, CA) that was equilibrated and eluted in 10 mM sodium acetate buffer, pH 4.6, at 30 mL/h at 22 °C. The A_{280nm} was measured on the 3.5-mL fractions. Fractions corresponding to the void volume were pooled. The amylase activity in the desalted microbial rennets was stable for at least 3 months at 4 °C.

Radioisotopic Procedures. ^{14}C-Starch (specific activity 1.4 mCi/mg) was obtained from New England Nuclear, Boston, MA. Scintillation fluid (Pico Fluor 15) was from Packard Instrument Co., Inc., Downers Grove, IL.

Amylase Colorimetric Assay. Reaction mixtures (1 mL) containing 5 mM buffer (sodium acetate, pH 4.9, or potassium phosphate, pH 6.1), 0.05 M sodium chloride, and 0.5% soluble starch (Sigma Chemical Co., St. Louis, MO) and aliquots of desalted rennets were incubated at 35 °C for 3 min. The reducing sugar formed was determined colorimetrically with glucose and maltose as standards as described by Hodge and Hofreiter (1962). One unit of α-amylase or glucoamylase produces 1 μg of maltose/min or 1 μg of glucose/min under these conditions.

Amylase Characterization. Fungal rennet amylase activity was characterized in reaction mixtures (0.2 mL) containing 0.1 M sodium acetate buffer, pH 4.6, chloramphenicol (40 μg), soluble starch (25 μg), and ^{14}C-starch (0.5 μCi). They were incubated with 5 μL of fungal rennet for 4 h at 22 °C. Aliquots (10 μL) were then spotted on 1 × 23 in. strips of 2043-A paper (Schleicher & Schuell, Keene, NH) and chromatographed in a descending manner in 1-butanol–absolute ethanol–water (2:1:1) for 20–24 h at 22 °C. Strips were either sectioned into 1-cm pieces, which were then counted in 7 mL of scintillation fluid in a scintillation counter, or were counted directly in a Packard Model 7220/21 radiochromatogram scanner (Packard Instrument Co., Downers Grove, IL). Identification of the hydrolysis products was established by comparison to standard chromatograms.

Heat Stability. The heat stability of each microbial rennet was determined by heating 80 μL of desalted *M. miehei* or desalted *E. parasitica* rennet or 40 μL of desalted *M. pusillus* rennet in 0.5-mL reaction mixtures containing 10 mM buffer (sodium acetate, pH 4.9, or potassium phosphate, pH 6.1), 0.5 mg of bovine serum albumin, and 0.1 M sodium chloride for the prescribed times at 50 °C. The aliquots were cooled on ice and were then mixed with 0.5 mL of a 1% soluble starch solution and incubated for 3 min at 35 °C. The reducing sugar formed was measured colorimetrically as described above.

The heat stability of each microbial rennet in whey was also determined. Skim milk was coagulated with 0.5% of each rennet for 30 min at 22 °C. The whey was collected by filtration through Miracloth (Calbiochem, San Diego, CA). Samples were heated in test tubes for 30 s at the appropriate temperature and were then cooled in ice. Amylase activity was determined in 0.2-mL reaction mixtures containing 0.1 mL of heated whey and ^{14}C-starch (0.5 μCi). Reactions were incubated for 4 h at 22 °C, chromatographed for 16 h, sectioned, and counted as described above. Amylase activity was estimated by summation of the radioactivity in the maltotetraose, maltotriose, maltose, and glucose from these chromatograms.

Amylase in WPC. Amylase activity was characterized as described in 0.2-mL reaction mixtures containing ^{14}C-starch and 5 mg of each WPC. They were incubated for 24 h at 22 °C. Aliquots (5 μL) were chromatographed as described above and were then scanned to locate the peaks of radioactivity.

Yield Value. Textural measurements were made by using a Haake Viscometer Model VT 24 (Haake Buchler Instruments, Inc., Saddle Brook, NJ) operated at speed 4 and equipped with an FL 100 vaned rotor totally immersed in the sample. The maximum torque required to initiate rotation of the rotor was obtained from the viscometer dial. Three measurements were made at depths from 1 to 7 cm and the readings averaged. Torque measurements were converted to yield values, expressed in dynes per square centimeter, by using a conversion factor supplied by the manufacturer for the rotor used. Higher yield values indicate a stiffer "body".

Table I. Amylase Activity in Desalted Fungal Rennets[a]

fungal rennet	trade name	amylase activity, units/mL pH 4.9	pH 6.1
Mucor pusillus	Emporase		
	lot 1	2.32	1.71
	lot 2	2.87	2.20
Mucor miehei	Marzyme		
	lot 1	0.51	0.41
	lot 2	0.41	0.39
Endothia parasitica	Sure Curd		
	lot 1	0.39	0.30
	lot 2	0.68	0.50

[a] Different lots of fungal rennet were desalted and assayed for amylase activity at pH 4.9 or pH 6.1 by using the colorimetric procedure described under Materials and Methods. Enzymatic activity is expressed as units in the starting material by correcting for the dilution during gel filtration.

RESULTS AND DISCUSSION

Amylase Characterization. Direct assay of the amylase activity in fungal rennets by colorimetric techniques (Hodge and Hofreiter, 1962) was confounded by the presence of reducing sugar in the preparations. These and other low molecular weight compounds were removed by gel filtration. Such partially purified desalted preparations could then be readily assayed for amylase activity by conventional means (Table I). Those rennets derived from *Mucor pusillus* contained the most amylase activity in agreement with Fergus (1969). The *Mucor miehei* and

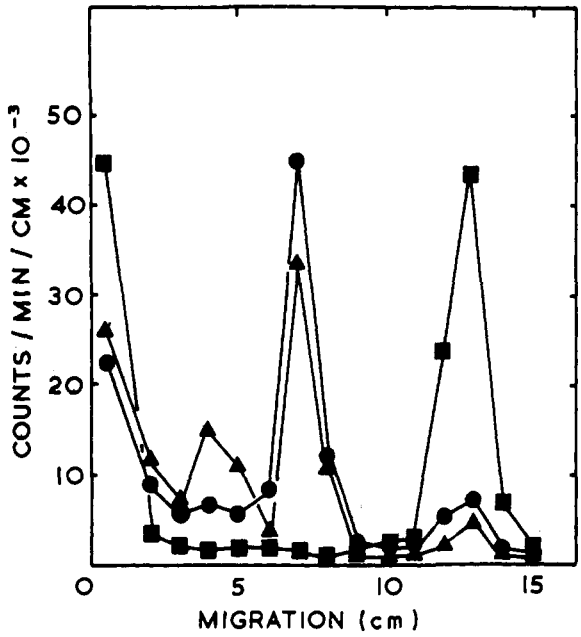

Figure 1. Starch hydrolysis by fungal rennets. Reaction mixtures containing ^{14}C-starch and fungal rennet [*Mucor pusillus* (●), *Mucor miehei* (▲), or *Endothia parasitica* (■)] were incubated, chromatographed, sectioned, and counted as described under Materials and Methods. Sugar standards established the migration of maltotriose to 4 cm, maltose to 7 cm, and glucose to 13 cm.

Endothia parasitica rennets contained equivalent amounts of amylase activity (Table I). Similar enzymatic activity was observed at pH 4.9 and at pH 6.1.

The availability of radioactive starch substrates facilitated the characterization of these fungal rennet amylase activities, which fell into one of two distinct classes (Figure 1). Those enzymes derived from *Mucor* species produced predominantly maltose with lesser amounts of maltotriose and glucose, typical of α-amylase (Bernfield, 1955). In contrast, rennets derived from *E. parasitica* contained glucoamylase enzymatic activity since glucose was produced exclusively from starch (Larner, 1960). Identical ^{14}C-starch hydrolysis patterns were observed between different lots of fungal rennet type.

Heat Stability in Buffers. The survival of these amylase activities was studied when heated in buffers at pH 6.1 and pH 4.9. Fresh sweet whey is normally at pH 6.1. Acid can develop in sweet whey if it is stored without pasteurization, and pH 4.9 was chosen to approximate this condition. Desalted fungal rennets were used to enable enzymatic survival to be followed by colorimetric procedures and also to remove enzymatic stabilizers that may have been included in the fungal rennets. A relatively mild heat treatment was chosen initially to illustrate differences among the rennets. Higher temperatures were included later to approximate processing conditions. Distinct differences were noted in the heat stability of these fungal rennets when heated at relatively low temperatures in buffered systems (Figure 2). The *M. pusillus* α-amylase activity was more heat stable than that derived from *M. miehei*. The *E. parasitica* glucoamylase activity was the least stable. Thunell et al. (1979) in their comparable study of the heat stability of the rennet proteases also found *E. parasitica* protease to be the least stable. However, they found, in agreement with Hyslop et al. (1979), that the *M. miehei* protease was much more stable than that from *M. pusillus*. The relative heat resistance of the *Mucor* amylase and protease activities is correlated with their thermophilic character (Chapman, 1974; Deploey, 1976). *E.*

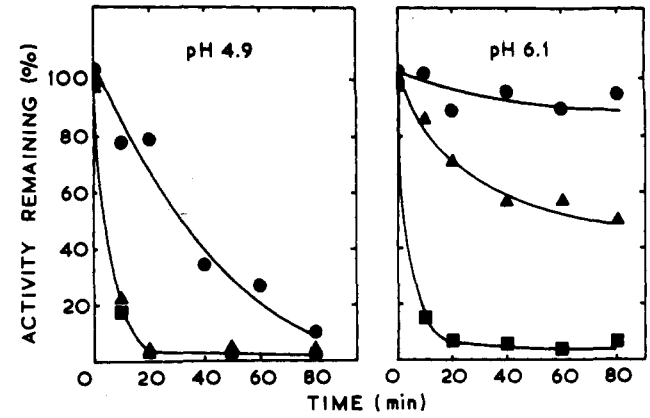

Figure 2. Heat stability of fungal rennet amylase activities. Desalted fungal rennet [*Mucor pusillus* (●), *Mucor miehei* (▲), or *Endothia parasitica* (■)] was heated in buffer at 50 °C for the indicated times and then assayed at pH 4.9 for residual amylase activity by using the colorimetric procedure described under Materials and Methods. Enzymatic activity is expressed relative to that of unheated controls.

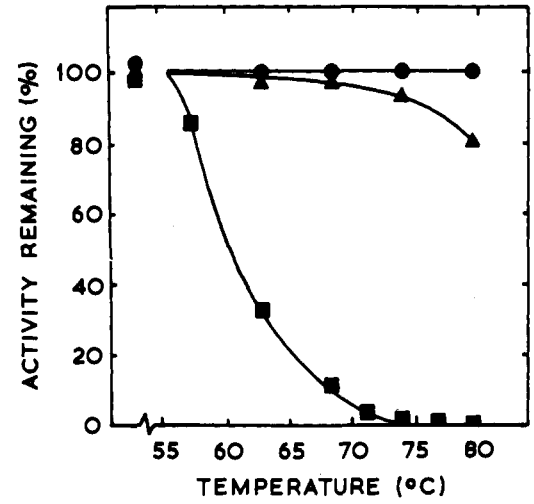

Figure 3. Heat stability of fungal rennet amylase activities in whey. Skim milk was coagulated with fungal rennet [*Mucor pusillus* (●), *Mucor miehei* (▲), or *Endothia parasitica* (■)]. The whey was heated and the amount of amylase activity remaining was estimated as described under Materials and Methods. Activity is expressed relative to that of unheated samples stored on ice prior to assay.

parasitica, in contrast, is a mesophile (Puhalla and Anagnostakis, 1971).

As expected, more rigorous heat treatments caused a greater loss in the enzymatic activity of each microbial rennet. The amylase activity in the desalted *M. miehei* and *E. parasitica* fungal rennets was completely destroyed by heating at 71 °C for 20 min, at pH 4.9 or pH 6.1. In contrast, only 53% or 35% of the amylase activity in the desalted *M. pusillus* fungal rennet was destroyed under these conditions at pH 4.9 or pH 6.1.

All rennet amylase activities were more stable to heat at pH 6.1 than at pH 4.9 (Figure 2). This suggests that inactivation of these enzymatic activities would be facilitated by lowering the pH prior to spray drying or whey pasteurization. Unfortunately, Thunell et al. (1979) found that the fungal rennet proteases showed exactly the opposite behavior, being more stable as the pH was lowered. Thus, processing conditions might need to be tailored to the intended use of whey powder or WPC powder if residual enzymatic activities are to be avoided.

Heat Stability in Whey. Further experiments were conducted to test the effect of processing conditions on

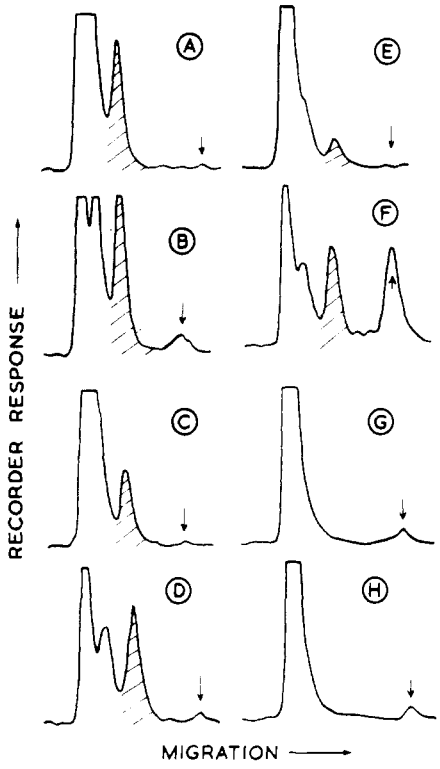

Figure 4. Residual amylase activities in WPC powders. Amylase activity was characterized as described under Materials and Methods [(A) pilot plant sample made by using *Mucor miehei* rennet; (B–F) commercial samples of unknown origin; (G) commercial sample made by using *Endothia parasitica* rennet; (H) no WPC addition]. The origin of each chromatogram is at the left. Sugar standards established the positions of maltose (hatched area) and glucose (arrow).

enzymatic stability in whey. Enzymatic survival was determined by the chromatographic separation of the reaction products generated from ^{14}C-starch upon enzymatic hydrolysis. The results (Figure 3) clearly showed once again the distinct heat sensitivity of the *E. parasitic* enzymatic activity even in whey and the superior resistance of the *M. pusillus* enzyme, in agreement with the observed heat stabilities in buffers. It is of practical importance to note that whey pasteurization conditions of 79 °C for 30 s would be sufficient to destroy the amylase activity in *E. parasitica* rennet but not in the *Mucor* rennets.

Amylase Activity in WPC. As might be expected, amylase activity was detected in WPC powder. Both laboratory and commercial preparations were tested for their ability to hydrolyze ^{14}C-starch. WPC made on a pilot scale with a *M. miehei* rennet contained α-amylase activity (Figure 4A). A substantial portion of the ^{14}C-starch was hydrolyzed to maltose. The excess of substrate remained at the origin of the chromatogram. This whey had been pasteurized at 79 °C for 30 s prior to spray drying. In light of this result, it was not surprising to find that most commercial samples also contained residual amylase activity. Maltose was the principle hydrolysis product (Figure 4B–D). Other commercial WPC samples (Figure 4F) gave complex hydrolysis patterns in which maltose and glucose were present in approximately equal proportions. Such a pattern might be produced by the joint action of both *Mucor* and *Endothia* amylase activities. In some cases (Figure 4E) very little amylase activity was detected. The origin of the rennets and the subsequent whey processing conditions were unknown in most cases (Figure 4B–F).

Table II. Effect of WPC on Starch-Based Salad Dressing Rheology[a]

WPC source	yield value, dyn/cm^2		
	1 day	7 days	14 days
amylase positive			
unheated	1850	1140	440
heated	1940	1940	1890
amylase negative			
unheated	1940	1940	1980
heated	1940	1940	1980

[a] WPC powder (50 g) was heated with 500 mL of water for 20 min at 100 °C. The solution was cooled and freeze-dried. Other samples of the same powder were dissolved and lyophilized but were not heated. The lyophilized material (1% w/v) was gently stirred into a starch-based salad dressing. The product was stored at 22 °C, and the yield values were determined at the indicated times. Amylase-positive and amylase-negative samples were those characterized in parts D and G of Figure 4, respectively.

However, a commercial sample that was known to be made by using exclusively *E. parasitica* rennet had no detectable amylase activity (Figure 4G).

We reasoned that these amylases might be sufficiently active to place starch-based food systems at risk. One of the commercial WPCs (Figure 4D) caused a rapid "thinning" when added to a starch-based salad dressing (Table II). This effect was not observed with an amylase free WPC powder nor when residual amylase activity was destroyed by heating the respective WPC powder in solution. Thus, the indiscriminate addition of WPC powder to starch-based systems may lead to product defects unless caution is exercised.

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Registry No. Amylase, 9000-90-2; glucoamylase, 9032-08-0.

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