THE ISOLATION AND CHARACTERIZATION OF THE EXTRACELLULAR PROTEASE OF PENICILLIUM ROQUEFORTI BP-13

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ABSTRACT

THE ISOLATION AND CHARACTERIZATION OF THE EXTRACELLULAR PROTEASE OF PENICILLIUM ROQUEFORTI BP-13

By

H. Wayne Modler

The extracellular protease of Penicillium roqueforti BP-13 (EC 3.4.4.99) was prepared by shake culturing the fungus in Czapek-Dox broth containing 0.5% of Proteose-Peptone No. 3. A cell free extract (CFE) was prepared and then concentrated by ultrafiltration and pervaporation. The enzyme was isolated from the concentrated CFE by fractionation over a series of Sephadex columns. The final enzyme preparation contained small amounts of peptides and/or amino acids which were present as either impurities or were formed as a result of autolysis. Attempts to remove these small molecular weight components by ion exchange, dialysis, electrodialysis and precipitation with ammonium sulfate were not successful.

The BP-13 protease had a pH optimum of 3.0 and 5.5 for bovine serum albumin and casein, respectively. The enzyme exhibited maximum stability to pH in the range of 3 to 6. The optimum temperature for activity was 45-46 C

when using 1% casein at pH 5.75; this optimum was based on a 9 min end point assay. An Ea of 8000 cal/mole for the hydrolysis of casein was calculated from an Arrhenius plot. Above 46 C, the enzyme was subject to irreversible first order thermal inactivation.

Serine and sulfhydryl protease inhibitors had no significant affect on enzymatic activity when compared to pepsin. Ethylenediaminetetraacetic acid did not alter the activity of the protease. Carboxyl modification with diazoacetoglycine methylester produced over 90% decrease in activity for both the BP-13 protease and pepsin, indicating the presence of aspartic and/or glutamic acid at the active site of the BP-13 protease.

Of 15 peptides and amino acid esters, only L-leucyl-L-tyrosine was hydrolyzed to any detectable degree. Even with this substrate, hydrolysis was too slow to be of value in an assay procedure. The action of the enzyme on the oxidized B chain of insulin resulted in the formation of 14 ninhydrin areas on a peptide map. This was interpreted to be an accurate reflection of the number of peptide bonds hydrolyzed when the very proteolytic nature of the enzyme is taken into consideration.

The two major casein components, α_s- and β-casein, were extensively hydrolyzed and no longer identifiable on polyacrylamide gel patterns of whole casein after 20 hr (30 C, pH 5.75) incubation with the BP-13 protease. Kappa casein appeared to remain relatively unchanged. When the

release of trichloroacetic acid (TCA) soluble nitrogen from a casein solution was followed over a period of 1 hr, striking differences were evident: the calf rennet displayed an initial specificity for the methionyl-phenylalanine linkage of κ-casein, followed by very slow and non-specific hydrolysis of casein in general. The BP-13 protease produced a rapid and nearly linear increase in TCA soluble nitrogen over the same time period. Milk clotting studies revealed that the fungal protease was 14 times as proteolytic as calf rennet for the same milk clotting activity. Milk never formed a smooth firm gel but tended to curdle. This was followed by syneresis and precipitation of the casein from suspension.

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By

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INTRODUCTION

The ripening of "Blue Type" cheeses relies mainly on the lipolytic enzyme system of Penicillium roqueforti according to most literature reports to-date. The typical peppery or piquant flavor of a good quality "Blue Type" cheese is due mainly to the oxidation of the fatty acids, by the spores of P. roqueforti, to methyl ketones, alcohols and minor flavor compounds. This area has been investigated in some depth and is well documented in the literature.

The proteolytic enzyme system of P. roqueforti was known to exist as early in the twentieth century. However, the importance of this system in the ripening of "Blue Type" cheeses has received only a cursory examination.

The main purpose of this investigation was to isolate the extracellular protease or proteases from P. roqueforti and characterize the enzyme(s) with respect to substrate specificity, proteolytic nature (serine, sulfhydryl or acid protease etc.) and its affect on the major casein components of milk (α_s-, β-, and κ-casein). Parameters such as molecular weight, optimum pH, stability to pH, optimum temperature, stability to temperature and

the affect of substrate concentration were an essential part of this investigation. In addition, the milk clotting ability and proteolytic activity of this enzyme were to be evaluated and compared to calf rennet.

LITERATURE REVIEW

The term "Blue" as applied to cheese refers to the blue or blue green veining which permeates the curd of certain mold-ripened cheese. Cheeses which are classified as "Blue" are ripened by the mold Penicillium roqueforti. Such cheese may take on one of many names depending on the country of origin: Stilton, United Kingdom; Gorgonzola, Italy; Roquefort, France; Cabrales, Spain; and Bleu or Blue, Denmark. These are only a few of the Blue type cheeses listed by Scott (1968). In North America, the term Blue is used to describe such cheese manufactured within this hemisphere.

Ripening of Blue Type Cheeses

Thibodeau and Macy (1942) indicate the typical flavor in Blue cheese is not solely a matter of hydrolysis of fat; this is only the first step of a much more complex ripening process. Subsequent oxidation of fatty acids to methyl ketones results in the piquant flavor characteristic of Blue cheese. When properly coordinated with the partial hydrolysis of protein by the proteolytic enzymes of P. roqueforti, a good quality Blue cheese is obtained.

Attempts to hasten the ripening of Blue cheese by adding lipase should also be balanced by adding protease at the same time according to Thibodeau and Macy (1942). These authors also suggest incorporating the lipase and protease enzymes of P. roqueforti into the curd of Blue cheese, providing these enzymes could be obtained in sufficient quantity to be of benefit. The addition of 6.0 g of mycelium per five lb of cheese was found to shorten the ripening period by 50%. Thibodeau and Macy (1942) noted that lipolytic and proteolytic enzymes are not readily liberated from the mycelium and suggested that such enzymes should be extracted, isolated and added to the curd in a more readily available form. This presumably would effect an even shorter curing time.

Variation of Proteolysis in Relation to Strain

Funder (1949) found a wide variation in the proteolytic properties of various strains of P. roqueforti. The difference in enzymatic properties was so great that extremely different results were obtained in cheese making experiments. Often the difference between morphologically similar strains was greater than between two widely different species of Penicillium. This author suggests that more attention should be paid to the physiological variation of the molds, their formation of mycelium and the reaction products formed during ripening of the cheese.

Proks et al. (1956) also noted a large difference in the proteolytic activity of nine strains of P. roqueforti isolated from good Roquefort cheese. The strains differed not only in quantity but also in the types of amino acid released when incubated with casein.

Salvadori et al. (1962a) divided 19 strains of P. roqueforti into three groups according to amino acid patterns obtained by two dimensional chromatography of casein hydrolysates. Based on proteolytic activities, this author established criteria for choosing a mold intended for use in the dairy industry. In a related series of experiments, Salvadori (1962b) studied three strains of P. roqueforti displaying weak, intermediate and strong proteolytic activity. Using paper chromatography and other techniques, Salvadori showed that histidine and methionine increased the proteolytic activity of all three strains. Great importance was attached to selecting the proper strain of Penicillium for the manufacture of high quality Gorganzola cheese.

Niki et al. (1966) observed that strains of P. roqueforti possessing low lipolytic activity, exhibited high proteolytic activity and vice versa. The most suitable strain for manufacturing blue cheese was found to be one which had moderate proteolytic and strong lipolytic activity.

Media for Culturing P. roqueforti

As early as 1910, Dox used a liquid medium commonly known as Czapeks broth to surface culture P. roqueforti. This medium consisted of: 1000 g water, 0.5 g magnesium sulfate, 1.0 g dipotassium phosphate, 0.01 g ferrous sulfate, 2.0 g sodium nitrate and 30 g sucrose. The mold was found to grow satisfactorily on this medium and was harvested after 10 days.

Naylor et al. (1930) found the medium best suited for growth of P. roqueforti and production of protease, contained in 1000 ml of solution: 0.5 g magnesium sulfate, 1.0 g dipotassium phosphate, 0.5 g potassium chloride, 0.01 g ferrous sulfate, 1.61 g ammonium chloride, 2.5 g sucrose and 10 g casein. The pH was adjusted to 5.6 and the mold allowed to grow on the surface of the medium for 10 days at 30 C. The mold mat was removed and the filtrate checked for activity.

Thibodeau and Macy (1942) noted that while some molds thrive on Czapek broth, P. roqueforti grows very poorly when surface cultured. Sodium nitrate, the nitrogen source in Czapeks solution, was one of the poorest sources of nitrogen for P. roqueforti. A modified Czapeks medium, containing less sodium nitrate and added skim milk, was found to satisfy more adequately the requirements for abundant growth and the formation of a thick resistant felt. The maximum proteolytic activity was obtained as soon as the culture had reached the stage of full sporulation.

Meyers and Knight (1958) developed a synthetic medium for the submerged growth of P. roqueforti. This medium contains the same basic ingredients as Czapek-Dox broth, except that sodium nitrate was replaced with ammonium sulfate, oleic acid was added and a small amount of trace metals included. For optimum growth the initial pH of the medium was adjusted to 4.0.

Niki et al. (1966) grew P. roqueforti on rennet whey at 20 C for seven days. For protease preparation, the whey was acidified with lactic acid to a pH of 4.0

Characteristics of P. roqueforti Protease

Naylor (1930) surface cultured P. roqueforti and checked the protease activity of the filtrate by incubating with casein for 48 hr at 30 C. Activity was based on the recovery of casein precipitated from solution as determined by the Kjeldahl method. The optimum pH for protease activity was 5.3 at 30 C.

Thibodeau and Macy (1942) found the optimum pH to be in the range of 5.8 to 6.3 for the protease of P. roqueforti. At this time the enzyme was thought to be trypsin-like but there was some controversy as to the true nature of the protease. The author states that the enzyme is not of the pepsin type and only one protease exists in the mycelium. When this fungus was surfaced cultured, Thibodeau and Macy (1942) noted the protease was released into the culture medium only when autolysis was occurring

in the mycelium. These authors postulated that lipases and proteases are difficult to obtain in solution, possibly because they are absorbed on the walls of the cells and are liberated only when the mycelial tissue is disrupted. The protease was precipitated by half saturation with ammonium sulfate.

Nishikawa (1957) reported the pH optimum for proteolytic activity of P. roqueforti protease to be 5.5 to 6.0 when cultured at 40 C and assayed in the presence of 0.6% casein sol. The enzyme was stable in the pH range of 5.0 to 6.0, but lower on either side of these limits. Maximum activity was observed at 40 C and at 45 C the enzyme was partially inactivated.

Niki et al. (1966) found that P. roqueforti produced an extracellular protease when grown on rennet whey at pH 4.0. In addition, an intracellular protease was also recovered from the mycelium when disrupted. Both proteases were found in varying quantities in different strains of P. roqueforti. One strain, designated BP-13, had the highest amount of proteolytic activity. Both the intracellular and extracellular proteases of the BP-13 strain had a pH optimum of 5.5 with the latter protease having a much narrower range of activity than the intracellular protease. Niki et al. (1966) states the extracellular protease contributes to casein breakdown for a short period during the initial stages of ripening of Blue

cheese, while the intracellular enzyme is active during the entire ripening period.

Motoc (1970) studied the proteolysis of a 1% solution of Hammersten casein by a preparation of proteolytic enzymes obtained by isopropanol extraction of a dried culture of P. roqueforti. Proteolysis was found to be greatest at pH 5.5 and 25 C. Some of the free amino acids released from casein by this enzyme included arginine, serine, aspartic acid, valine and norvaline.

Nomenclature of Rennet and Rennet Substitutes

The term rennet, as used today, refers to the enzyme system extracted from the fourth stomach or abomasum of a suckling calf. Rennin is the major enzyme in this system but as the calf ages, the ratio of rennin to pepsin decreases.

Foltmann (1970) refers to the major milk-clotting enzyme from the abomasum of young calves as chymosin rather than rennin. He indicates that there are three reasons for using the term chymosin: the word rennin resembles renin and leads to confusion with the latter enzyme; more papers are published about renin; and, the name chymosin is 50 years older than rennin.

Sardinas (1968) defined rennet as any crude enzyme preparation of animal, plant or microbial origin which curdles milk. Pure milk clotting enzyme per se, regardless of origin, were designated as rennin. The nomenclature of

Sardinas will be used in this dissertation with the exception that chymosin will refer specifically to calf or veal rennin. Unless otherwise designated, pepsin will refer to the extract from porcine stomach.

Criteria for Choosing Calf Rennet Substitutes

Sardinas (1968), Kikuchi and Toyoda (1970) noted that the supply of calf rennet varied seasonably and is becoming progressively more scant as the result of increased cheese production, decreasing slaughter of calves and diminished exports of calf rennet to the USA by countries which retain the enzyme for home use.

The non-acceptability of products of animal origin to vegetarian populations, particularly in countries like India and Israel, has also stimulated research for microbial and plant rennet substitutes according to Babbar et al. (1965) and Dewane (1960). This latter writer states that a calf rennet substitute should have the following characteristics:

a. Yield of curd comparable to rennet
b. Curd should possess physical properties comparable to rennet curd
c. Loss of fat in the whey should be minimal
d. No detectable flavor defects

Vanderpoorten and Weckx (1972) state that the microbial rennets, whose proteolytic properties most closely resemble those of veal rennet, offer the best chances of

success in cheese manufacture. The microbial protease should act in a similar manner during all three phases of action on casein:

a. Phase one is very specific and is designated the primary reaction. Calf rennet cleaves a glycomacropeptide from κ-casein resulting in destabilization of the "calcium sensitive α_s-casein."

b. During the second phase the casein precipitates.

c. The third phase is slow and consists of unspecific proteolysis. This phase progresses simultaneously with the primary and secondary reaction and during the ripening of the cheese.

Schulz and Thomasow (1970) consider coagulation of milk to be only a small part of the role of the coagulating enzyme in cheese making. These workers attach greater importance to the breakdown of α- and β-casein and other proteins in cheese ripening. In the estimation of these authors, two properties need to be considered when evaluating calf rennet substitutes; the breakdown of κ-casein in the milk and hydrolysis of proteins in the cheese. The breakdown of κ-casein is evaluated by determining the rennet strength. Additional criteria of coagulating properties are elasticity of the coagulum and the ability to separate the curd from the whey. Hydrolysis of the α- and β-casein depends on the nature of the coagulum and the type of cheese being manufactured.

Schulz and Thomasow (1970) also stipulate that the nature of the cheese being manufactured dictates the requirements of calf rennet substitute. For example, in butter or cream cheeses which are ripened at 7 C, the coagulum plays an essential role in maturation. With a cheese such as Emmental the curd is scalded in the whey to temperatures of 50 to 58 C for 20 to 40 min which results in partial destruction of the calf rennet added. In this case, thermolabile rennet substitutes will have little influence on protein breakdown with maturation depending mainly on the enzymes of thermobacteria and propionic acid organisms. Kylä-Siurola and Antila (1970) confirmed these results when the fungal rennets of Mucor pusillus Lindt and Endothia parasitica were used. Schulz and Thomasow (1970) also noted that in soft cheeses such as Camembert, Tilsit and Limburger, smaller amounts of rennet are generally used with ripening being primarily a function of the organism smeared on the surface of the cheese.

The bitterness often associated with calf rennet substitutes may be produced as a result of a different kind of casein breakdown or increased hydrolysis of whey protein (Schulz and Thomasow, 1970). Temperature of ripening, pH and minerals may also affect the production of bitter flavors. On the other hand, the increased proteolytic effect of rennet substitutes is important for accelerated cheese ripening. In view of this, Schulz and Thomasow (1970) feel that future research should be concerned more

with proteolysis by calf rennet substitutes since this may in turn lead to quicker ripening as well as improved cheese flavor. In the future, veal rennet substitutes may have to be designed for a particular variety of cheese, rather than one rennet for all cheese types.

Calf Rennet Substitutes

Animal

Calf rennet-pepsin blends.--Babel (1967); Chapman and Burnett (1968); Thomasow (1971a); Dan and Jespersen (1970); and Emmons et al. (1971) reported similar results when using pepsin-calf rennet blend. Cheese prepared with such a mixture (1:1) of these milk coagulants graded comparable to cheese manufactured with calf rennet. Pepsin alone was not considered an ideal milk coagulant and ripening agent according to Mickelsen and Fish (1970).

Ernstrom (1961) demonstrated the sensitivity of porcine pepsin to pH change in milk while other workers determined proteolysis by chymosin (El-Negoumy, 1968), calf rennet (Sherwood, 1935), and pepsin (Melachouris and Tuckey, 1964; and Sherwood, 1935) in milk, and/or cheese. Results showed pepsin to be less proteolytic than calf rennet or chymosin.

Dan and Jespersen (1970) reported the chief objection to the use of procine pepsin, when used as the sole coagulant, have been defects of taste, consistency

and texture of the resultant cheese. He also noted a slower breakdown of the casein in cheese produced with a 1:1 mixture of calf rennet and porcine pepsin than in a cheese made with only calf rennet.

Both El-Negoumy (1968) and Ledford et al. (1968) observed that β-casein is evidently more resistant to calf rennet proteolysis than α_s-casein. Cerbulis et al. (1960) noted that pepsin does not hydrolyze β-casein extensively. Itoh and Thomasow (1971) reported that pepsin had the lowest proteolytic activity on casein fractions when compared to calf rennet and other microbial rennets. Procine pepsin hydrolyzes most of the protein substrates strongly at its pH optimum of about 2.0 but Fruton (1970) demonstrated that pepsin did not react strongly as a protease above a pH of 6.0. However, pepsin reacts easily on κ-casein near pH 6.5 and converts it into "para κ-casein" which subsequently causes clotting in milk according to Fruton (1970). Melachouris and Tuckey (1964), and Veringa (1961) believe that these properties are the reason for the somewhat slow ripening of the pepsin cheeses. Sherwood (1935) found that it took three times as much porcine pepsin as calf rennet to produce the same total protein breakdown.

Bovine pepsin.--Fox and Walley (1971) made cheese using commercial calf rennet, commercial mixture of calf rennet/pepsin (1:1) and bovine pepsin. All cheese graded

special and no signs of bitterness were evident in the cheeses. Bovine pepsin produced the slowest formation of soluble nitrogen. Electrophoretic patterns of Cheddar cheese were identical after 11 months of ripening, suggesting similar proteolysis had occurred in each cheese. The bovine pepsin did show one additional well resolved peptide not present with either of the other two coagulating agents. Bovine pepsin is currently being used in combination with calf rennet as a milk coagulant for cheese manufacturing in Canada (Emmons, 1972).

Fungal Rennets

The molds Mucor pusillus Lindt, Mucor miehei and Endothia parasitica are currently being used to produce microbial rennets on a commercial basis as a substitute for calf rennet.

M. pusillus.--Tuasaki et al. (1967a, 1967b) studied the properties of the crude chymosin-like enzyme from M. pusillus F-27. This acid protease had a pH optimum of 3.5 for digesting casein. The milk clotting and proteolytic activity of this enzyme resembled that of calf rennet more so than most proteases of fungal origin. In addition, the enzyme was more heat stable and more resistant to pH changes than its traditionally used counterpart. Milk clotting activity was affected by calcium (Ca^{++}) ion concentration to a greater extent than was calf rennet. Curves showing the release of trichloroacetic (TCA) soluble

nitrogen indicated the fungal rennet was less specific than calf rennet. Moving boundary electrophoretic patterns of α_s-casein were similar.

Richardson et al. (1967) found the rennet extract from M. pusillus produced a greater increase in non-protein nitrogen (NPN) in casein sols and in cheese, than either pepsin or calf rennet. Further work revealed the fungal protease activity was dependent on (Ca^{++}) concentration to a greater extent than calf rennet. Normal curd tension could be restored by adding 0.015% calcium chloride to the cheese milk or by adding 10 to 12% additional microbial rennet. The difference in curd tension could also be obviated by using a longer set time. Curd tension can also be increased by raising the "setting" temperature; however, as the temperatures are increased the fungal rennet clot tends to become progressively weaker after a critial maximum of 32.2 C is reached.

When Cheddar, Brick and Parmesan cheese were manufactured by Richardson et al. (1967) employing the fungal rennet of M. pusillus, an increase in the fat content of whey from 0.30 to 0.42% was observed. This is apparently related to slow set and fragility of the curd.

Richardson et al. (1967) found the protease extract of M. pusillus to be very stable in the dry form or in saline solution; however, severe loss in activity was observed when this enzyme was blended and stored with calf

rennet in the liquid form. Results indicate the fungal rennet hydrolyzed the veal rennet.

Trop and Pinsky (1971) found that when chymosin and M. pusillus rennin were mixed together, the coagulation activity increased over 200%. This author concludes that one enzyme system appears to be a synergist to the other, suggesting two separate coagulating mechanisms.

Itoh and Thomasow (1971) reported that the protease of M. pusillus caused no significant increase in NPN when incubated with α_s- and β-casein but demonstrated by starch gel electrophoresis that this enzyme degraded α_s- and β-casein more than did chymosin or pepsin.

E. parasitica.--Sardinas (1968) compared fungal rennet from E. parasitica to animal rennet and found striking similarities with respect to molecular weight, amino acid composition, isoelectric point, pH stability and clotting activity. In addition, this fungal protease was mildly proteolytic, which is an essential attribute for the production of high quality aged cheese.

Berridge (1954) noted that when animal rennet is added to bovine milk below 15 C, no curd is formed, though some alteration of the milk occurs. If the temperature of this milk is increased to 37 C, the milk will quickly clot. The protease of E. parasitica acts in an identical manner, according to Sardinas (1968).

Shovers and Bavisotto (1967) manufactured a wide variety of cheeses with partially purified E. parasitica rennin and found all products to be equal or superior to control cheeses made with animal rennet. Alais and Novak (1970) confirmed these results, although some differences in coagulating and proteolytic activity were observed.

Morris and McKenzie (1970) manufactured Cheddar cheese using microbial rennet derived from E. parasitica. Coagulation times were slightly prolonged when greater than 50% substitution of calf rennet was made. In addition, cheese manufactured with 75% microbial rennet and 25% calf rennet graded slightly lower than controls manufactured strictly from calf rennet.

Nadassky (1972) manufactured Edam cheese from "sure curd" (E. parasitica rennet) and found the course and duration of renneting were the same for both types of coagulants. On this basis, the rennet from E. parasitica was considered suitable for use in Dutch type cheese manufacture.

M. miehei.--A recent report by Thompson (1972) indicates that M. miehei rennet has commercial significance. This author manufactured Cheddar cheese from Mucor rennet and found this enzyme preparation to be equal to calf rennet in all respects: clotting and cutting time, curd firmness and knitting characteristics, cooked curd size,

final curd texture, protein breakdown, body, texture and flavor. In addition, bitterness did not develop when excess rennet was added.

Research by Edelsten and Jensen (1970) revealed that M. miehei rennet contained at least three different coagulation-active components, each with its own characteristic temperature dependence. Sternberg (1971) also indicated the presence of more than one protease in M. miehei.

Comparison of Fungal Proteases

Birkkjaer and Thomsen (1970) manufactured Samsoe and Danho cheese with "Noury-Rennet" (M. pusillus) and "Suparen" (E. parasitica) using "Hansens Fifty-Fifty" animal rennet (50% calf rennet and 50% pepsin) as the control. Both of the microbial rennets took 10-15 minutes longer to form a gel than did the animal rennet. Cheese of good quality could be manufactured with the M. pusillus rennet providing calcium chloride was added but the E. parasitica rennet produced a cheese described as mealy, hard, bitter, acid, off flavor and short in body.

Edwards (1969) manufactured Cheddar cheese with various milk coagulants and found E. parasitica rennet and animal rennet to produce bitter flavors but cheese manufactured with M. pusillus protease did not suffer from this flavor defect. Casein was implicated as the source of the bitter peptides.

In a study by Mickelsen and Fish (1970) the protease from M. pusillus Lindt and E. parasitica both showed greater proteolytic activity than did veal rennet or pepsin on whole, α_s- and β-casein. Of the two fungal rennets, the extract from E. parasitica was more proteolytic on the casein preparations. In general, the fungal rennets produced more NPN when incubated in casein sols and more soluble nitrogen when mixed with cheese paste than did calf rennet or pepsin. Electrophoretic patterns of major casein fractions subjected to the proteolytic action of the enzymes, revealed considerable more proteolysis by the fungal rennets.

Labuschagne and Jaarsma (1970) manufactured Gouda and Cheddar cheese using E. parasitica, M. miehei and two strains of M. pusillus. Results showed the microbial rennets were an acceptable substitute for calf rennet providing slight variations were made in the manufacturing procedure. The milk clotting activity of the microbial rennets was influenced by variations such as ionizable calcium, acidity, temperature of the milk and conditions of pasteurization. These variables were observed to affect animal rennet to a lesser extent. The microbial rennets tended to produce a soft curd and the problem was counteracted by adding calcium chloride to the milk and ripening to slightly higher acidity. Firmer curd could not be obtained by adding more fungal rennet. There was a tendency for the cheese made with microbial rennet to have

higher moisture content. In addition, fat losses in the whey were higher with Cheddar cheese.

Edwards and Kosikowski (1969) manufactured Cheddar cheese using commercially available microbial rennets obtained from the organisms, E. parasitica, M. pusillus and M. miehei. Electrophoretic patterns showed that calf rennet attacked mainly α-casein; E. parasitica acted mainly on β-casein while the two Mucors hydrolyzed both α_s- and β-casein to the same degree. In addition these workers noted that bitter cheese was produced with calf rennet and the Endothia rennet but not with the Mucor rennets.

Vanderpoorten and Weckx (1972) compared the same three commercial fungal rennets with respect to their affect on casein, casein components and casein in cheese. Generally the microbial rennets liberated more NPN from whole casein, α_s- and β-fractions, than did veal rennet. E. parasitica was the most proteolytic towards all casein fractions except for κ-casein. In this instance, calf rennet and the Mucor rennets displayed stronger activity when the release of NPN was followed over a period of two hr. Electrophoretic analysis of casein digests revealed that α_s- and β-casein produced a characteristic pattern for each coagulating agent. Gel patterns indicated that E. parasitica was the most proteolytic except in the case in κ-casein. M. pusillus Lindt and M. miehei exhibited the greatest proteolytic activity on κ-casein.

By electrophoresis of 4 week old Gouda cheese, Vanderpoorten and Weckx (1972) revealed that microbial rennets produce gel patterns which can be clearly differentiated from the electropherogram of Gouda cheese made with veal rennet. All three fungal rennets produced an additional band ahead of the α_s-casein that was not evident when calf rennet was used. E. parasitica produced an extra band behind the β-fraction which could not be demonstrated when the Mucors and calf rennet were used as coagulants. This phenomenon was also observed by other workers (Mickelsen and Fish, 1970; Edwards and Kosikowski, 1969).

Thomasow (1971b) observed that all three commercial fungal proteases and animal rennet attacked κ-casein. The remaining caseins were not attacked by animal rennet to any extent but the fungal proteases had a greater affect on the α_s- and β-caseins. This investigator also observed that pH variation in the milk had the least affect on E. parasitica while pepsin-calf rennet mixtures were most strongly influenced.

Tam and Whitaker (1972) compared the rates and extent of hydrolysis of α_s-, β-, and κ- and whole casein at pH 3.0, 3.5, 5.5 and 6.0 by crystallized chymosin, crystallized pepsin and purified proteases of M. pusillus and E. parasitica. The results obtained can be summarized as follows:

a. E. parasitica protease proved to be more active than the other three enzymes on all substrates at each pH assayed.

b. With α_s-, κ- and whole casein, the initial rates and extent of hydrolysis tended to decrease as the pH was lowered from 6.0 to 3.0

c. All enzyme preparations hydrolyzed β-casein more extensively at pH 3.5 than at 3.0.

d. At pH 6.0, all four enzymes hydrolyzed κ-casein the most rapidly. This was followed in turn by α- and β-casein.

e. The initial rates of hydrolysis of β-casein at pH 6.0 was slow by all enzyme preparations.

f. β-casein was hydrolyzed more rapidly at pH 5.5 than at 6.0 by all of the coagulating enzymes.

Bitterness Associated With Calf Rennet Substitutes

In the preceding section, the data reported by several authors indicates that bitterness is often associated with fungal proteases while others contend that such problems do not exist.

Kikuchi and Toyoda (1970) manufactured Edam, Gouda and Cheddar type cheeses from M. pusillus Lindt and Bacillus polymyxa. Curd produced by the microbial rennets was softer and shattered more easily at cutting when compared to calf rennet. A bitter taste was frequently found in cheese made with microbial rennets, especially in

cheeses made with crystalline enzymes. These authors concluded that the bitter taste in cheeses made with microbial rennets was not caused by the action of contaminant proteases but was an inherent characteristic of the primary enzymes.

In contrast to Kikuchi and Toyoda's theory (1970) Organon (1971) indicates that non-specific enzymes present in the microbial rennets of Mucor, Endothia, Rhizopus, Monascus and Colletotrichum are responsible for bitter flavor. The non-specific enzymes can be removed by adsorption to silicate. The ratio of coagulating activity to proteolytic activity can be increased from a range of 6,000-11,000, to a range of 14,000-20,000 following such treatment.

Richardson and Nelson (1968) reported that fungal milk clotting enzymes produced more bitterness in cheese paste than did rennet or pepsin. Dulley and Kitchen (1972) demonstrated that bitterness can also be produced by calf rennet and is due to the release of simple peptides rather than phosphopeptides as implied by earlier workers.

Despite problems with bitterness, Kikuchi and Toyoda (1970) indicate that microbial rennets could be used to replace the conventional calf rennet in cheese making if certain aspects of manufacture were modified. These included setting temperature, cutting time and cooking method. Optimizing these factors would probably

have a greater affect on curd characteristics rather than actually eliminating the bitterness problem.

Potential Calf Rennet Substitutes

Bacterial Rennets

Bacillus Species.--A bacterial protease produced by B. polymyxa has also received some attention as a calf rennet substitute. Itoh and Thomasow (1971) noted that milk clotting enzyme hydrolyzed casein fractions (α_s, β and κ) extensively at pH 6.5. Proteolysis was not specific but continued as general proteolysis. This was reflected in NPN as well as electrophoretic patterns of each of the major casein components. This bacterial protease has a pH optimum of 8.0 and its proteolytic activity on casein decreases markedly below pH 7.0. Itoh and Thomasow (1971) concluded that application of this protease to cheese making would require proper pH control to prevent over-proteolytic action. If proteolysis of α_s- and β-casein were extensive, bitterness was encountered.

Thomasow (1971b) studied the milk clotting activity of B. polymyxa and Bacillus subtilus in addition to extracts from several fungi. The bacterial proteases exhibited very strong proteolytic activity which resulted in a weak curd. Extensive protein breakdown caused off-flavor and poor consistency in the cheese.

Dutta et al. (1971) examined the protease from B. subtilus a spore forming bacterium. The milk clotting activity (MCA) of this enzyme was affected greatly by the concentration of calcium chloride with the pH optimum for clotting and proteolytic activity being 6.0 and 8.0 respectively. No conclusions were drawn as to the acceptability of this rennet as a calf rennet substitute.

Stefanowa-Kondratenko et al. (1971) prepared a rennet extract from Bacillus mesentericus (strain 76) and studied its suitability for manfacturing Bulgarian cheese from sheep and cows' milk. Results showed the keeping quality and taste of the experimental cheese to be equal to those of conventionally manufactured control cheese.

Melachouris and Tuckey (1968) isolated a milk-clotting enzyme from Bacillus cereus. The clotting activity of this microbial rennet was less sensitive to pH changes of the substrate than calf rennet. This enzyme resembled calf rennet with respect to optimum temperature for clotting and inactivation. The microbial rennet was more proteolytic than calf rennet and degraded casein fractions continuously and non-specifically with β-casein being the most susceptible to hydrolysis. The action of this enzyme on κ-casein was similar to that obtained with calf rennet.

Fungal Rennets

Aspergillus niger.--Osman et al. (1969a, 1969b) studied a number of fungi and found that A. niger (Isolate no. 58) produced extracellular proteases, one of which had high MCA and low proteolytic activity. The course of proteolysis in the first stage of enzymatic action was similar to that of calf rennet.

Basidomycetes.--Kawai and Mukai (1970) and Kawai (1970a, 1970b) surveyed the milk clotting enzyme produced by a large number of Basidiomycetes. Research revealed that rennet extract from two strains, Irpex lacteus (Fr.) and Fomitopsis penicila (Fr.) Karst, could be employed to produce Cheddar cheese of good quality. Rennet of the latter type produced a slightly bitter taste after five months. Like many other microbial proteases, these acid proteases are also affected by (Ca^{++}) ion concentration. I. lacteus had MCA to proteolytic activity ratio which resembled the mucor rennet. Kawai (1970a) indicates the protease of I. lacteus is the most promising Basidiomycete substitute for calf rennet.

Plant Rennets

Papain.--Balls and Hoover (1937) studied the milk clotting action of papain but did not describe its use as a rennin substitute. Dewane (1960, citing Nasher) points

out that when papain is used alone it is not a satisfactory calf rennet substitute.

Ficin.--Whitaker (1959) suggested that Ficin, a sulfhydryl protease similar to papain, could be used in cheese making. In an earlier paper, Krisnamurti and Subrahmanyan (1949) reported that in cheese making trials ficin compared favorably to rennet as a coagulant in that cheeses of equal quality were produced. Ficin is more versatile than calf rennet as it will clot not only animal milk, but also soya milk.

Cardoon.--Other vegetable proteases used include rennet extracts from the flowers of Cardoon (Cynara cardundulus). This extract was traditionally used by farmers of Portugal in making Serra cheese according to Sá and Barbosa (1970a). Cardoon extract was regarded as a satisfactory substitute for animal rennet and was considered more suitable than calf rennet for coagulating sheep milk.

In a related study, Sá and Barbosa (1970b) observed that Cardoon rennet showed higher proteolytic activity than calf rennet and did present some technological problems in Edam cheese making. However, it was a satisfactory clotting enzyme for soft bodied cheeses like Serra and Roquefort although there was some loss in yield with the latter. Sá and Barbosa (1970c) concluded that Cardoon

extract and animal rennet behaved almost identically in both cows' and sheep milk.

Microbial Rennets in General

Behnke (1967) surveyed 20 different rennet preparations of animal and microbial origin. The microbial proteases exhibited high rennet strength and were less dependent upon pH than were calf rennet and porcine pepsin. Generally, microbial proteases have 10 to 100 times the proteolytic activity of pepsin and rennin. Some microbial proteases did not show any primary reactions despite normal rennet strength.

Srinivasan et al. (1970), without specifying the source of microbial rennet, produced good quality soft varieties of cheese from both cows' milk and buffalos' milk. The body, texture and flavor of the cheese produced was found to depend more on the source of the milk than on the type of rennet used.

Kylä-Siurola and Antila (1970) also compared microbial rennets to calf rennet using the normal manufacturing method for Finnish Edam and Emmental cheese. No differences were observed between acidity, dry matter, fat and total nitrogen. In addition, the free amino acid content was about the same and differences in soluble nitrogen at the end of six weeks tended to diminish with continuing proteolysis. There were no differences in the

electrophoretic patterns of cheeses made with animal rennet and the microbial rennets.

Additional Calf-Rennet Substitutes

The vast literature on this subject precludes the practicality of presenting an exhaustive review of calf rennet substitutes. This review has attempted rather to describe the major proteases that are, or have the potential of being, acceptable calf rennet substitutes.

Hundreds of other sources of calf rennet substitutes have been sought and are described or cited by various authors including: Matsubara and Feder (1971), Babbar et al. (1965), Dewane (1960), Labuschagne and Jaarsma (1970), Veringa (1961), Behnke (1967), Sardinas (1968), Kawai and Mukai (1970), Osman et al. (1969a), Arima and Tamura (1967), Abel-Fattah et al. (1972), Sannabadth et al. (1970), Knight (1966), Arima et al. (1970), Genin (1968), Oruntaeva and Seitov (1971), Chaudhari and Richardson (1971).

EXPERIMENTAL PROCEDURES

Microbiological Techniques

Selection of Cultures

Two strains of P. roqueforti were obtained from American Type Culture Collection (ATCC); 6987, 10110. A third strain was provided by Snow Brand Milk Products (Tokyo, Japan) and was designated BP-13 by Niki et al. (1966).

All cultures were carried on Czapek-Dox agar containing 0.75% (w/v) Proteose-Peptone no. 3 (Difco) and 0.75% (w/v) sodium caseinate.

Detection of Proteolytic Activity

Four types of media, poured into disposable plastic petri dishes, were used to qualitatively detect proteolysis.

Type I: This was an improved medium used by Martley et al. [(1970), (Appendix)] to detect proteolysis by a wide variety of organisms.

The sodium caseinate was dissolved in 300 ml of 0.015 M sodium citrate and added to the Standard Methods Agar (SMA) which had been hydrated in 700 ml of the same buffer. The medium was autoclaved at 121 C for 15 min and

just before pouring into sterile petri dishes, 20.0 ml of sterile 1 M calcium chloride was added to a liter of the liquid medium.

Type II: The composition of this medium was the same as Type I except the casein was sterilized using hydrogen peroxide and heat.

Ten g of sodium caseinate was dissolved in 300 ml of water containing 4.41 g of trisodium citrate. To the casein solution, 0.4% hydrogen peroxide was added (v/w) and heated to 55 C for 15 min. The solution was then cooled to 30 C and 0.5 ml of sterile catalase (Nutritional Biochemical Corporation) was added and the solution allowed to stand at room temperature for 8 hr. Residual hydrogen peroxide was assayed by adding saturated potassium iodide solution. The SMA (obtained from BBL), calcium chloride and water were mixed in the same proportion as in the Type I medium, sterilized, cooled to 50 C, then mixed with the sterile casein solution. Sterility of the casein solution was determined by Standard Plate Count.

Type III: Thirty-five g of Czapek-Dox Broth (Difco) was dissolved in 700 ml of water (Appendix). To this, 15.0 g of agar was added and autoclaved to obtain sterility. The casein was prepared as with the Type II medium.

Type IV: The composition of this medium, devised by Meyers and Knight (1958), is listed in the Appendix. After formulation of the medium, 1.5% (w/v) agar was added. The solution was autoclaved, cooled and then mixed

with the peroxide sterilized casein solution as previously described. Prior to pouring, 20 ml of sterile 1 M calcium chloride solution was added.

The plates, containing Types I through IV media, were held at 30 C for 48 hr and stored at 4 C until required for use. Proteolytic activity by each of the three strains of P. roqueforti was detected by streaking 0.1 ml of a standardized spore count (in a 0.01% sterile soap solution) on the surface of each type of agar medium and incubating at 25 C for three to four days.

Standard Plate Counts

Standard plate counts were determined as described by the American Public Health Association (1960).

Spore Counting

Penicillium spores were removed from Czapek-Dox agar slants by adding 100 ml of a 0.01% (w/v) sterile soap solution and shaking gently. An estimate of spore count/ml was made by means of a Spencer bright line hemacytometer.

Shake-culturing

Culturing was carried out at 225 rpm on a gyrotatory shaker (New Brunswick Scientific) held at 25 to 27 C for 72-78 hr. One ℓ erlenmeyer flasks were used for shake-culturing. The broth consisted of 35 g of Czapek-Dox broth and 5.0 g of Protease-Peptone no. 3 per ℓ of solution. Prior to sterilization at 121 C for 15 min, the pH of the

broth was adjusted to 4.0 with 1 N hydrochloric acid. Three hundred ml of broth was added to each flask. Following sterilization, an inoculum of 90 to 100 x 10^6 spores was placed in each flask.

Preparation of Cell Free Extract (CFE)

The P. roqueforti (BP-13), which had been shake-cultured at 225 rpm for 72-78 hr, was added to 250 ml polycarbonate flasks and centrifuged in a swinging bucket type head (International Model K) at 1000 G for 20 min. The spores and mycelial mass compacted at the bottom of the flasks allowing the supernatant to be decanted easily. The CFE was prepared by passing the above supernatant through two Millipore filters, 0.8 and 0.45 μ, then storing in sterilized flasks until needed at 4 C.

Preparative Procedures

Buffers

Universal Buffer.--To obtain effective buffering capacity over a wide pH range, requires a multi-component buffer with pK values approximately 2 pH units apart. Coch Frugoni (1957) describes the preparation of a Universal buffer of constant ionic strength covering the pH range of 2 to 12 at integral pH units. This buffer is 0.04 M with respect to phosphoric, acetic and boric acid (Appendix).

Additional Buffers.--Citrate and other buffers were prepared as described by Dutta and Grzybowsk (1961).

Casein

Sodium Caseinate.--Casein was precipitated from freshly separated skimmilk (0.04% butterfat) at pH 4.6, with 1 N hydrochloric acid and 30 C. After washing the precipitate with copious amounts of distilled water, the protein was redissolved by adding 1 N sodium hydroxide to bring the pH of the suspension back to 7.0. The precipitation and washing process were repeated. The casein was then freeze dried to a final platen temperature of 33 C by means of a Virtis model 42 freeze drier.

Hammersten Casein.--The method of Dunn (1949) was followed for the preparation of Hammersten casein. Sodium caseinate was prepared as previously described with the final casein precipitate being suspended in 95% ethanol. Following a series of ethanol and ether washes to remove moisture and fat, the purified product was held at room temperature for 8 hr to remove the ether and attain moisture equilibrium. The final product contained 6.73% moisture as determined by the vacuum oven method (A.O.A.C., 1960). Hammersten casein was used in all assays for protease activity.

Enzyme Substrate.--A 1% casein sol was prepared by dissolving 10.72 g of Hammersten casein in 800 ml of 0.03 M citrate buffer at pH 8.1. The suspension was placed in a boiling water bath for 15 min, cooled, pH adjusted to 5.75 with 1 N hydrochloric acid and ionic strength brought up to 0.3 with sodium chloride. Following dilution to 1 ℓ with 0.03 M citrate buffer (pH 5.75, μ=0.3) the suspension was filtered through a 0.45μm Millipore filter to remove microorganisms and insoluble material. The 1% casein sol was stored in a sterile flask at 4 C.

Dialysis Tubing

The method of McPhie (1971) was used to prepare the dialysis tubing. Approximately 50 feet of 2.5 cm tubing was placed in 2 ℓ of 50% ethanol and simmered for 1 hr. The ethanol was drained and this treatment repeated. This was followed by two repeated immersions, 1 hr each, in 10 mM sodium bicarbonate. After being submerged in 1 mM EDTA for another hr, the tubing was rinsed in two changes of distilled water for 1 hr each. The tubing was stored at 4 C in distilled water containing 0.02% sodium azide (w/v).

Dialysis

Samples requiring dialysis were placed in 4 ℓ of deionized distilled water and stirred continuously at 0 C by means of a magnetic stirrer.

Pervaporation

Sols in dialysis tubing were pervaporated at room temperature by means of a fan placed 12 to 16 inches from the sample being concentrated.

Lyophilization

Approximately 15 to 30 ml of sample was placed in a 50 ml round bottom flask fitted with a 35/25 ball socket. The flask was then attached to a Rinco evaporator by means of an (24/40 $ - 35/25 ball) adaptor and shell frozen in a dry ice-acetone bath. When solidified, the samples were connected to a glass "udder-type" lyophilizer (Kontes) by means of another adaptor (34/45 $ - 35/25 ball). Vacuum was attained with a Cenco Hyvac 14 pump. A cold finger containing dry ice and acetone was used to trap moisture sublimed from the frozen samples. After 12 to 16 hr the lyophilized samples were removed and stored at 4 C in a desiccator.

Standardized Assay Procedure for Proteolytic Activity

The Lowry modification of the Folin reagent was used for determining proteinase activity (McDonald and Chen, 1965). This modified procedure incorporates a pretreatment with alkaline copper sulfate. Substances that give a positive biuret reaction also produce color with the Folin reagent. Without the copper treatment, only substances containing tyrosine and tryptophane produce color when the

Folin-Ciocalteau reagent is added, according to Herriott (1941).

Assay Procedure

To measure proteolysis, 0.5 ml of the proper dilution of enzyme was added to 2.0 ml of a 1% sol of Hammersten casein incubated at 30 C for 9 min. The reaction was terminated by the addition of 3.0 ml of 6% trichloroacetic acid (TCA). Blank determinations were made by adding TCA to the casein substrate, mixing, then adding the enzyme solution. After standing 20 min the sol was filtered through Whatman no. 44 filter paper. One-half ml of filtrate was then mixed with 5 ml of alkaline copper sulfate-sodium tartrate solution (Appendix). The solution was allowed to stand 10 min at room temperature. One-half ml of 1 N Folin-Ciocalteau reagent was added, and the solution was mixed within 2 sec on a Fisher mini-shaker. The pH of the solution was 9.9 to 10.1 which is the optimum for color development according to McDonald and Chen (1965). The color was developed at room temperature for 1 hr or at 28 C for 30 min. Absorbance was read at 600 or 750 nm depending on color intensity.

Casein Standard Curves

Two standard curves were prepared from Hammersten casein using the Lowry-Folin procedure for color development. From these two curves it was determined that absorbances in the range of 0.0 to 0.19 (0 to 50 µg casein)

and 0.16 to 0.45 (50 to 200 µg casein) would be read at 750 and 600 nm respectively when determining proteinase activity. The two standard curves for casein were prepared by peptizing Hammersten casein in 0.03 M citrate buffer and selecting appropriate alliquots to cover the range desired. To 0.5 ml of the casein sol, 2.0 ml of alkaline copper sulfate-sodium tartrate solution (Appendix) were added with the alkali being 0.2 N sodium hydroxide in 2% sodium bicarbonate. Color was developed as described in the "assay procedure."

Bovine Serum Albumin (BSA) Standard Curves

Two standard curves were prepared in the same manner as described for casein. Absorbances in the range of 0.0 to 0.215 (0 to 50 µg of BSA) and 0.160 to 0.570 (50 to 200 µg BSA) were read at 750 and 600 nm respectively.

Enzyme Unit

One unit of enzymatic activity was defined as that amount of enzyme which would produce a Lowry-Folin absorbance increase of 0.01/min when read at 600 nm or ΔA of 0.012/min at 750 nm when using a 1% sol of Hammersten casein at pH 5.75 as substrate.

Determination of Variables Involved in Assay

Optimum pH

Activity was measured at various pH levels using sols of 1% BSA and Hammersten casein dispersed in Universal buffer. Assays were conducted as previously described.

Stability to pH

In order to determine the stability of the BP-13 protease to pH, enzyme sols were adjusted to integral pH units from 1 to 12 by means of hydrochloric acid or sodium hydroxide. The enzyme sols were held 48 hr at 4 C, the pH adjusted to 5.75 and assayed for residual activity.

Optimum Temperature

The relative activity of the BP-13 protease was determined at 20, 23, 25, 30, 35, 40, 45, 50 and 60 C. This was a nine min end point assay. In a related experiment, activity was assayed at 3, 6 and 9 min to determine the rate of hydrolysis at temperatures of 20, 25, 30, 35, 40, 46, 48, 50 and 55 C.

Stability to Temperature

The protease was incubated at temperatures of 25.0, 30.0, 35.0, 40.0, 45.0, 50.1, 53.2, 56.0 and 59.9 C. For temperatures up to and including 45 C, 0.5 ml samples of enzyme were taken at 10, 20 and 30 min. At temperatures greater than 45 C, samples were removed at 3, 6 and 9 min due to rapid inactivation of the enzyme. In each case

the enzyme was incubated at the above temperatures at pH 5.0 in citrate buffer (0.03M, μ = 0.3). After exposure to various temperatures for the required time interval, 0.5 ml of the enzyme sol was pipetted into test tubes packed in ice. For purposes of assaying for activity, 2 ml of 1% casein (pH 5.75) at 30 C were added to 0.5 ml aliquots of enzyme sol incubated at the same temperature.

Effect of Substrate Concentration

The rate of hydrolysis of two casein sols, 0.5 and 1.0% casein (pH 5.75), were followed over a period of 30 min with the TCA soluble nitrogen being determined by the Lowry-Folin procedure at time intervals of 3, 6, 9, 15, 20, 25 and 30 min. Hydrolysis of a 0.5% casein sol (pH 4.0) was also studied in a similar manner. The casein was dispersed in 0.04 M Universal buffer (μ = 0.3).

Study of Autolysis

An enzyme sol collected from G50/G100 Sephadex columns was incubated at 30 C with samples being taken at 2, 18, and 30 hr. Each aliquot was then analyzed for TCA soluble nitrogen, protein and activity.

Purification of BP-13 Protease

Precipitation of Enzyme

Solvents.--Ethanol, methanol and acetone were added to 50 ml of CFE until solvent concentrations of 60,

70, 80 and 90% (v/v) were obtained. After 12 hr of storage at 4 C the suspensions were centrifuged at 30,000 G for 30 min in a refrigerated International centrifuge (Model HR-1). The supernatant was decanted off and the precipitate dissolved in Universal buffer at pH 5.75. Both fractions were assayed for activity.

Salt.--Ammonium sulfate was added to 50 ml of CFE to obtain final saturations of 40, 50, 60, 70, 75, 80, 85 and 90% at 25 C. The samples were refrigerated (4 C) overnight and then centrifuged and assayed as described in the preceding paragraph.

Ultrafiltration

The CFE was concentrated in an Amicon thin channel filtration system (TCF-10). The membrane (UM 10) was 90 mm in diameter with an apparent pore diameter of 1.5×10^{-3} μm. The manufacturer indicates the membrane has a cut off of 10,000 Daltons when calibrated with polyethylene glycol. Ultrafiltration was conducted under 40 psi of nitrogen pressure in a cold room held at 0 C. Approximately 1000 ml of CFE were concentrated to 50 ml.

Gel Filtration

Preparation of Sephadex.--G50 fine and G100 Sephadex were hydrated in 80 to 90 C citrate buffer (0.04M, pH 5.0, μ=0.3). The beads were allowed to settle five min, then the supernatant and fines were siphoned off. This process

was repeated. Following deaeration of the Sephadex solution, the beads were allowed to hydrate for 12 hr. The K25/45 and K16/100 columns (Pharmacia) were poured as described by Fischer (1971).

Application of Sample.--Fifteen ml of concentrated CFE was applied to the top of the G50 Sephadex column by means of a 10 ml syringe. After all the concentrate had entered the gel, a small amount of buffer was used to elute the sample from the top of the column. The column was then filled with the same citrate buffer and connected to the eluate reservoir. A constant hydrostatic head pressure of 15 to 18 cm was maintained on both the G50 and G100 columns by means of a Mariotte flask. The flow rate was regulated to obtain 1 drop/12 sec or approximately 15 ml/hr.

The partially purified enzyme obtained from the G50/G100 Sephadex was applied in a similar manner to the K16/100 (1.6 x 100 cm) column containing G100 Sephadex.

Chromatography.--The G50 column was run downflow into the top of the G100 Sephadex by means of a flow adaptor. An LV-4 valve (Pharmacia) was placed between the two columns so a major portion of the Proteose-Peptone fraction could be diverted from the G100 Sephadex column (Figure 15). Eluate from the G100 Sephadex column passed through a flow regulating value, and was then monitored at 254 nm using an ISCO dual beam UA-2 analyzer. From the monitor, the effluent travelled through a flow interrupter

valve designed to momentarily stop the flow while the fraction collector changed tubes. Drops of effluent from the flow interrupter valve were recorded as they passed through the drop counter to the ISCO fraction collector (Model 326). After 75 drops (5 ml) the delay timer on the fraction collector was activated. This device compensated for the time the effluent took to travel from the photocell to the flow interrupter valve. This was set for 100 sec for the two K25/45 columns and 150 sec for the K16/100 column. When the delay timer reached 0 sec, the flow interrupter valve would close and the shuttles of the fraction collector advanced a new test tube into position. Each time tubes were changed, this event appeared as a dot on the left side of the recorder paper.

Removal of the Nucleic Acids

Certain preparations of CFE contained excessive quantities of nucleic acids which tended to obstruct the G50 Sephadex column. Rhodes et al. (1971) outlines a procedure for removing nucleic acids. This method involves the use of 10% (v/v) of a 2% (w/v) solution of protamine sulfate added to the CFE adjusted to pH 7.0. After stirring for 20 min the suspension was centrifuged at 15,000 G for 20 min. The supernatant was decanted off, concentrated by ultrafiltration, then applied to the G50 Sephadex as previously described.

Further Purification of BP-13 Protease

Additional fractionation procedures were used in an attempt to remove small molecular weight components from the purified BP-13 protease.

Ion Exchange

Several types of ion exchangers were employed: DEAE, SE and P cellulose (Bio-Rad); IRC-50 and IR-120 Amberlite (Rohm and Haas). The functional group, pK and method of regeneration for each type of ion exchange are listed in Table 8.

Preparation of Celluloses.--DEAE cellulose was hydrated in 1 M sodium hydroxide for 30 min at room temperature. This was followed by 500 ml washes of 1.0 M sodium hydroxide, water, 0.5 M hydrochloric acid, water and 1.0 M sodium hydroxide. The SE and P celluloses did not require this pretreatment.

One gram of each ion exchanger was stirred in a 80 ml of buffer. After settling 15 min, the cloudy supernatant was discarded. Forty ml of the same Universal buffer were added, stirred and treated as before. Two additional repetitions of this experiment prepared the ion exchanger for use.

Preparation of Resins.--Amberlite IRC-50 and IR-120 were prepared for use following the procedure of Bailey (1967). This involves a series of washes with hydrochloric

acid or sodium hydroxide of various strengths, water, and finally buffer for equilibration.

Preliminary Ion Exchange Experiments.--Pilot studies were performed in order to determine the optimum pH for absorption of the enzyme to the ion exchanger. This entailed mixing enzyme and ion exchanger at several pH levels in Universal buffer and then determining the residual activity in the supernatant.

Preliminary experiments were carried out with only the DEAE and SE cellulose at integral pH units of 3 to 7 and 3 to 6 respectively. Twenty ml of Universal buffer (μ = 0.1), 1.0 g of ion exchanger, and approximately 100 units of purified enzyme were mixed and centrifuged. The supernatant was then assayed for activity.

Ion Exchange Experiments.--Each of the ion exchangers was used under varying conditions of pH and/or ionic strength (Table 10) in an attempt to further purify the BP-13 protease. The cellulosic exchange resins were packed in 0.9 x 15 cm columns (K15/30, Pharmacia) to a height of 8 to 10 cm. A flow rate of 5 to 10 ml/hr was established for all columns. pH and ionic strength gradients were prepared by means of a GM-1 gradient former (Pharmacia).

Electrodialysis

Electrodialysis equipment was fabricated from a 1 gal Nalgene container, Plexiglass, nylon bolts and stainless steel (Figure 25). The bottom end of the dialysis tubing (7.5 cm, flat width) was doubled back 2 cm, then placed on the 1/4 inch Plexiglass frame. The nylon bolts were tightened down and the frame was then inserted into the slotted Plexiglass head fitted on top of the Nalgene container. Two stainless steel electrodes (0.065 inches in diameter) were centered 1.5 inches from the dialysis tubing. Protein sols were injected into the cellulose tubing through a slot in the upper portion of the Plexiglass frame. The electrodes were connected to a Heathkit high voltage power supply (Model lP-32) with a maximum potential of 400 V at 150 ma. The surface of the dialysis tubing was continuously flushed with deionized distilled water obtained from a 20 ℓ Mariotte flask. Following electrodialysis the enzyme was freeze dried.

Analytical Procedures

Protein

Lowry-Folin.--Protein was determined in the same manner as described for the preparation of the standard curves. An absorbance of 0.214 at 750 nm was equivalent to 51 µg of purified BP-13 protease as determined by the Kjeldahl procedure.

Kjeldahl.--From 5 to 50 mg of protein were mixed with 4.0 ml of digestion mixture (Appendix), heated for 2 hr, then cooled. After the addition of 1 ml of 30% hydrogen peroxide the digestion was continued for 2 hr. Upon cooling, the sides of the flasks were rinsed down with distilled water. The digestion mixture was then neutralized with 20 ml of 40% NaOH and approximately 35 ml of distillate was collected in 15 ml of 4% (w/v) boric acid. This solution was then titrated with 0.060 N HCl to the same pH as the blank. Recoveries of nitrogen were checked with DL tryptophan which had been desiccated over phosphorus pentoxide for one month. This method for nitrogen determination is a modified AOAC procedure (1960).

Carbohydrate

Carbohydrate was qualitatively determined by the Molish test (Clark, 1964). This consists of adding 0.5 ml of a 0.5% (w/v) protein sol of BP-13 protease to 0.1 ml of 5% alcoholic α napthol and mixing. Without agitation, 1 ml of concentrated sulfuric acid was layered beneath the aqueous solution. Glucose was used as a control in this experiment.

Acrylamide Gel Electrophoresis

All electrophoretic analyses were made using an EC 470 vertical gel electrophoretic cell (E.C. Apparatus) equipped with a buffer pump to circulate electrode buffer from the lower to upper chamber (Jordan and Raymond, 1967).

Alkaline Gels.--Alkaline gels were prepared and protein samples electrophoresed by the method of Melachouris (1969). This is a discontinuous system in which the 8% (w/v) running gel (Cyanogum 41, E.C. Apparatus) was dissolved in 0.38M Tris-HCl buffer at pH 8.9 while the 5% spacer gel (w/v) utilized 0.062 M Tris-HCl buffer at pH 6.7. After adding 0.1% (v/v) N N N'N' Tetramethylethylene-diamine (TEMED) the gel solution was filtered through Whatman no. 44 filter paper and stored at 4 C until required for use. Gels were warmed to 25 C and polymerized with ammonium persulfate; 0.5 ml of a 2% solution (w/v) per 50 ml of gel.

Spacer and running gels were poured using the procedure described by Jordan and Raymond (1967). This method consists of occluding the bottom of the column with running gel by placing the cell at an angle of 45° while 50 ml of running gel polymerized. Excess buffer was removed from the surface of the blocking gel by means of a sponge strip. This was followed by the addition of running gel which is allowed to polymerize with the cell in the vertical position. Again the excess buffer was removed from the surface of the running gel. The electrophesis cell was then placed in the horizontal position, slot former inserted, and chamber filled with spacer gel. Following polymerization, the excess spacer gel was removed. After the addition of 2 ℓ of electrode buffer to both the upper and lower chambers, the 8 place teflon slot former was

removed and samples applied by means of a 50 µl syringe. Sample size ranged from 15 to 20 µl of a 5% protein sol dissolved in spacer gel buffer diluted 1:1. When urea was not employed, 5% sucrose was added to increase the specific gravity of the samples. Bromphenol Blue (J. T. Baker) was also added as a marker to each protein suspension. Electrophoresis was performed at 225 v for 2.5 to 3.0 hr.

Alkaline gels were also run utilizing 6M urea in both running and spacer gels. When 6M urea was added to the protein samples, sucrose was omitted.

Acid Gels.--A discontinuous system described by Jordan and Raymond (1970) utilizes 0.2 M tris-citric acid in 12% (w/v) acrylamide gel and 0.37 M glycine-citric acid in the electrode chamber. Twelve percent acrylamide was found to be too high and this was reduced to 8% in all acid gels. The catalyst system is composed of 0.7% ascorbic acid, 0.0025% ferrous sulfate with 0.03% hydrogen peroxide (30%) added immediately prior to pouring. Coolant was circulated through the cell to remove the heat of polymerization; otherwise the gel would contract on cooling, leaving air spaces between the gel slab and cooling plates. A spacer gel was not poured. Basic Fuschin (Sigma) was used as a marker. Electrophoresis was conducted at 225 v for 3.0 to 3.5 hr.

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis for Determination of Molecular Weight.--Five percent acrylamide gels containing 0.1% SDS (Pfaltz and Bauer Inc.) were used for the determination of molecular weight. This is a continuous buffer system which utilizes 0.1 M phosphate buffer at pH 7.1 (Shapiro, 1967). Protein samples were dispersed in phosphate buffer containing 1% SDS and denatured by heating to 37 C for 3 hr. A short prerun of 15 min was made prior to introducing protein samples into the slots. Electrophoresis was performed at 75 v (95 ma) for 12 hr. Protein standards included aldolase, ovalalbumin, chymotrypsinogen A, ribonuclease A (Pharmacia) and pepsin (Calibiochem).

Staining and Destaining of Acrylamide Gels

Amido Black 10B.--Gels prepared by the method of Melachouris (1969) were stained for 8 min in a solution of 0.25% Amino Black 10B (E.C. Apparatus). The dye was dissolved in methanol, water and glacial acetic acid (5:5:1).

The same acrylamide gels were destained electrophoretically in 7% (v/v) acetic acid.

Coomassie Blue.--Prior to staining with Coomassie Blue, the gels were fixed for 1 hr in a solution of 15% TCA. Staining required 6 to 8 hr immersion in a 1%

aqueous solution (w/v) of Coomassie Blue diluted 1:10 with 15% TCA.

The excess stain was removed by soaking in 15% TCA for 12 to 20 hr. Both acid and SDS gels were stained and destained by this procedure.

Absorption Spectra

Enzyme and nucleic acid-protein peaks were scanned from 300 nm down to approximately 240 nm with a Beckman scanning spectrophotometer (ACTA). The peaks were scanned at the rate of 0.1 nm/sec with the recorder set at 20 nm/inch.

Analysis of Insulin (B Chain) Hydrolysates

Hydrolysis.--Ten mg of oxidized B chain of Insulin (Mann Research Laboratories) was dissolved in 1.0 ml of 0.1 M ammonium acetate buffer, pH 7.2. When all the substrate was in solution, the pH was lowered to 4.25 with 0.5 N acetic acid. After the addition of 100 µg of enzyme, the solution was incubated at 30 C for 20 hr. Following centrifugation to remove insoluble material, the solution was concentrated to approximately 0.25 ml.

High Voltage Electrophoresis.--Forty to 50 µl of concentrated hydrolysate was spotted on Whatman 3 MM paper (46 x 57 cm) and dried by a stream of hot air. The entire solid support, except for the area dried, was dipped in a

pyridine, acetic acid and water (0.1:1.0:109) solution at pH 3.6. The paper was removed and dried to approximately twice the original weight. The same buffer system was used in the electrophoretic run, with varsol being used as the coolant. Electrophoresis was carried out at 2500 v (approximately 150 ma) for 1 hr on a High Voltage Electrophorator, Model D (Gilson Medical Electronics).

Descending Paper Chromatography.--After drying the electropherogram at 80 to 90 C, descending chromatography was conducted at 90° to the previous run. The mobile phase consisted of butanol, acetic acid and water (4:1:5). The three solvents were mixed together with the two phases being partitioned by means of a separatory funnel. The light phase was used in the descending trough while the heavy phase was placed in the lower portion of the chromatographic chamber. The chromatogram was allowed to develop for 15 to 18 hr, then removed and dried at 80 to 90 C. After spraying with ninhydrin reagent (Appendix) the chromatogram was heated to 100 C in a closed cabinet, to activate the color reaction.

Hydrolysis of Synthetic Substrates

Nine substrates were peptides blocked on the N terminal end by a carbobenzoxy or acetyl group. Four peptides were not blocked on the N terminal end while two substrates were arginine esters. Hydrolysis of all substrates, except the two ester derivatives, was assayed

using ninhydrin. Each substrate was made up to a concentration of 10 mM by dissolving in dilute NaOH and warming to 35 C. All substrates are listed in Table 11.

Peptides.--The reaction mixture consisted of 0.5 ml of substrate, 0.45 ml of 0.1 M citrate buffer at pH levels of 3, 4 and 5 (μ = 0.4) and 0.05 ml of enzyme solution (1 μg/μl). Following incubation at 30 C for 1 hr, 2.0 ml of ninhydrin solution (Appendix) were added. Glass marbles were placed on the test tubes with color development being carried out at 100 C for 20 min. After cooling, 5.0 ml of 50% n-propanol were added. Absorbance was read at 520 nm in a Beckman DU-2 spectrophotometer. This is a modified method of Clark (1964).

Esters.--

1. p-Tosyl arginine methyl ester (TAME)

The BP-13 protease was assayed at pH levels of 3, 4 and 5 (Universal buffer) using a substrate concentration of 1.05×10^{-3}M. One hundred μg of enzyme was added to 3.0 ml of the substrate and the reaction followed over a period of 10 min at 25 C. Hydrolysis of TAME results in an increase in absorbance at 247 nm.

2. N-Benzoyl-L-Arginine ethyl ester (BAEE)

Again the protease was assayed at the same pH levels as TAME using 1.0×10^{-2}M BAEE. After incubation at 30 C for 1 hr the samples were titrated with 0.0498 N sodium hydroxide to the initial pH of each reaction mixture.

Determination of Molecular Weight by Gel Filtration

Molecular weight standards used for calibration of the G100 Sephadex column (1.6 x 100 cm) included aldolase, ovalalbumin, chymotrypsinogen A and ribonuclease A with molecular weights of 158,000, 45,000, 25,000 and 13,700 respectively. Five mg of aldolase, chymotrypsinogen and 30 mg of sucrose were added to 1.5 ml of 0.05 M phosphate buffer (pH 6.88, μ = 0.4) mixed and allowed to stand for 10 min. After centrifugation at 1000 G for 10 min, 0.75 ml of the clear supernatent was added to the top of the G100 Sephadex. The two standards were eluted with the same phosphate buffer. A second solution consisting of ovalalbumin and ribonuclease A was treated in an identical manner. The void volume was determined using 0.5 ml of a 0.5% Blue Dextran 2000 solution. Kav were calculated by:

$$Kav = Ve - Vo \; / \; V_T - Vo$$

where Ve = elution volume

Vo = void volume

V_T = total volume

Enzyme Inhibitors

Serine Protease Inhibitor

The activity of trypsin (Mann Research Laboratories, 2x crystallized) porcine pepsin (Calbiochem, 3x crystallized) and purified BP-13 protease were assayed in the presence of

1.0×10^{-3} and 1.25×10^{-3} M concentrations of phenylmethyl sulfonylfluoride (Calbiochem, B grade). The serine protease inhibitor was dissolved in 2-propanol then diluted 1:10. Trypsin was dispersed in Universal buffer (pH 8.0) containing 5% 2-propanol and 1.5 mM calcium chloride while pepsin and BP-13 protease were dispersed in the same solvent system buffered to pH levels of 2.5 and 3.0, respectively. Ten ml of each enzyme sol (60 µg/ml) was added to 10 ml of inhibitor at concentrations of 2.0×10^{-3} and 2.5×10^{-3}M, then incubated for 20 min at 25 C. The substrate (1% BSA) used for assaying proteolytic activity was adjusted to the same pH as the enzyme sol. When assays for activity were conducted above pH 4.0 the BSA was denatured by heating for 5 min in a boiling water bath. Activity was assayed as previously described.

Sulfhydryl Inhibitors

Iodoacetamide and p-chloromercuribenzoate (PCMB) were used at concentrations of 2.4×10^{-7}M in enzyme solutions of pepsin, BP-13 protease and papain (Calbiochem) adjusted to pH levels of 2.5, 3.0 and 8.0 respectively. Five ml of inhibitor (4.8×10^{-7} M) and 5.0 ml of enzyme (60 µg/ml) were mixed and incubated at 30 C for 1 hr and assayed at the appropriate pH using 1% BSA as substrate.

Carboxyl Inhibitor

Synthesis.--Diazoacetoglycine methylester was synthesized by the procedure of Riehm and Scheraga (1965). A solution of 9.1 g of glycylglycine methylester hydrochloride and 5 g of sodium nitrate in 40 ml of 2 M sodium acetate was cooled in an ice bath. Two ml of glacial acetic acid was added and the reaction mixture allowed to stand for 2.5 hr at 0 C. The diazoacetoglycine methylester was extracted with 25 ml aliquots of chloroform. Petroleum ether (30 - 60 C) was added to the combined chloroform extracts until the solution became turbid. The mixture was allowed to stand overnight at room temperature and the resulting precipitate was filtered and dried over phosphorous pentoxide for 12 hr. This material was then recrystallized from chloroform by the addition of petroleum ether and dried as before.

Reaction With Enzymes.--The BP-13 protease, trypsin and pepsin were dialyzed against distilled water for 72 hr to remove inorganic ions such as chloride and sulfate which can promote decomposition of diazoacetoglycine methylester, according to Means and Feeney (1971b). Perchloric acid was then used to adjust all enzyme sols (100 µg/ml) to pH 5.0. Two mg of inhibitor was added to 5 ml of each enzyme sol and incubated for 12 hr at 30 C. Pepsin, BP-13 protease and trypsin were then assayed for activity at pH levels

of 2.5, 3.0 and 8.0 respectively using 1% BSA in Universal buffer.

Effect of EDTA and Calcium

Sols of the BP-13 protease were made 10 mM with respect to disodium EDTA and calcium chloride, then assayed for activity using 1% BSA in Universal buffer at pH 3.0.

Milk Clotting and Proteolytic Activity of BP-13 Protease

Milk Clotting

The assay for milk clotting activity was based on the time in sec required to form curd fragments when 1 ml of enzyme sol was added to 10 ml of a 5% sol of skimmilk powder. The low temperature non-fat dry milk was dispersed in 0.03 M acetate buffer at pH 5.5 (μ = 0.3) containing 10 mM calcium chloride. Milk clotting activity (MCA) was defined as

$$MCA = \frac{2400}{t\ (sec)} \times \text{dilution factor}$$

where "t" is the time in sec required for curd formation (Kawai and Mukai, 1970).

TCA Soluble Nitrogen

As soon as the milk had clotted, 2.5 ml of the coagulated solution was added to 3.0 ml of 6% TCA. The soluble nitrogen for both calf rennet and the BP-13 protease were determined by the Lowry-Folin method.

Proteolytic activity of the two milk coagulants was followed over a period of 1 hr by incubating an appropriate aliquot of enzyme with a 1% sol of casein at pH 5.75 and sampling at intervals of 1, 5, 10, 20, 40 and 60 min. Enzyme concentrations were adjusted so initial rates of hydrolysis were approximately the same after 1 min. TCA soluble nitrogen was determined as above.

Gel Electrophoresis of Casein Hydrolysates

Approximately 100 units of enzyme was added to 10 ml of a 5% casein solution which was incubated at 30 C. One-half ml samples were taken at 1 min, 5 min, 10 min, 30 min, 3 hr and 20 hr and added to 0.5 ml of spacer gel buffer containing 7 M urea. Thirty-five µl samples was applied to the slots with the exception of the control in which case 15 µl of a 5% casein sol were added. Both the 5% spacer gel and 8% running gel contained 6M urea. Alkaline gel electrophoresis was then performed as previously described.

Evaluation of Milk Clotting Ability

The BP-13 protease was evaluated as a calf rennet substitute in small scale cheese making experiments. Twenty lb batches of whole milk (pasteurized at 145 for 30 min) were added to stainless steel containers and incubated at 30 C. The milk was inoculated with 1% starter culture (Streptococcus lactis) and after an increase of 0.02% in titratable acidity, the milk was divided into

two 10 lb batches. At this point, 0.9 ml of single strength calf rennet was diluted with 50 parts of water and added to one lot of milk. The amount of BP-13 protease added to the remaining batch of milk was equated to calf rennet on the basis of TCA soluble nitrogen produced in nine min using the standard assay procedure previously described. Five ml of CFE concentrate, obtained by ultrafiltration, corresponded to approximately 0.5 ml of single strength rennet in terms of proteolytic activity.

RESULTS AND DISCUSSION

Enzyme Production

Detection of Proteolysis

When proteolysis of casein occurs in agar medium, a white zone of precipitation is formed due to the deposition of insoluble para-caseins mainly "para-K-casein." The extent of proteolysis exhibited by the Penicillium roqueforti is reflected by both the size and type of precipitation (Martley et al., 1970).

The four types of media are compared with respect to clarity of zone of precipitation and amount of mold growth (Table 1). Types III and IV media supported the best growth of all strains of P. roqueforti. This is to be expected as both media are formulated specifically for fungal growth. The Martley media (Types I and II) are used mainly for the detection of proteolytic organisms in total bacterial counts.

Type I medium was autoclaved and this resulted in some visible precipitation of the casein. As a consequence, the zone of precipitation was masked to a certain extent and was not as sharp as in the case of Type II and III media where the casein was sterilized by a combination of hydrogen peroxide and low heat treatment.

TABLE 1.--Qualitative detection of proteolysis by P. roqueforti (BP-13) when grown on various types of media.

Medium	Growth of P. roqueforti (BP-13)	Clarity of Zone of Precipitation
I. Martley (Autoclaved)	++[a]	Sharp
II. Martley (H_2O_2)	++	Very Sharp
III. Czapek-Dox (H_2O_2)	+++[b]	Very Sharp
IV. Meyers (H_2O_2)	+++	Medium White: Zone of Precipitation Unclear

[a] ++ Very good.

[b] +++ Excellent.

Type IV medium was unacceptable for detecting proteolysis due to opaqueness which formed as soon as the calcium chloride was added to the liquid medium. Trace quantities of metals such as Zn, Cu, Mn and Mo in the medium may have contributed to the partial precipitation of casein at this point. When P. roqueforti was streaked on this medium, the zone of precipitation was unclear.

Type III (Czapek-Dox) was selected as the best medium for detecting proteolysis. All three strains of P. roqueforti (ATCC 10110, ATCC 6987 and Japan BP-13) produced a zone of precipitation on the agar medium. This zone extended from 0.5 to 1.0 cm beyond the mycelial growth,

indicating that all strains were producing an extracellular protease or proteases. The zone of precipitation was approximately the same size with each of the three strains; however, the BP-13 strain exhibited slightly more proteolysis within the immediate vicinity of the mycelium. This was evident from the clearing of the precipitated casein.

The BP-13 strain was selected for shake culturing experiments on the basis of the above results and earlier reports by Niki et al. (1966) in which the authors indicated this strain produced large amounts of extracellular protease.

Choice of Broth for Shake Culturing

The BP-13 protease was grown on Czapek-Dox and Meyers broth at pH increments of 4, 5 and 6. Although the medium devised by Meyers and Knight (1958) was specifically formulated for the growth of P. roqueforti, no major differences were evident when compared to Czapek-Dox broth (Table 2). Both types of broth supported the best growth of fungi at pH 4.0. The Czapek-Dox broth was selected for culturing experiments on the basis that it was easier to prepare and could also be purchased commercially.

Shake-Culturing Experiments

The fungi was cultured using varying amounts of added Tryptone and Proteose-Peptone No. 3 (Figure 1 and Figure 2, respectively). Analysis of the CFE for activity revealed that the broth containing 2.0% added Tryptone had

TABLE 2.--Effect of media and pH on the growth of P. roqueforti (BP-13).

Broth	Growth of P. roqueforti (BP-13)		
	pH 4	pH 5	pH 6
Czapek-Dox	+++[b]	++[a]	++
Meyers	+++	++	++

[a] ++ Very good.

[b] +++ Excellent.

approximately three units/ml after 86 hr of culturing while 0.5 and 1.0% Protease-Peptone induced the release of over 4.5 units of protease activity per ml during the same time period. When inducer was omitted from the Czapek-Dox broth, little proteolytic activity could be detected in the CFE.

In succeeding culturing studies, 0.5% Proteose-Peptone was added to the liquid broth for two reasons:

a. Isolation of an extracellular protease would entail removal of added inducer.

b. This concentration produced 97% as much protease, after 86 hr of culturing, as did 1% inducer.

The data in Figure 3, compares the various strains of Blue cheese mold with respect to their ability to produce extracellular protease. Both ATCC cultures failed

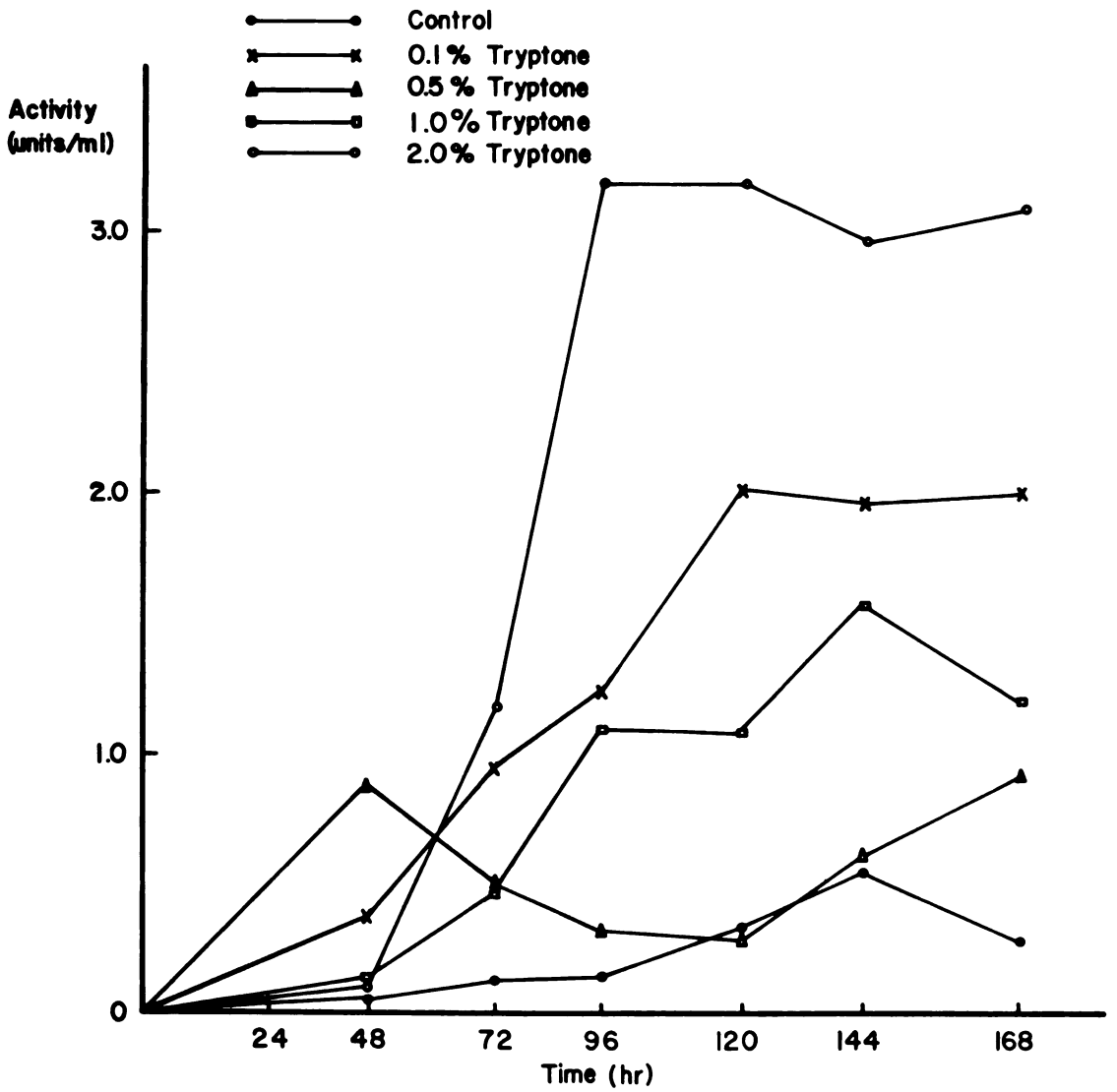

Figure 1. Effect of Tryptone on production of extracellular protease by P. roqueforti (BP-13) when shake cultured in Czapek-Dox broth.

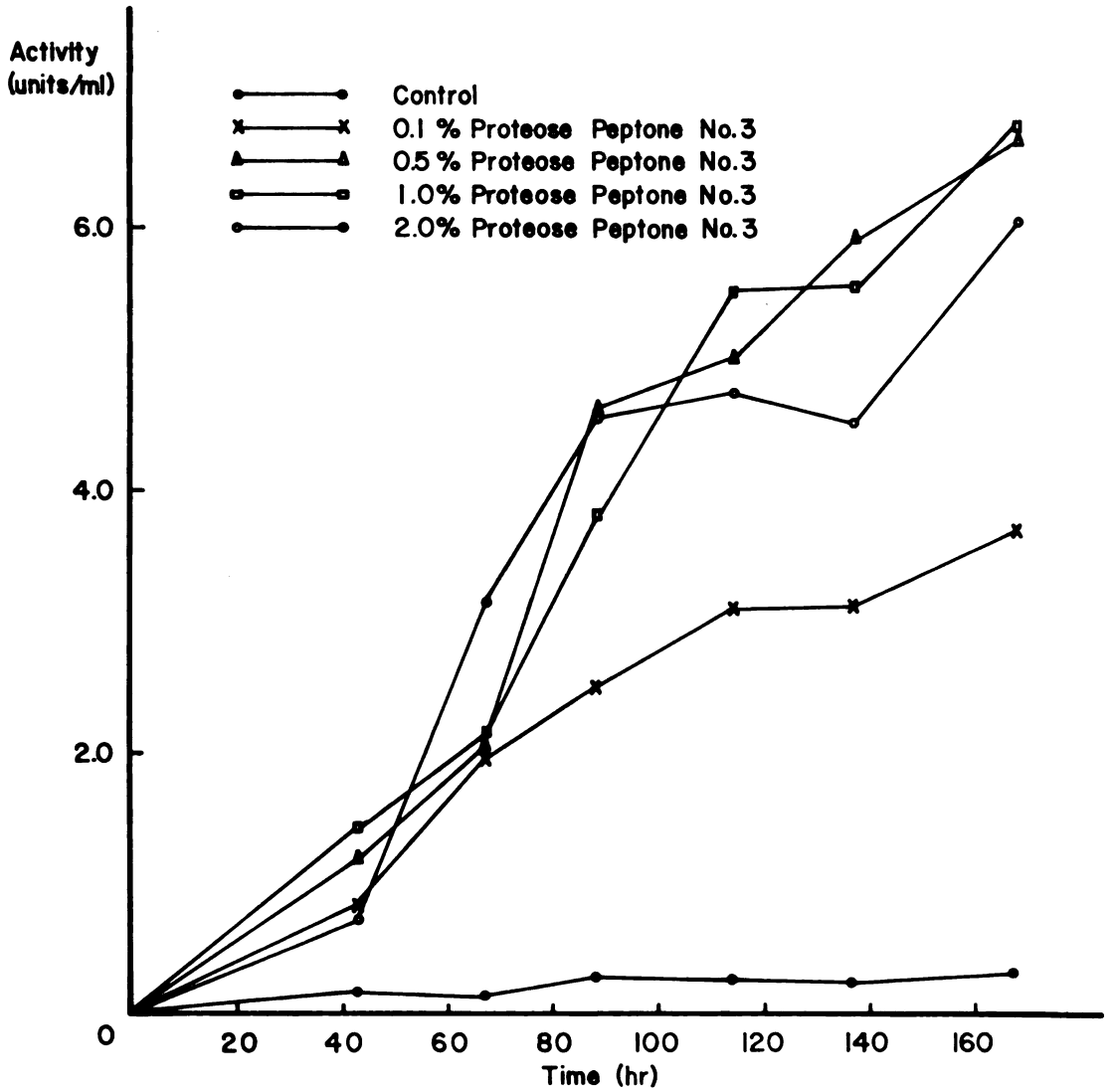

Figure 2. Effect of Proteose-Peptone no. 3 on production of extracellular protease by P. roqueforti (BP-13) when shake cultured in Czapek-Dox broth.

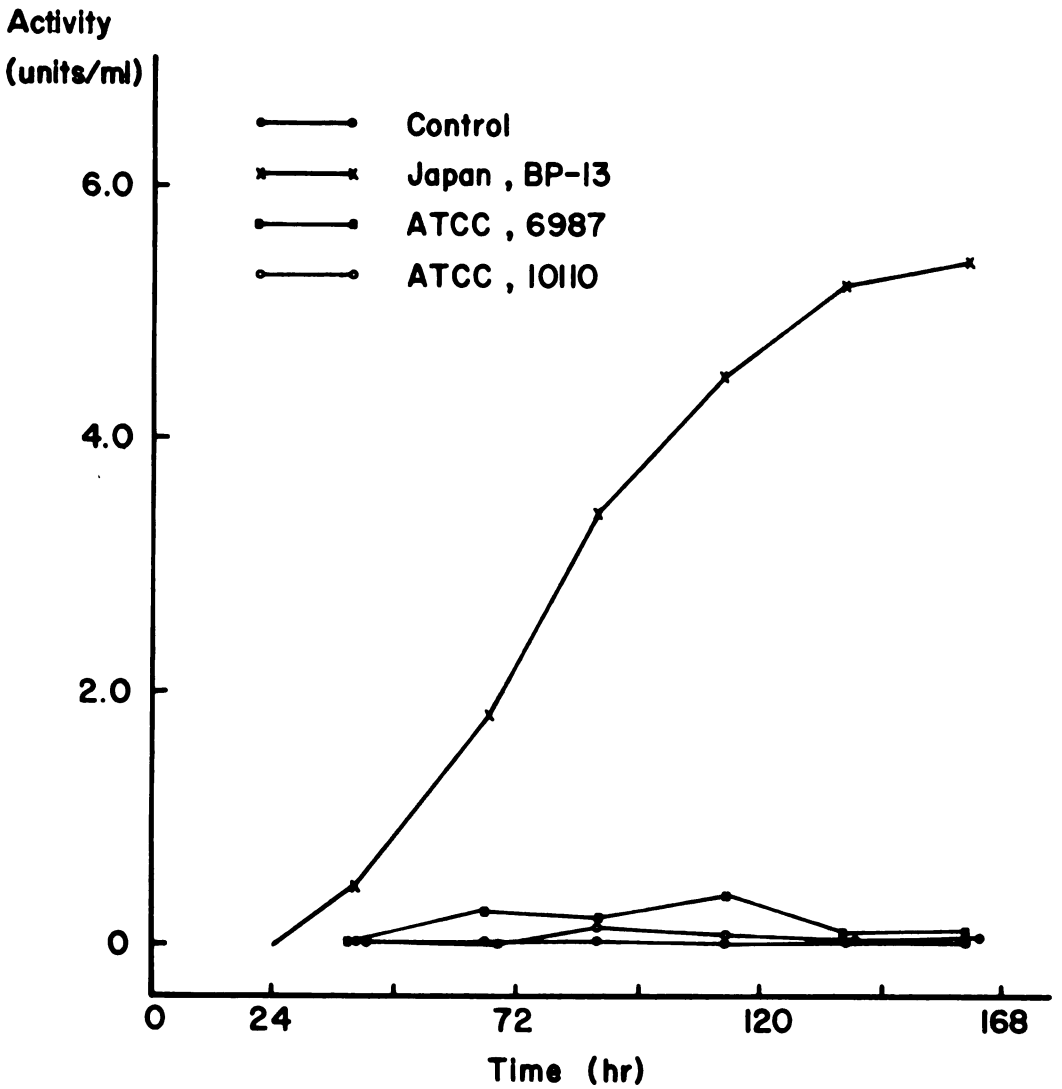

Figure 3. Production of extracellular protease by various strains of P. roqueforti during shake culturing in Czapek-Dox broth containing 0.5% Proteose-Peptone no. 3.

to produce significant quantities of protease while the Japanese strain showed high activity in the CFE.

The BP-13 strain of P. roqueforti began to sporulate after 60 to 66 hr of shake culturing at 225 rpm and 25 to 27 C (Figure 4). Culturing was terminated after 72 to 78 hr. If culturing was extended beyond 80 hr, mycelial breakdown was extensive and preparation of the CFE was extremely slow due to clogging of the millipore filters. Prolonged culturing resulted in the release of excessive amounts of nucleic acids which tended to plug the G50 Sephadex column. This resulted in poor resolution of the three fractions. Problems with preparing a CFE were also encountered if culturing was terminated before sporulation had commenced. In addition, the yield of extracellular protease was also reduced (Figure 4).

Infrequent transferring of the culture (once/3 months) resulted in a reduced amount of proteolytic activity in the CFE. Transferring weekly on Czapek-Dox agar containing 0.75% casein and 0.75% Proteose-Peptone no. 3, greatly increased the proteolytic activity but also created problems with respect to filtering and excessive amounts of nucleic acid. Sporulation was also delayed by 48 to 96 hr when the cultures were transferred on a weekly basis. A time interval of 3 to 4 weeks between each transfer was found to be ideal for maintaining proteolytic activity and minimizing the problems of filtering and excessive nucleic

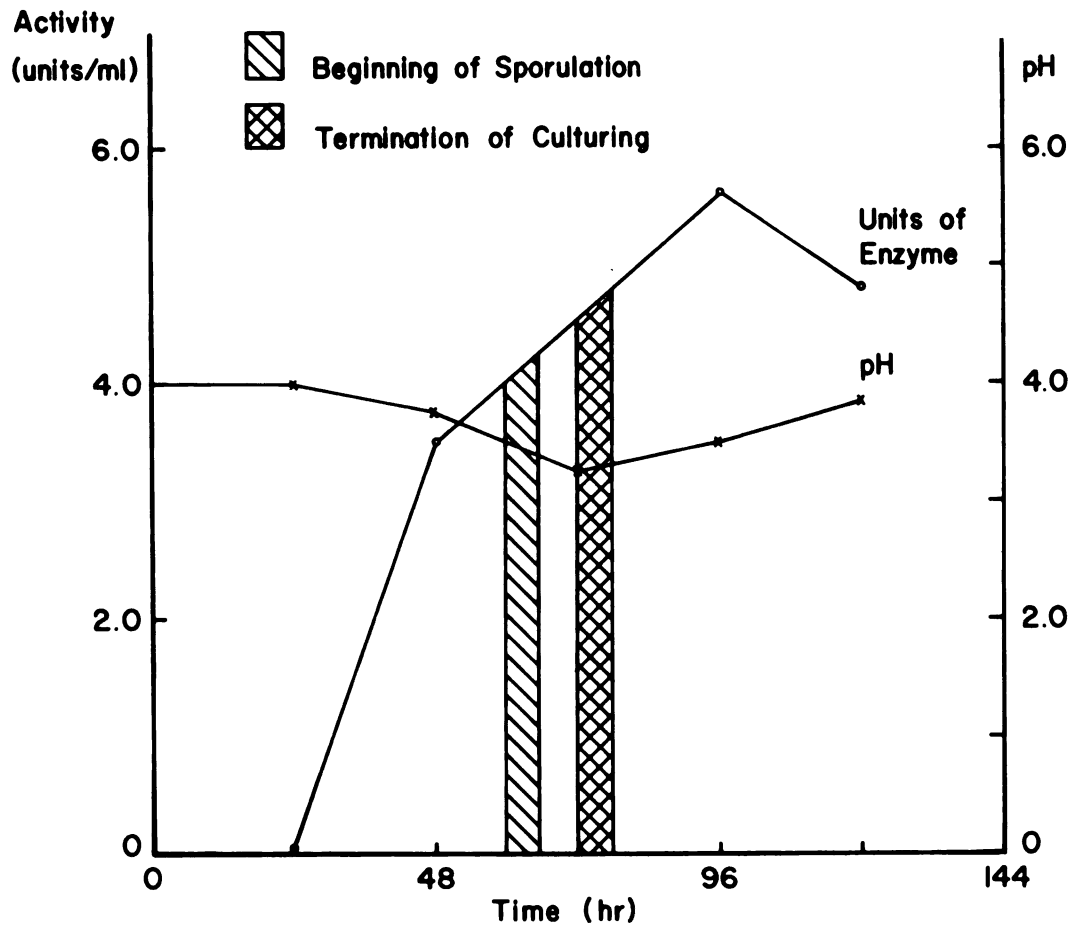

Figure 4. Production of extracellular protease and change in pH by P. roqueforti (BP-13) during shake culturing in Czapek-Dox broth containing 0.5% Proteose-Peptone no. 3.

acid production. Protamine sulfate partially removed the nucleic acids but also inactivated the protease.

A spore count of 90 to 100 x 10^6/300 ml of broth produced the desired amount of growth. Inoculations using a lower count resulted in prolonged culturing and the production of excessive amounts of nucleic acids. This latter problem appeared to be a consequence of mycelial lysis. Normally 15 one ℓ flasks, each containing 300 ml of broth, were shake cultured at a time. The yield of CFE varied from 3.5 to 4 ℓ. The pH of the CFE was in the range of 3.2 to 3.5 (Figure 4).

Determination of Assay Parameters

Effect of Substrate Concentration and pH on Protease Activity

The rate of hydrolysis of 0.5% casein at pH 4.0 was too slow to be considered a useful assay procedure for proteolytic activity (Figure 5). Using the same protein concentration at pH 5.75 resulted in the release of considerably more TCA soluble nitrogen. This may be attributed to approaching the pH optimum for the enzyme more closely or could possibly be a function of the amount of protein in suspension. At pH 4.0, a major portion of the casein had precipitated out of solution. When the enzyme was assayed at pH 5.75, in the presence of 1.0% Hammersten casein, even larger amounts of TCA soluble nitrogen were released. This curve was more linear than when 0.5% casein was assayed at the same pH.

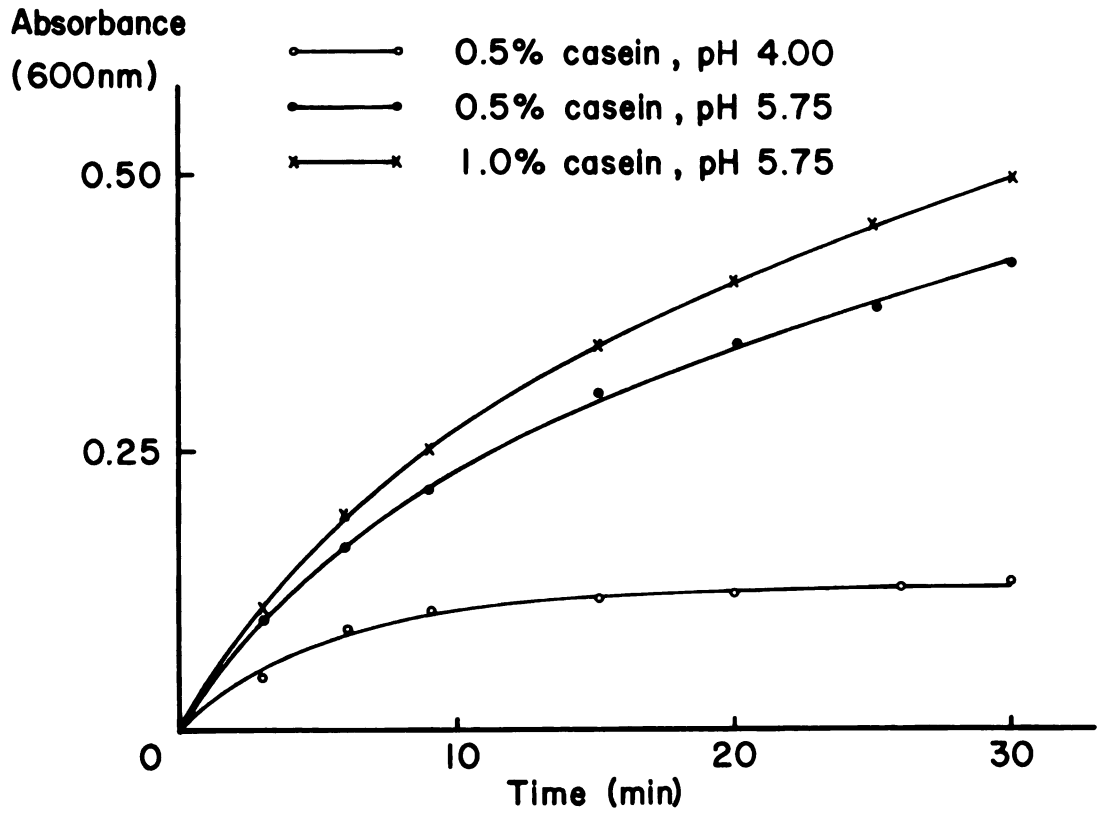

Figure 5. Increase in TCA soluble nitrogen (Lowry-Folin) resulting from hydrolysis of casein by the BP-13 protease (CFE) at 30 C.

A decision was made to use 1.0% casein at pH 5.75, on the basis of the results in Figure 5. The reaction was terminated after 9 min at 30C. Allowing hydrolysis of casein by the BP-13 protease to extend beyond 9 min resulted in pronounced non-linearity of the curve. Assay times less than 9 min resulted in less product (TCA soluble nitrogen) being formed and a corresponding increase in experimental error. The enzyme solution being assayed was diluted to the extent where a ΔA of 0.25 to 0.30 would not be exceeded in 9 min (100 to 120 µg of TCA soluble nitrogen). This was to insure that zero order kinetics would be approached as closely as possible throughout the entire assay procedure. When working with purified enzyme preparations, a ΔA of 0.25 in 9 min corresponded to approximately 8 µg of enzyme; however, this was solely dependent on the specific activity of the enzyme being assayed.

Optimum pH and Stability to pH

The optimum pH, when using 1% BSA as substrate, was 3.0 (Figure 6). With casein, a pH of 5.5 appeared to be optimum (Figure 7). This same optimum was reported earlier by Niki *et al.* (1966). At pH 5.25 the casein sol became turbid, with considerable precipitation occurring at pH 5.0. This results in occlusion of a large number of cleavage sites. Consequently, less TCA soluble nitrogen is released per unit of time even though the pH optimum may lie in this range. Obtaining 100% relative activity at pH

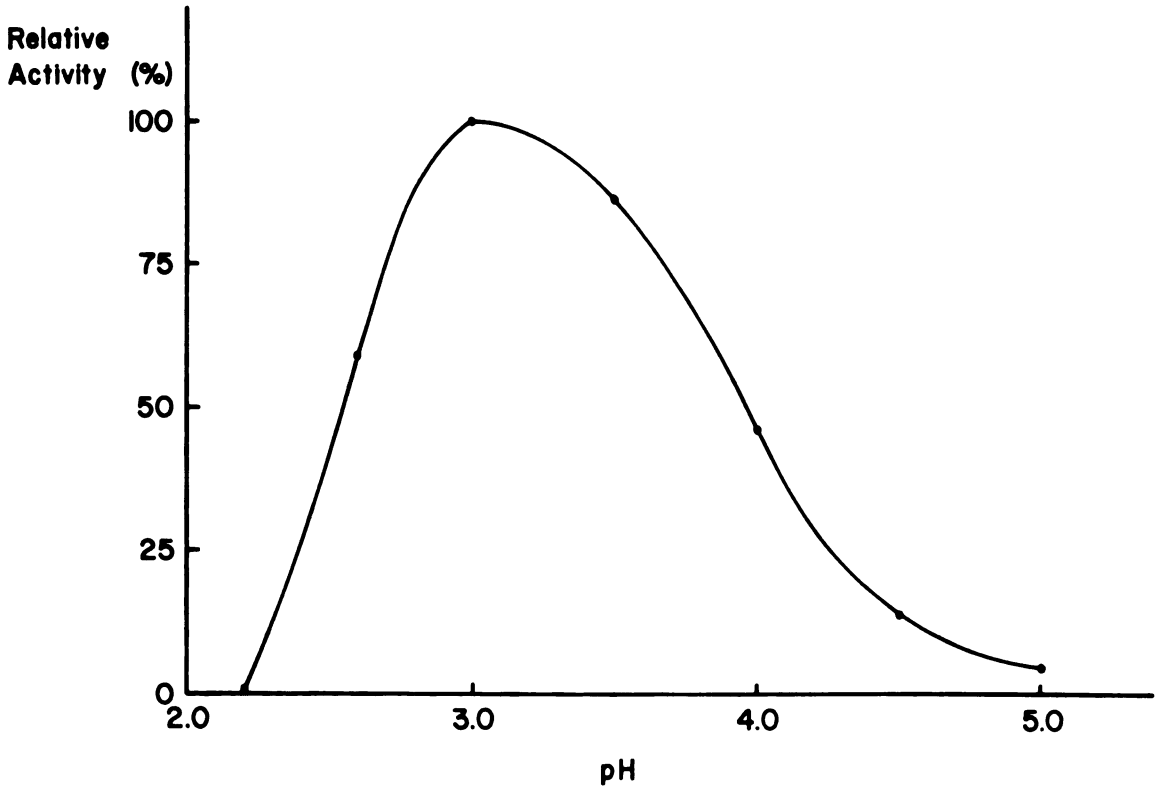

Figure 6. Activity of BP-13 protease (CFE) at various pH levels using 1% BSA as substrate.

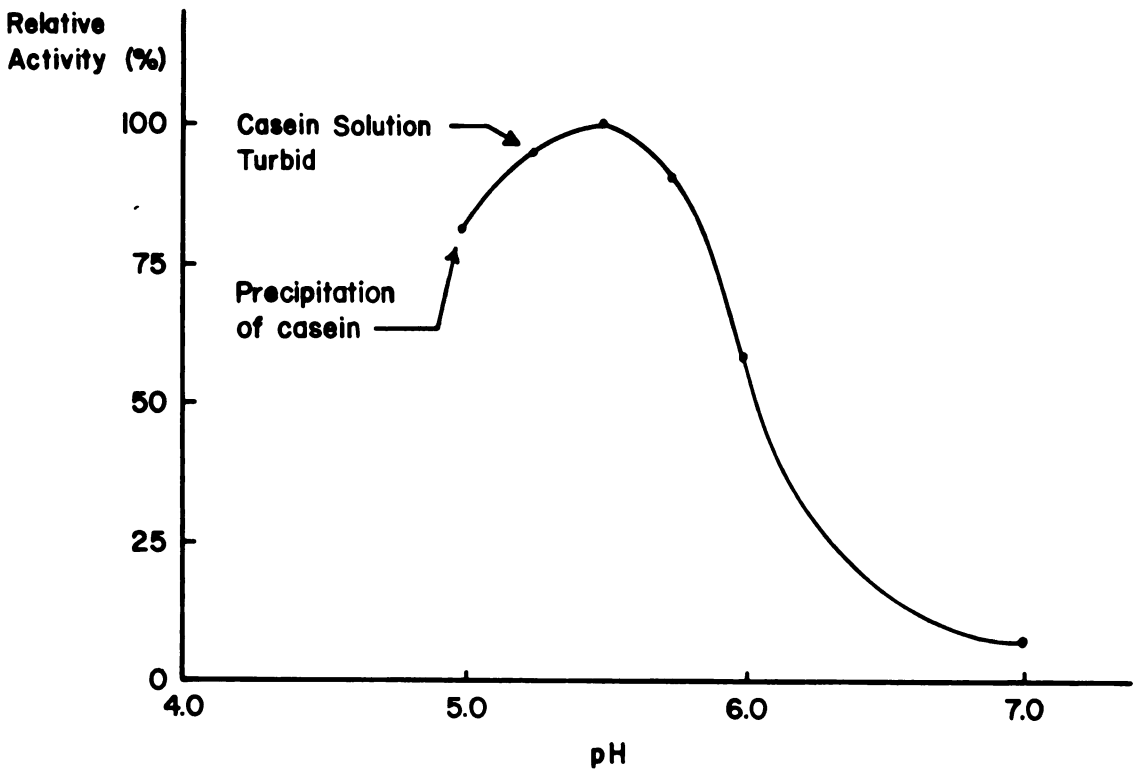

Figure 7. Activity of BP-13 protease (CFE) at various pH levels using 1% casein as substrate.

5.5 may be a true optimum or a reflection of the amount of casein in suspension.

The BP-13 protease was rapidly inactivated below pH 3.0 and above 6.0 when stored for 48 hr at 4 C (Figure 8). When the curves for optimum pH are compared with stability to pH, a marked similarity is evident: the portions of the BSA curve (Figure 6) between pH 2 and 3 and the casein curve between 5.5 and 7.0 (Figure 7) correspond closely to the curve for stability to pH in Figure 8. This indicates that activity below pH 3.0 and above 6.0 is reduced due to denaturation of the enzyme. A decrease in activity between 3.0 and 5.0 when using BSA, may be due to a true reversible affect of the velocity, or an affect of pH on the affinity of enzyme for substrate. This same reasoning may also apply to the pH optimum curve for casein (Figure 7) but an additional factor, the precipitation of substrate, must also be taken into consideration.

Optimum Temperature and Stability to Temperature

The relative activity of the BP-13 protease at various temperatures appears in Figure 9. The enzyme has maximum activity at 45 C when using the standard 9 min end point assay. Above 45 C the enzyme is rapidly inactivated. This is more clearly shown by Figure 10 in which the rate of hydrolysis was followed at time intervals of 3, 6 and 9 min at each temperature assayed. The rate of hydrolysis

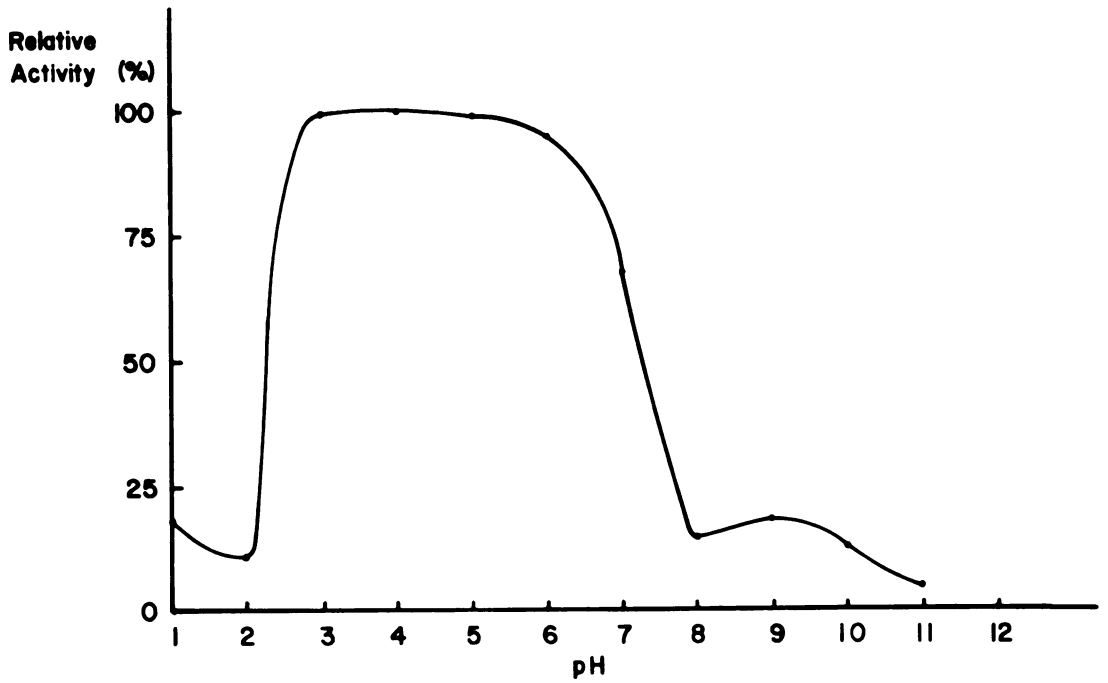

Figure 8. Stability of BP-13 protease (CFE) to pH.

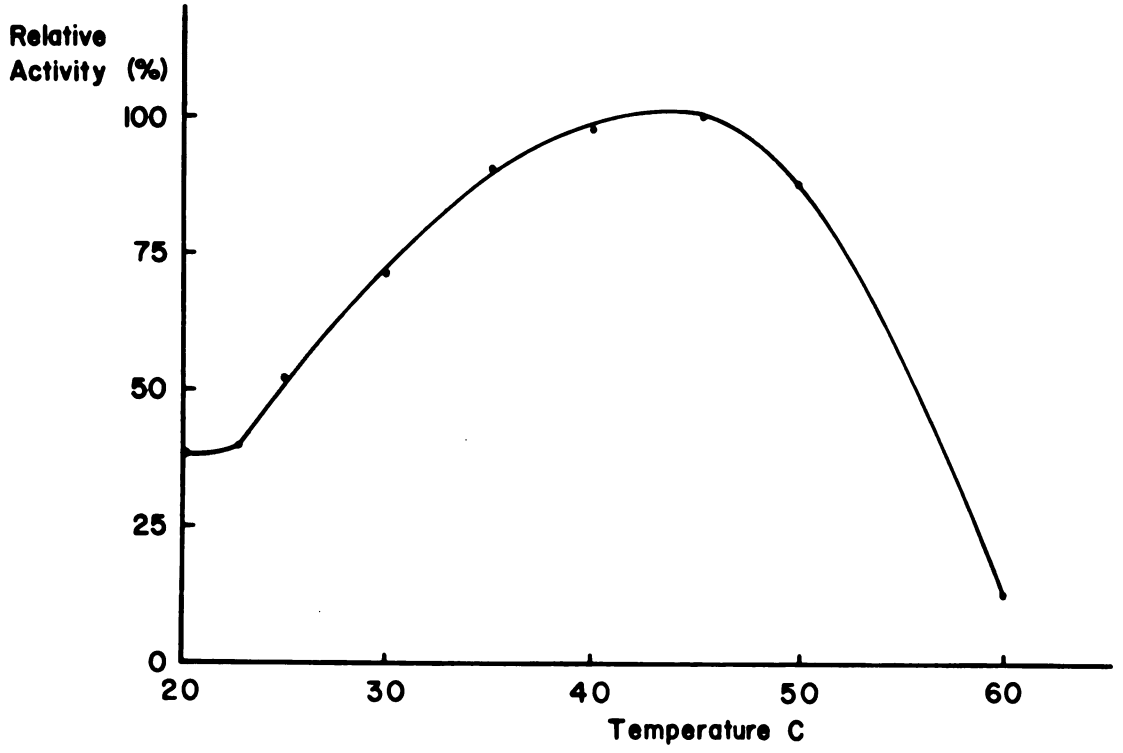

Figure 9. Relative activity of the BP-13 protease (CFE) at various temperatures.

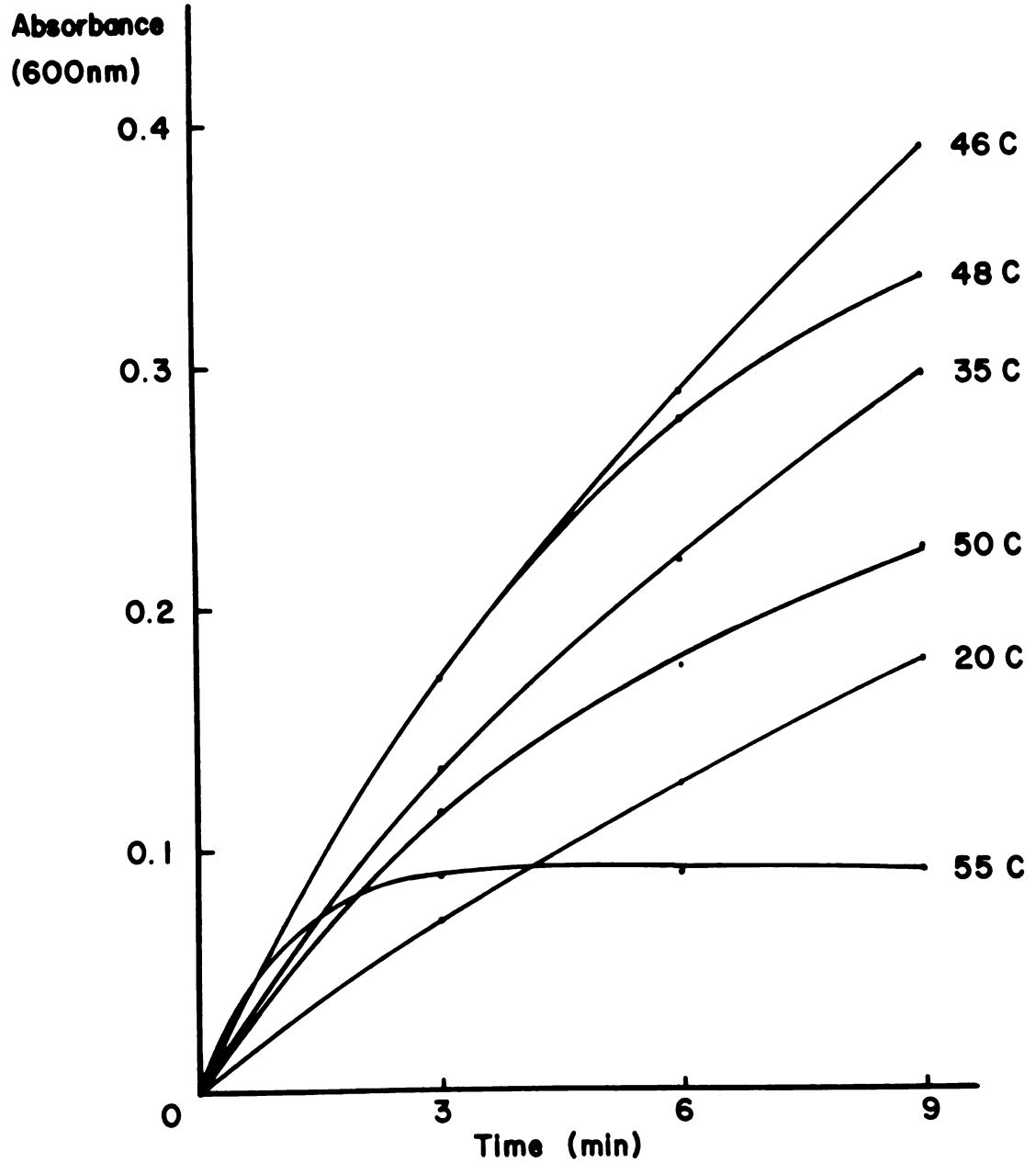

Figure 10. Rate of hydrolysis of casein at various temperatures by the BP-13 protease (CFE).

proved to be linear up to 46 C but at 48 C some inactivation had occurred.[1] At 55 C the enzyme is rapidly inactivated. Although the true initial velocity increases steadily as the temperature is raised, the amount of substrate transformed at any finite time first rises and then falls, giving an apparent optimum temperature. This optimum temperature is not constant but decreases as the time interval increases: at 55 C an assay time of 30 sec or less would be required, but at 46 C the assay time can be lengthened to 9 min (Figure 10). Dixon and Webb (1964a) indicate the effect of temperature on the velocity of enzyme reactions may be due to several different causes including, stability of the enzyme, enzyme-substrate affinity, velocity of breakdown of the enzyme-substrate complex, alteration of pK values, pH functions of any or all of the components and affinity of the enzyme for activators or inhibitors.

Temperatures up to and including 45 C for 30 min had no effect on the activity of the BP-13 protease (Figure 11). Beyond 45 C the enzyme was rapidly inactivated. A plot of $\log_e$ of residual activity after 3, 6 and 9 min of incubation at temperatures of 50.1, 53.2, 56.0 and 59.9 C, indicates that the denaturation is of the first order and irreversible (Figure 12), when exposed to heat at pH 5.0

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[1]Hydrolysis was also linear with time at temperatures of 25, 30 and 40 C but these curves were eliminated from Figure 10 for the sake of clarity.

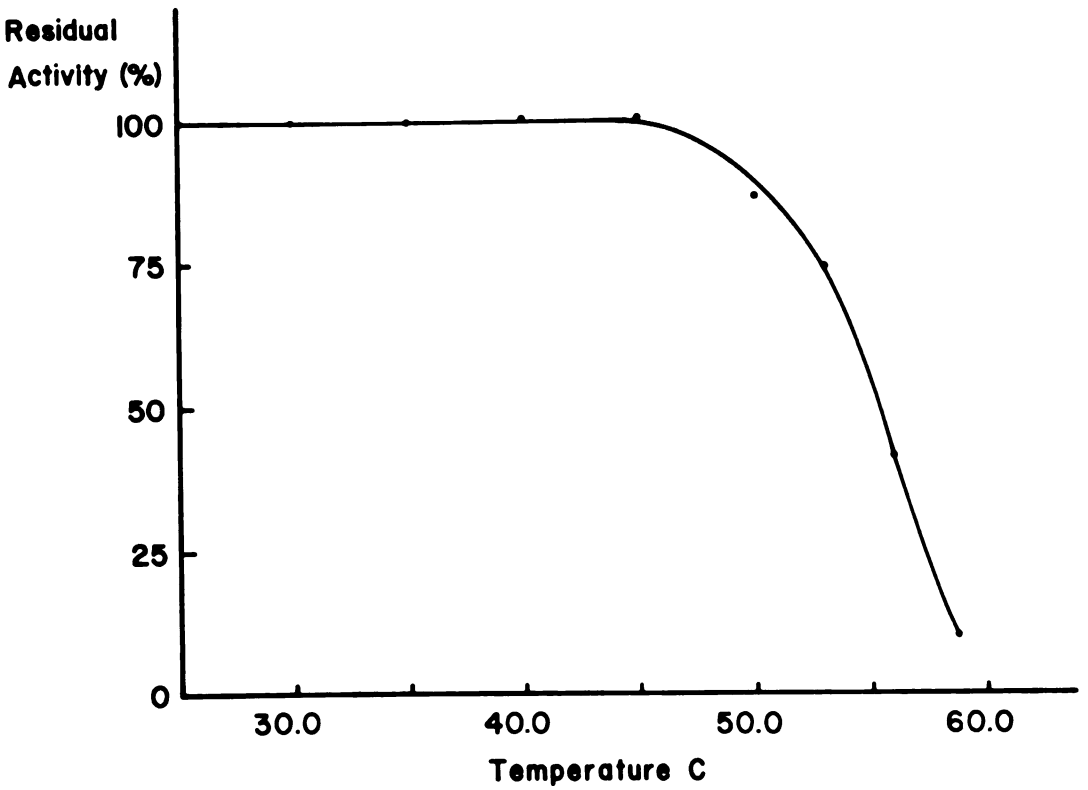


Figure 11. Stability of purified BP-13 protease to temperature (Enzyme incubated 30 min for temperatures up to 45 C; above 45 C, samples were incubated 9 min).

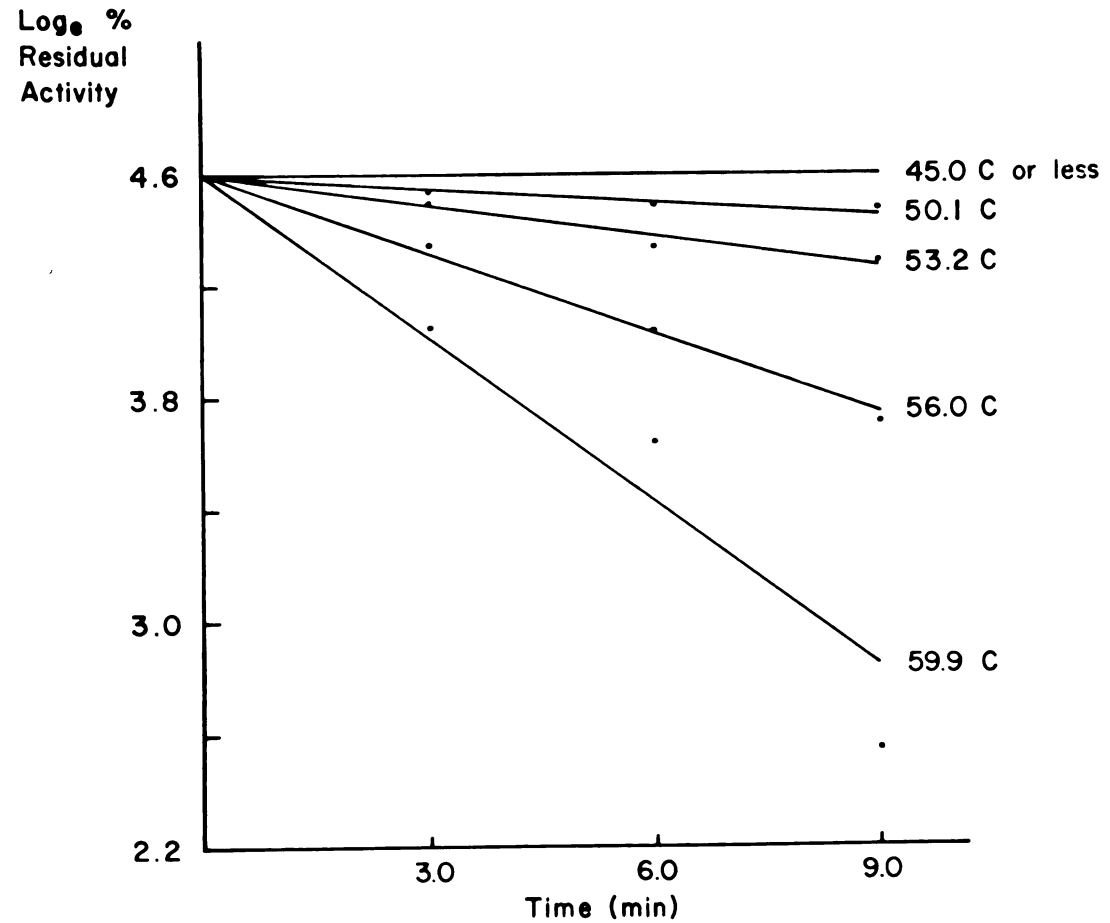


Figure 12. Rate of thermal inactivation of BP-13 protease at various temperatures.

in 0.04 M citrate buffer (μ = 0.3). Because inactivation parameters vary greatly with pH, Dixon and Webb (1964b) indicate that caution should be exercised in drawing general conclusions from observations at only one pH. Other variables such as ionic strength, protein concentration and inhibitors also have to be considered.

The amount of enzyme denaturated at any given time (T) for a specific temperature, can be calculated from the slope of the lines in Figure 12. A $T_{0.5}$ (50% destruction of enzyme activity) of 41.3, 18.7, 7.35 and 3.57 min for temperatures of 50.1, 53.2, 56.0 and 59.9 respectively, were calculated from the relationship

$$T_{0.5} = \frac{1}{k} \ln 2$$

where K is the rate of inactivation.

There were insufficient data in Figure 12 to calculate a meaningful energy of denaturation (Ea) for the BP-13 protease.

Activation Energy for Hydrolysis of Casein

The rate of hydrolysis (k) of casein increases in an exponential manner with the temperature according to the Arrhenius equation.

$$k = A\, e^{-Ea/RT}$$

A is a pre-exponential factor, Ea is the activation energy, k a gas constant and T the temperature in degrees Kelvin. When the log of the above equation is taken

$$\log_e k = \frac{-Ea}{R} \left(\frac{1}{T}\right) + \log_e A$$

and $\log_e$ k vs $\frac{1}{T}$ is plotted, the Ea can be easily calculated. The $\log_e$ of the initial velocities at temperatures of 20, 30, 35, 40, 46 and 55 C (Figure 10) were plotted against $\frac{1}{T}$. An Ea of 8000 cal/mole, for the hydrolysis of casein by the BP-13 protease, was calculated from the slope of the curve presented in Figure 13.

Purification of BP-13 Protease

Concentration

Several precipitating reagents were used in an attempt to remove the extracellular protease from the CFE (Table 3). Despite the fact that ethanol, methanol and acetone precipitated significant quantities of protein, a major portion of activity in both the precipitate and supernatant was destroyed. This can be attributed to denaturation of the protease by these solvents.

Salt fractionation with ammonium sulfate (90% saturation) yielded a recovery of 56.9% of the initial activity. This compares very closely to a recovery of 56.5% when the CFE was concentrated by ultrafiltration and pervaporation (Table 5). When the same precipitated enzyme

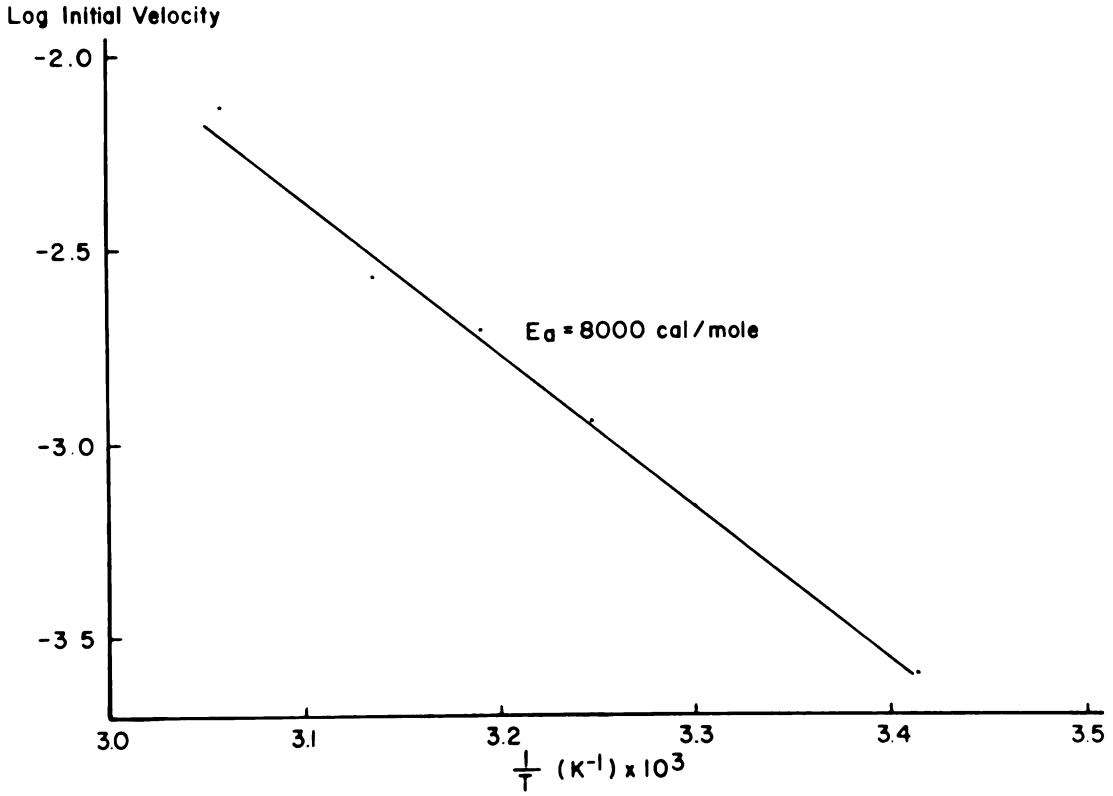


Figure 13. Arrhenius plot for purified BP-13 protease using 1% casein (pH 5.75) a substrate.

TABLE 3.--Recovery of BP-13 protease activity after precipitation with various reagents.

Precipitating Reagent	% v/v	% Recovery[a]	
		Precipitate	Supernatant
Ethanol	60	n.d.[b]	20.9
	70	18.5	n.d.
	80	31.8	n.d.
	90	19.8	n.d.
Methanol	60	n.d.	1.8
	70	n.d.	1.8
	80	n.d.	n.d.
	90	n.d.	n.d.
Acetone	60	n.d.	1.2
	70	22.7	n.d.
	80	23.1	n.d.
	90	24.2	n.d.
Ammonium Sulfate	% Saturation		
	40	1.7	60.6
	50	2.3	56.4
	60	3.5	49.0
	70	13.1	39.4
	75	38.7	23.9
	80	45.0	2.4
	85	53.9	2.5
	90	56.9	2.0

[a]From 50 ml of CFE.

[b]Not detectable.

and retentate concentrate were fractionated on G50/G100 Sephadex, the specific activity of the latter was much higher than with the ammonium sulfate cut. In view of these results, ultrafiltration and pervaporation were selected as a means of concentrating the enzyme preparation.

When using the UM 10 membrane (1.5×10^{-3} µm pore diameter) in the Amicon ultrafiltration unit, from 20 to 30% of the original activity could be detected in the ultrafiltrate. When the PM 10 membrane (1.8×10^{-3} µm pore diameter) was employed, up to 80% of the original activity was present in the ultrafiltrate. These results would indicate that the BP-13 protease had a molecular weight of less than 10,000; however, experiments in gel chromatography and SDS gel electrophoresis indicated a molecular weight of 49,000 and 45,000, respectively. For a molecule of this size to pass through a membrane with a molecular weight cut-off of 10,000, may indicate:

a. High axial ratio for the enzyme
b. Enzyme is composed of sub-units
c. Reduction in molecular size due to shearing stress in ultrafiltration.

No evidence was found to support either "a" or "b." There was about 20% loss in activity due to shear denaturation of the molecule. According to Charm and Lai (1971) this is to be expected. For a molecule to be reduced from a molecular weight in the range of 45,000-49,000 to less than 10,000

and still retain activity seems only remotely possible. No attempt was made to determine the molecular weight of the enzyme present in the ultrafiltrate. Pepsin has a molecular weight of approximately 35,000 and is retained to the extent of at least 95% on the PM 10 membrane while the retention on the UM 10 membrane is greater than 99%.

After the CFE was concentrated from 1 ℓ to approximately 50 ml, further concentration was achieved by placing the retenate in dialysis tubing and pervaporating to a volume of 10 to 15 ml. This final concentration step required 6 to 8 hr. At this point the sample was deaerated and applied to the top of the G50 Sephadex column. Concentrated samples, exhibiting high viscosity, were dialyzed for 12 hr at 0 C and pervaporated back to 15 ml prior to application to the G50 Sephadex. If a highly viscous sample was applied to the column, the eluate would drain away from the top of the G50 gel before all of the enzyme concentrate was delivered. This was attributed to the lack of an air tight seal between the sample applicator and the inside wall of the column. The viscosity of the concentrate appeared to be a function of the amount of sucrose not utilized by the fungus during shake culturing.

Purification of the BP-13 Protease by Gel Filtration

A picture of the columns (2.5 x 45 cm and 1.6 x 100 cm), monitor, fraction collector and additional accessories used in purifying the BP-13 protease appears in

Figure 14. A schematic of the same apparatus appears in Figure 15, showing the distribution of the three major peaks during the fractionation procedure. By the time the Proteose-Peptone fraction had reached the bottom of the G50 Sephadex column, the nucleic acid-protein peak was coming off the bottom of the G100 Sephadex with the enzyme being distributed between these two major peaks. At this point the LV-4 valve was rotated 90° so the Proteose-Peptone fraction would bypass the G100 column. A second eluate flask containing the same 0.04 M citrate buffer at pH 5.0 (μ = 0.03) was connected to the LV-4 valve and automatically fed the G100 Sephadex column when this valve was given a quarter turn.

The results in Figure 16 are a plot of A_{254}, enzymatic activity and protein concentration (Lowry-Folin) for each fraction collected. The first peak displays high absorbance at 254 nm in relation to the amount of protein present, indicating the presence of nucleic acids. The second peak represents the enzyme fraction. There is an excellent correlation between A_{254}, protein and activity for this peak. This suggests that a high degree of purification has been achieved at this point in the fractionation procedure. The third peak contained the Proteose-Peptone which was originally added to the Czapek-Dox broth. The size of this peak depended on the amount of Proteose-Peptone allowed to reach the G100 column.

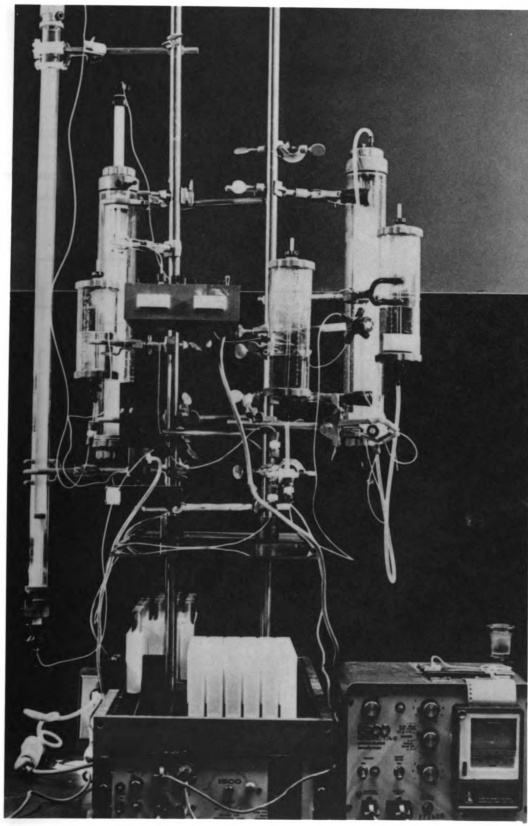


Figure 14. Gel filtration equipment used to purify the BP-13 protease: monitor, fraction collector, recorder, columns and ancillary equipment.

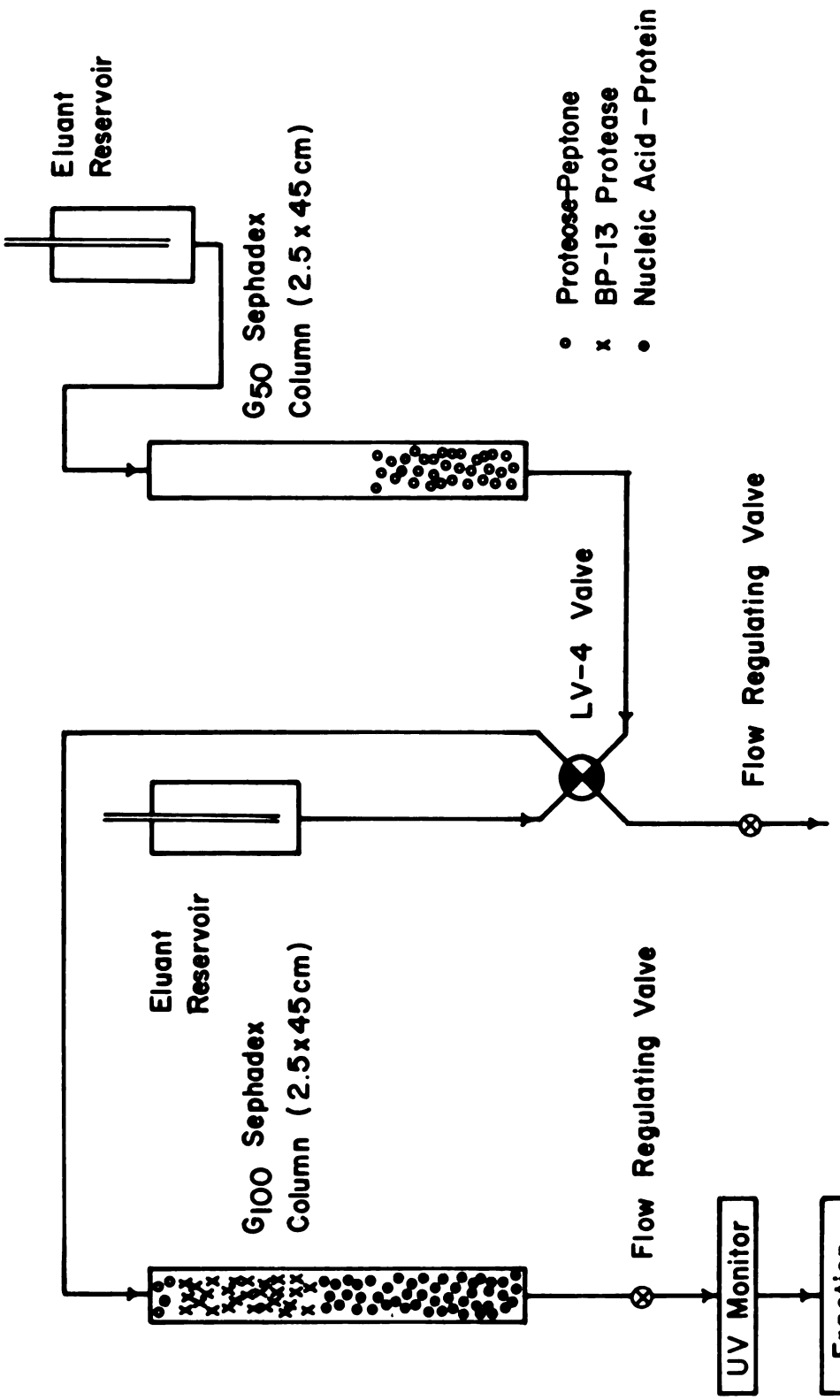


Figure 15. Schematic of gel filtration equipment used for purifying BP-13 protease.

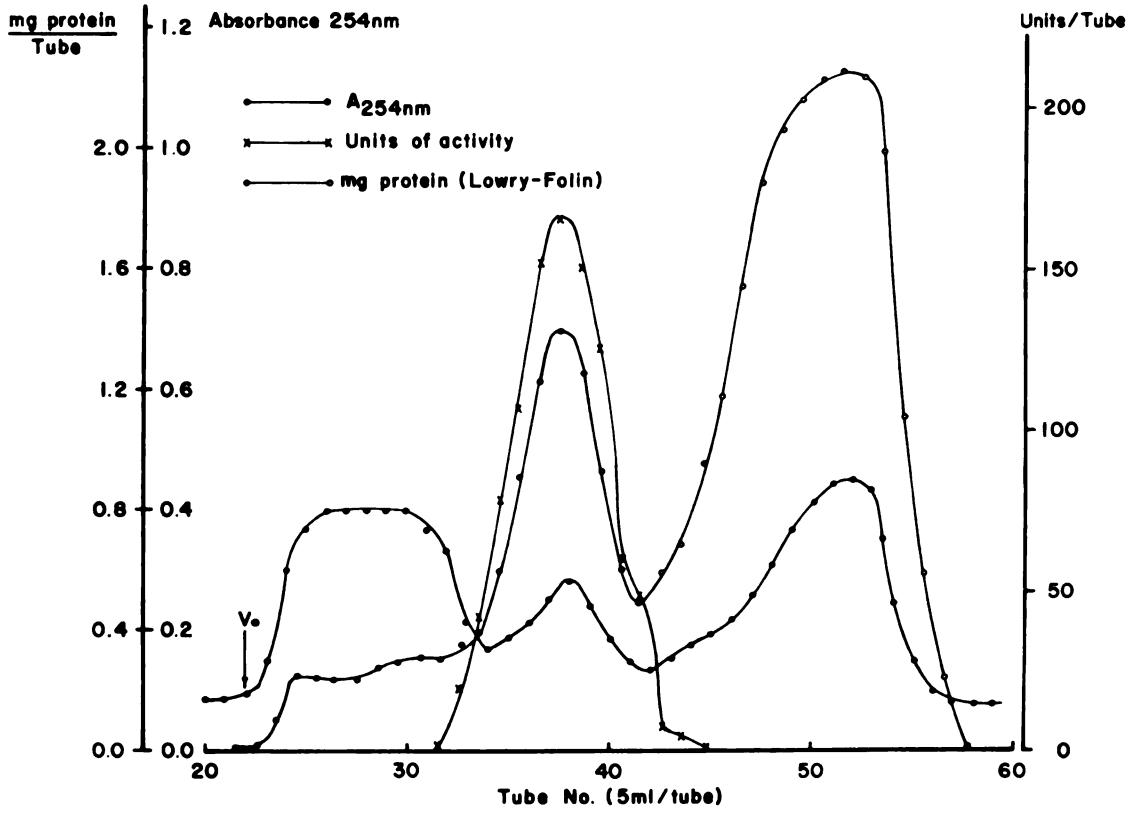


Figure 16. Purification of BP-13 protease by gel filtration with G50 and G100 Sephadex.

Further purification of peak 2 was achieved by applying this fraction to a second G100 Sephadex column (1.6 x 100 cm). The results in Figure 17 shows the presence of two well resolved peaks, with the first fraction being the enzyme. This purification step served to almost double the specific activity of the protease (Table 4). The second elution peak obtained from the G100 column (Figure 17) contained a minor fraction that had no proteolytic activity. The steps required in the purifying of the BP-13 protease appear in Figure 18.

A purification summary for the BP-13 protease appears in Table 4. The CFE was arbitrarily assigned a purification fold of 1.0. A major portion of the Proteose-Peptone fraction was removed from the CFE by ultrafiltration. This resulted in a 2.69-fold purification of the concentrated retentate. The G50/G100 Sephadex chromatography proved to be a powerful purification step: the specific activity increased from 8.85 to 285, reflecting in an 87.0-fold purification. Again this was due mainly to the removal of the Proteose-Peptone. The final G100 column served to increase the specific activity to approximately 465 which represents a 141-fold purification from the initial CFE.

The 280/260 ratio of the final enzyme preparation was 2.01. Normally a pure protein has a 280/260 ratio of 1.75 but a higher value of 2.01 may be a reflection of a

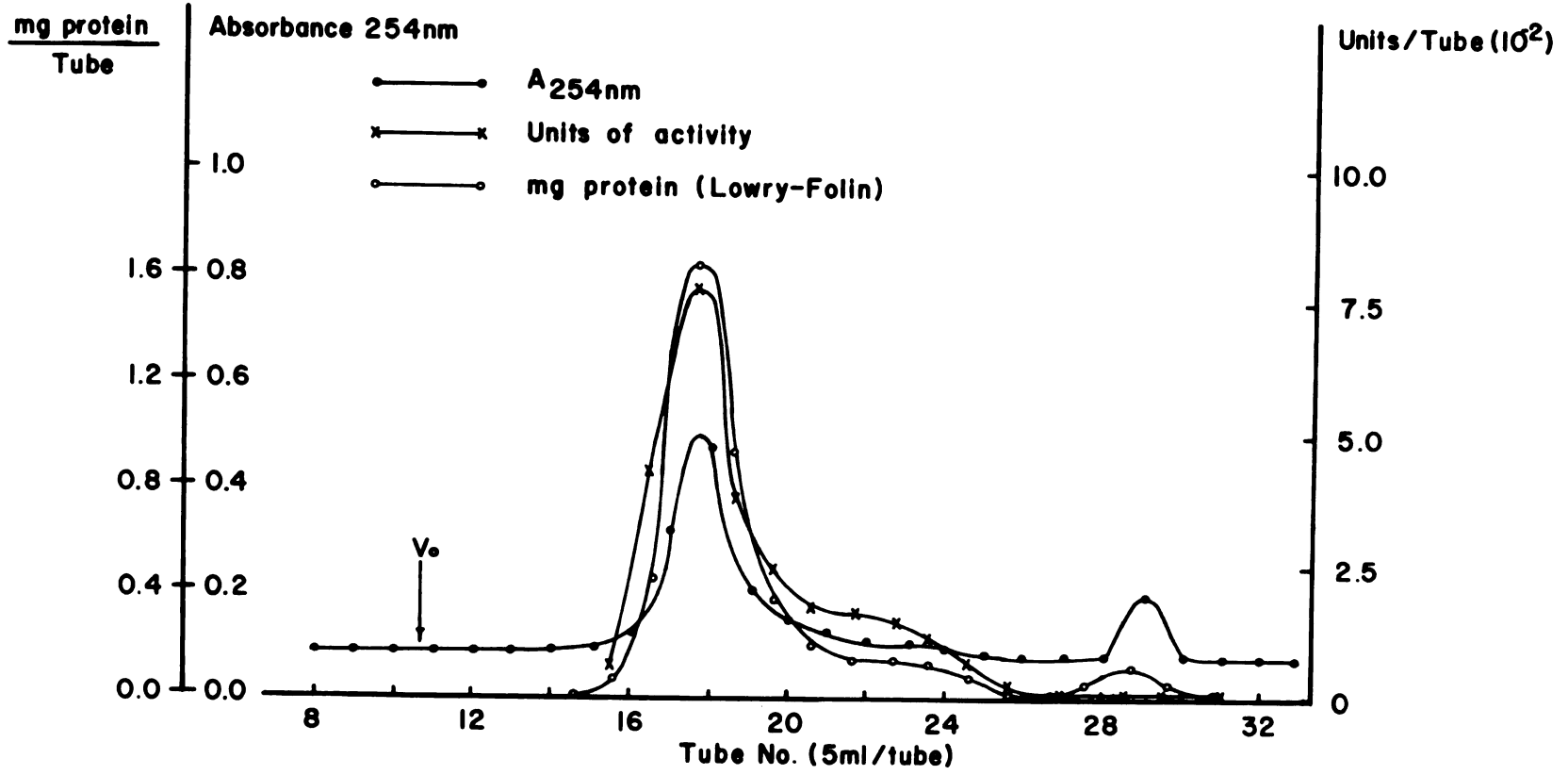


Figure 17. Purification of BP-13 protease by gel filtration with G100 Sephadex.

TABLE 4.--Enzyme purification summary of BP-13 protease.

Step	Volume (ml)	Activity			Protein		Purification		
		Units per ml	Total Units	Percent Recovery	Mg per ml	Total mg	Specific Activity	Fold Purification	280/260
Cell Free Extract	1000	9.4	9400	100	2.86	2,860	3.29	1.00	. .
Ultrafiltration and Pervaporation	15.0	354	5310	56.5	40.0	600.0	8.85	2.69	. .
G50/G100 Sephadex	40.0	84.4	3380	36.0	296 (µg)	11.84	285	87.0	. .
G100 Sephadex	25.0	91.0	2280	24.3	196 (µg)	4.90	496	141	2.01

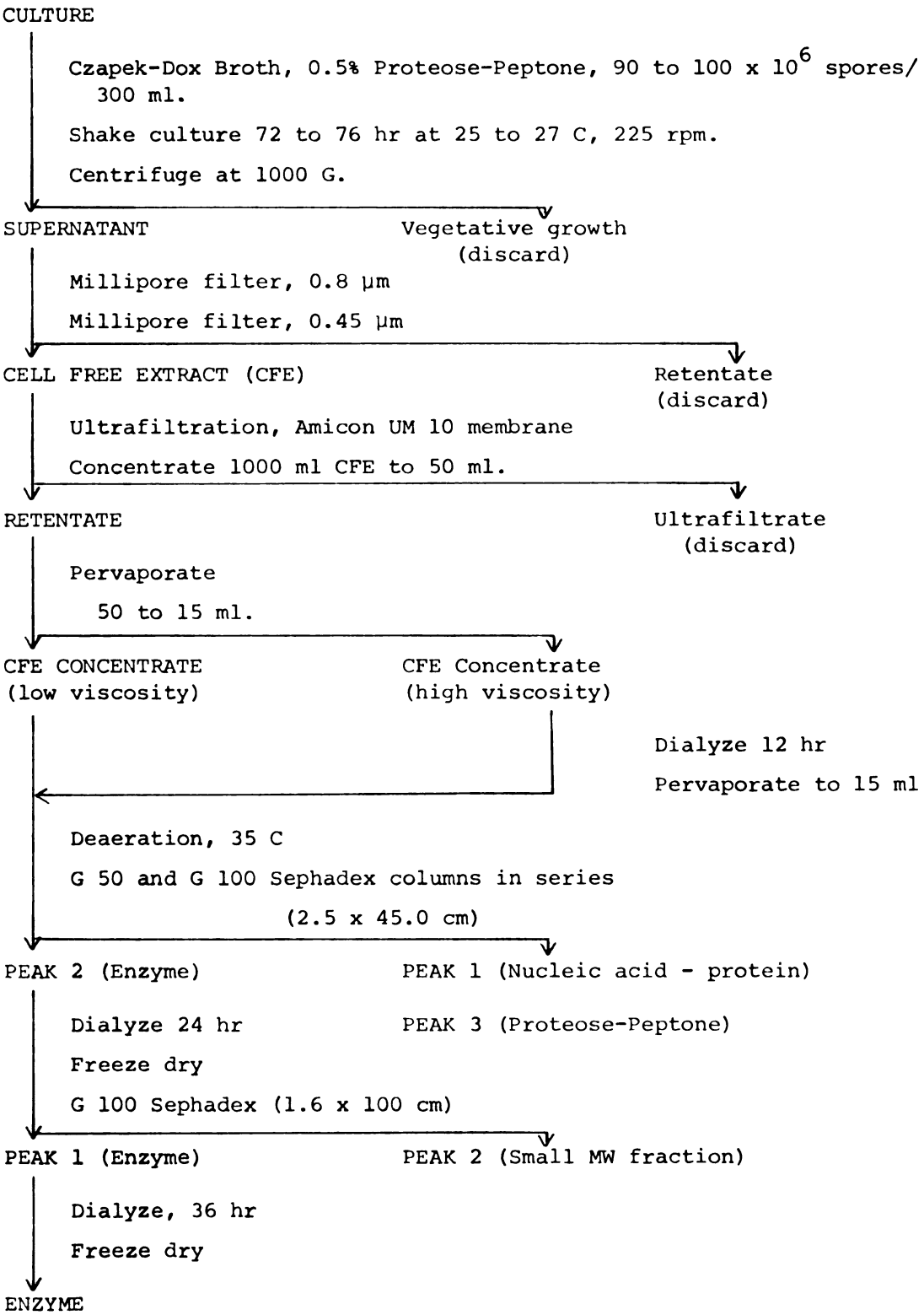


Figure 18. Steps in the purification of BP-13 protease.

higher tyrosine and/or tryptophan content. The 280/260 ratio was determined using a Beckman DU-2 spectrophotometer.

Absorbance Spectrum of Nucleic Acid-Protein and Enzyme Peaks

A scan of the first peak obtained off the G50/G100 columns showed the presence of two overlapping peaks with adsorbance maxima near 262 and 275 (Figure 19). This corresponds closely to the absorbance maximum of nucleic acid and protein respectively. A slight decrease in absorbance in the range of 266 to 270 nm indicates the area of overlap of the two spectrums.

The BP-13 protease had an absorption maximum at 280 nm. This is characteristic of most pure proteins.

Standard Plate Counts

Standard plate counts were made on several enzyme preparations after each operation in the purification procedure (Table 5). The CFE, UM 10 retentate and pervaporated enzyme preparation had a count of less than 30/ml. The CFE is essentially sterile: the 0.45 μm Millipore filter removes all microorganisms with the exception of some viruses. From this point on, microbial counts are mainly a function of sanitation and pH of the enzyme solution. The very low microbial count for the UM 10 retentate and pervaporated enzyme preparation can be partially ascribed to the pH of the solution (3.2 to 3.5).

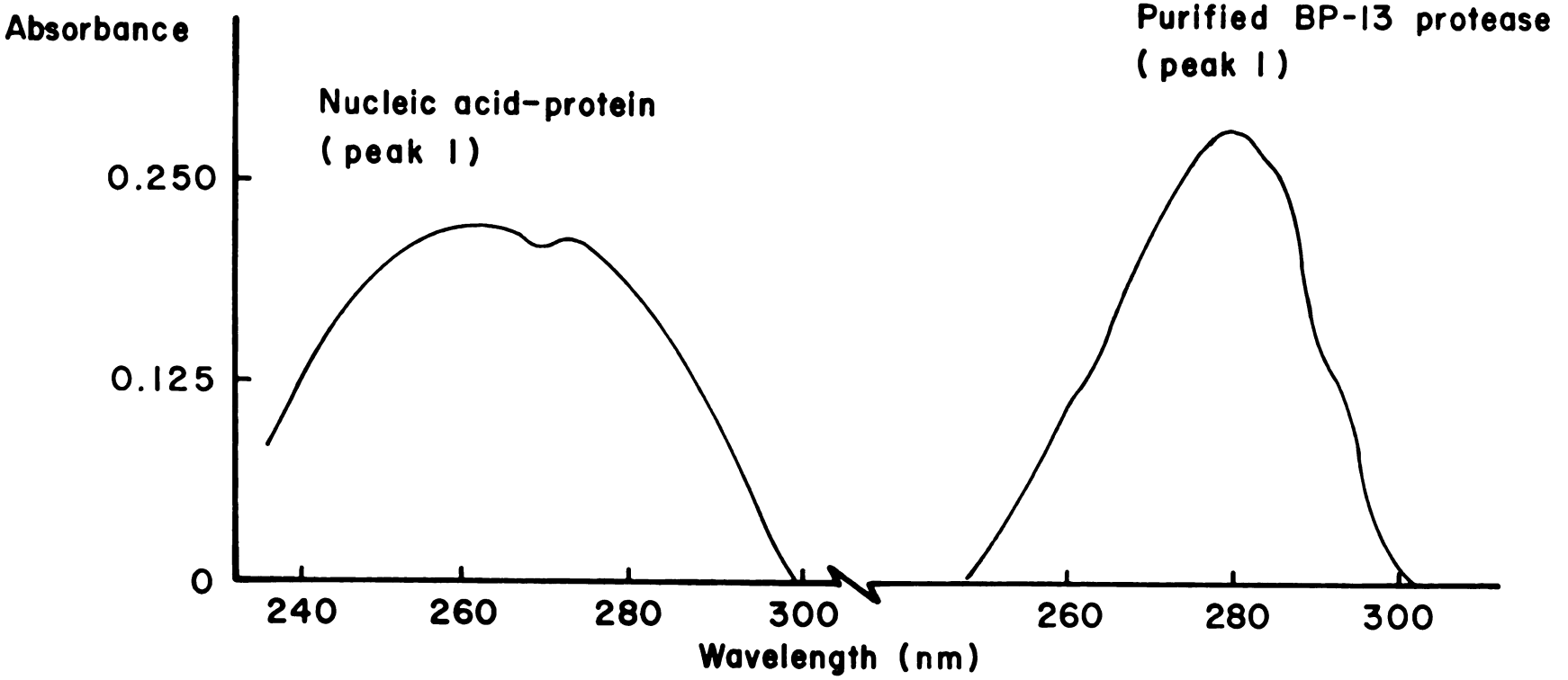


Figure 19. Absorption spectrum of nucleic acid-protein (G50/G100 Sephadex--peak 1) and purified BP-13 protease (G100 Sephadex--peak 1).

TABLE 5.--Standard plate count of BP-13 protease during purification procedure.

Purification Step	Count/ml
BP-13 (CFE)	< 30
UM 10 Retentate	< 30
Pervaporation	< 30
G50/G100 Sephadex	50 - 200
G100 Sephadex	380 - 760

Low microbial counts in the fractions obtained from the various columns is to a large degree, a reflection of proper sanitation. Microbial counts for the final enzyme preparation ranged from 380 to 760 per ml.

Electrophoresis of Various Fractions Resolved by Gel Filtration

The components of the first two peaks collected from the G50/G100 columns were subjected to alkaline acrylamide gel electrophoresis (Figure 20) using the system described by Melachouris (1969). The nucleic acid-protein peak (PK 1) was resolved into two very light bands (indicated by arrows in Figure 20) differing only slightly in electrophoretic mobility. Large quantities of sample (65 µl of a 5% nucleic acid-protein solution) had to be introduced into the sample slot in order to achieve the straining effect presented in Figure 20.

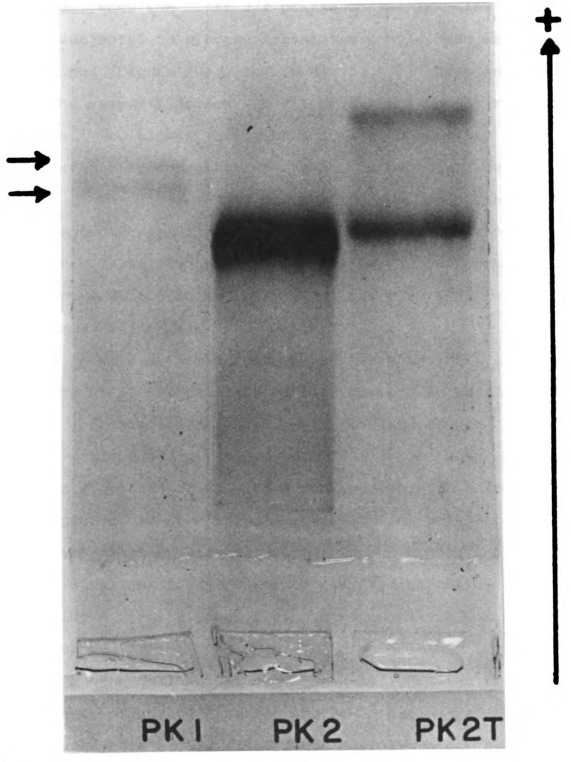


Figure 20. Alkaline acrylamide gel electrophoresis of fractions collected from G50/G100 Sephadex. Nucleic acid-protein fraction (PK 1); first 3/4 of enzyme fraction (PK 2), tail of enzyme fraction (PK 2T).

When the first 3/4 of the enzyme fraction (PK 2) was subjected to electrophoresis, a single band was obtained (Figure 20). The remaining 1/4 (based on volume) of the enzyme fraction (PK 2T) produced two well resolved bands. The slower moving band possessed activity but the faster moving component was unable to hydrolyze casein.

The final enzyme preparation was homogeneous when subjected to acid, alkaline and SDS gel electrophoresis (Figure 21). SDS gel electrophoresis was performed by adding 0.2% SDS to the enzyme sol prior to electrophoresis in an alkaline gel.

When 0.1% mercaptoethanol was added to a 5% sol of the BP-13 protease and electrophoresed in an alkaline gel, approximately 14 bands were resolved (Figure 22). This would suggest the presence of a large number of inter-chain disulfide bands. To have a proteolytic enzyme composed of 14 or more polypeptide chains covalently linked to each other by disulfide bonds, seems highly improbable. Determination of the number of polypeptide chains would require N terminal analysis. This experiment was not performed.

The Molish test qualitatively indicated the presence of carbohydrate in the BP-13 protease. According to Purkayasta et al. (1967), the carbohydrate prosthetic group of κ-casein causes the formation of multiple bands when this milk protein is subjected to electrophoresis.

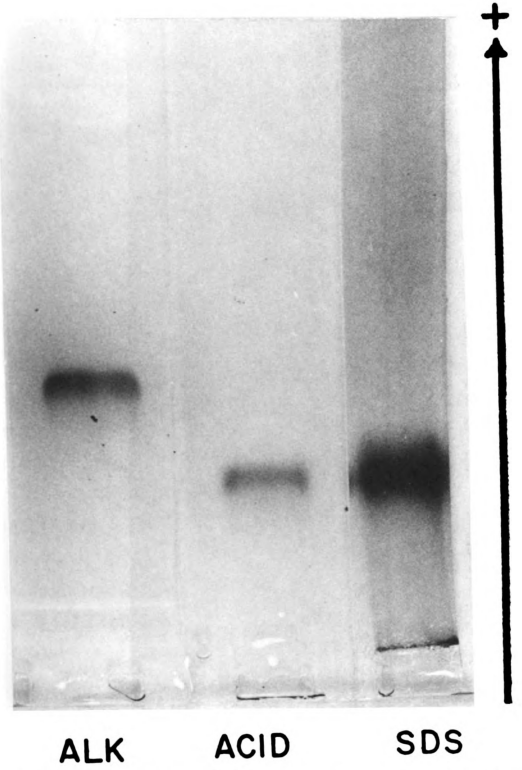


Figure 21. Alkaline (ALK), acid and SDS acrylamide gel electrophoresis of purified enzyme preparation.

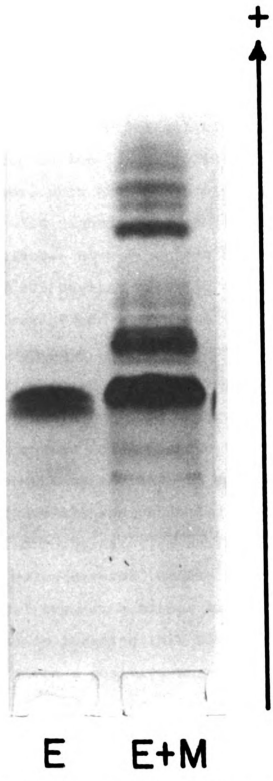


Figure 22. The affect of 2-mercaptoethanol (M) on the BP-13 protease (E).

The carbohydrate of the BP-13 protease may also be producing a similar affect in this case.

Further Purification of the BP-13 Protease

Alkaline, acid and SDS gel electrophoresis of the final enzyme preparation indicated the protein was homogeneous. However, when the same protein sample was reapplied to the G100 Sephadex column (1.6 x 100 cm), two peaks were obtained. Both fractions had the same Kav as in the previous run but the specific activity of the enzyme had decreased from 477 to 343 (Table 6). In addition, the size of the second peak increased considerably while the quantity of enzyme decreased accordingly. The component(s) in the second peak did not possess proteolytic activity. When this fraction was added back to the enzyme, represented by Peak 1, there was no increase in activity. When the second peak (Figure 17) was subjected to either acid or alkaline gel electrophoresis, no stained areas could be located on the gel. Reversal of the electrodes and staining each gel type with either Amido Black or Coomassie Blue also failed in locating this component. This fraction had previously given a positive response to the Lowry-Folin procedure for measuring protein. These results indicated this non-staining species was a very small molecular weight component which was present as an impurity or formed due to autolysis of the enzyme.

TABLE 6.--Effect of repeated chromatography on specific activity of BP-13 protease.

Step	Gel Type	Column Size (cm)	Specific Activity
1	G50/G100 Sephadex	2.5 x 45	350
2	G100 Sephadex	1.6 x 100	477
3	G100 Sephadex	1.6 x 100	343

Autolysis was studied by incubating the BP-13 protease at 30C for 30 hr. Protein, activity and TCA soluble nitrogen were determined at time intervals of 2, 18 and 30 hr. The results in Figure 23 show the protein level decreased approximately 9% after 30 hr but there was only a 4% drop in activity over the same time period. No reason can be given for this decrease in the protein level. In addition, the TCA soluble nitrogen continued to decrease throughout the incubation period. This negates the possibility of autolysis occurring during incubation in 0.04 M citrate buffer at pH 5.0 (μ = 0.3). The amount of TCA soluble nitrogen should remain the same in the absence of autolysis. No plausible explanation can be given for this decrease.

A Kav of 0.89, for the second peak obtained off the long G100 Sephadex column, indicated this fraction had a molecular weight of less than 1000 (Figure 26). When the BP-13 protease was subjected to descending chromatography, five ninhydrin positive areas were detected (Figure 24).

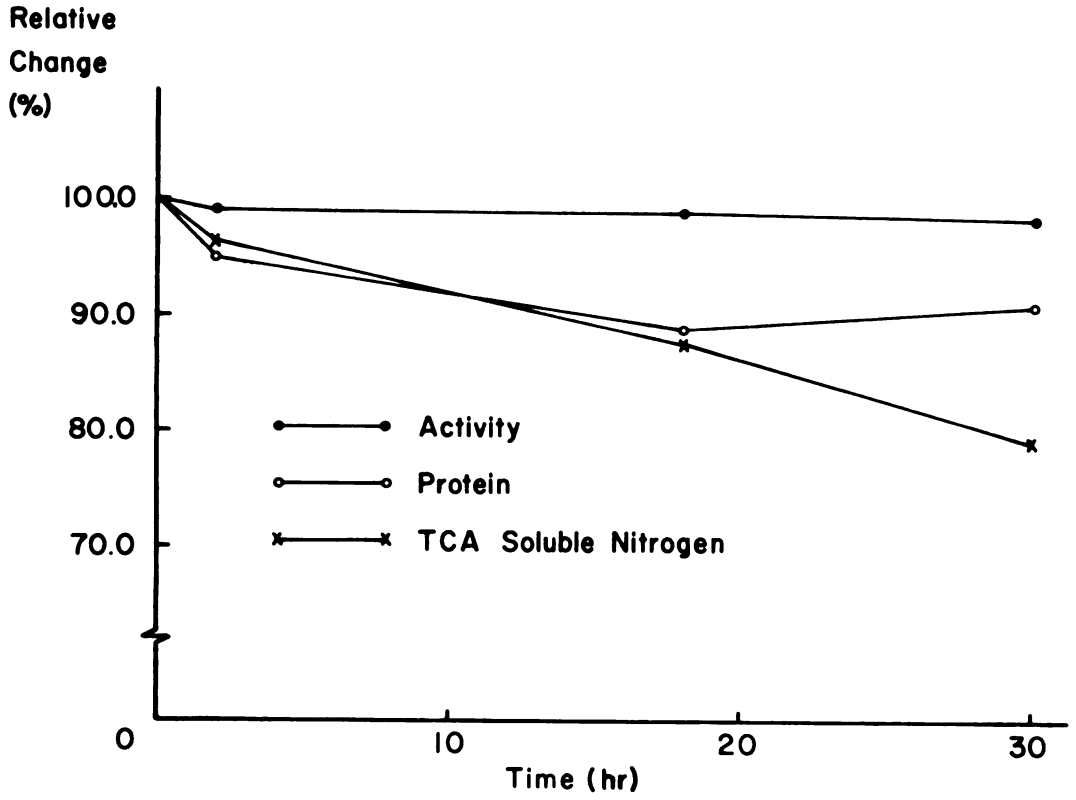


Figure 23. Change in characteristics of purified BP-13 protease after incubation in 0.04 M citrate buffer (pH 5.0, μ = 0.3) for 30 hr at 30 C.

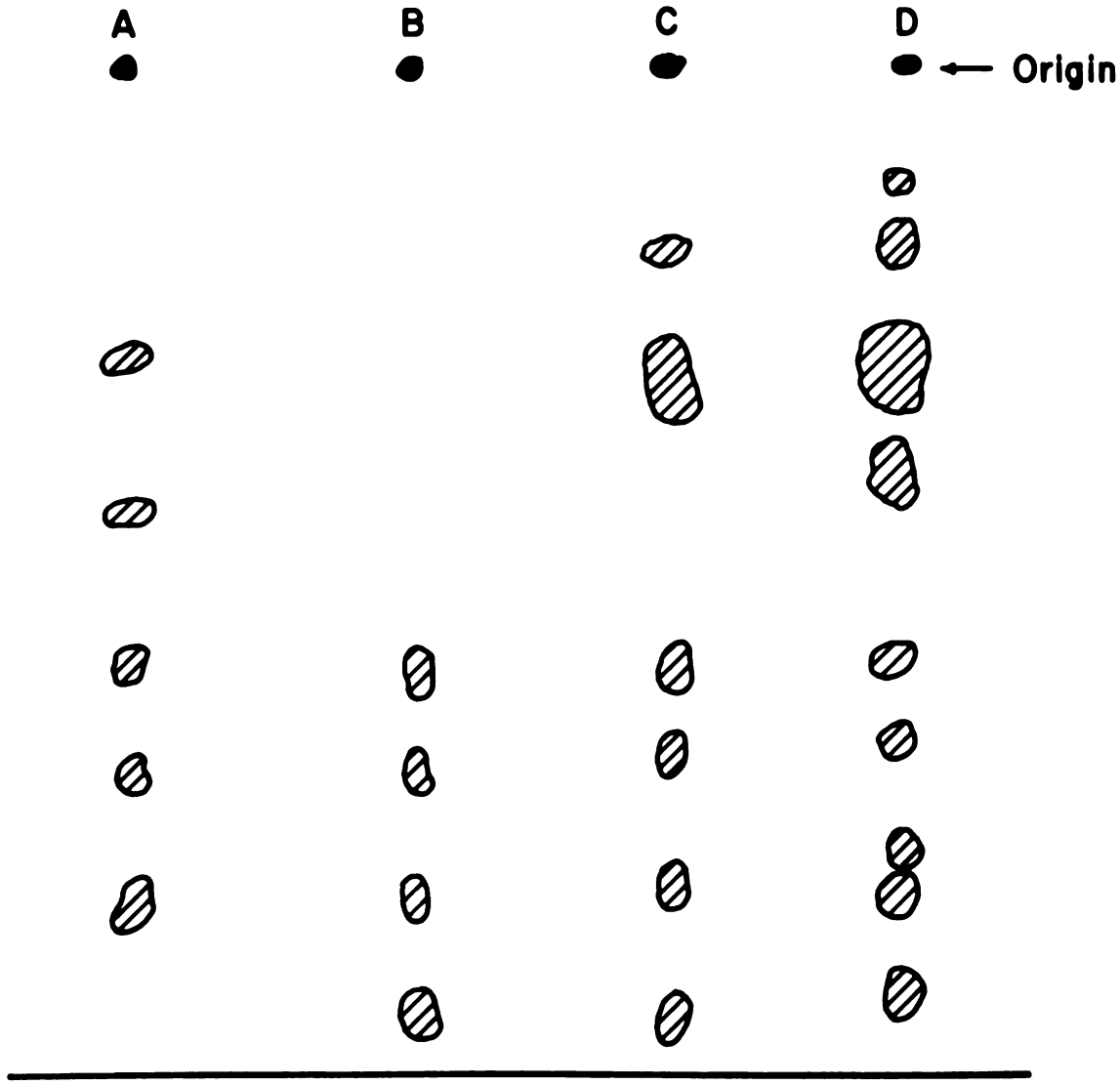


Figure 24. Descending paper chromatogram of purified protease after various treatments: A, dialysis, B, precipitation with ammonium sulfate (85% saturation at 25 C); C, electrodrolysis at 225 V for 3 hr; and D, electrodialysis at 400 V for 18 hr.

The small molecular weight components would then appear to be either amino acids and/or small peptides.

Electrodialysis and precipitation of the protease with ammonium sulfate (85% saturation at 25 C) failed to remove the amino acids and/or peptides (Figure 24). Electrodialysis at 225 V for 3 hr had no affect on the specific activity of the BP-13 protease (Table 7) but did produce an additional spot on the descending chromatogram (Figure 24). A potential of 400 v for 18 hr reduced the specific activity over two-fold (Table 7) and also resulted in the formation of nine ninhydrin positive areas on the descending chromatogram (Figure 24). Electrodialysis at high voltage for a prolonged period of time results in precipitation of the enzyme from solution. This suggests the enzyme is a globulin. A decrease in activity of the BP-13 protease by electrodialysis can then probably be attributed to alteration of the secondary and teritary structure of the enzyme rather than disruption of covalent bonds. The equipment used for electrodialyzing the protease appears in Figure 25.

An attempt was made to further purify the BP-13 protease by using several types of ion exchangers (Table 8). Preliminary experiments were conducted to determine the optimum pH for adsorption of the enzyme to DEAE and SE cellulose. The results in Table 9 indicate that a pH of 7.0 was conducive to total binding of the enzyme to DEAE cellulose while 91.5% of the protease is adsorbed by the

TABLE 7.--Effect of electrodialysis on specific activity of BP-13 protease.

	Time (hr)	Voltage	Specific Activity
Dialysis	24	. .	500
Electrodialysis	3	225	500
Electrodialysis	18	400	220

SE cellulose at pH 3.0. The isoionic point of the enzyme then lies somewhere between pH 3.0 and 7.0. Approximately 97% of the enzyme was adsorbed to the DEAE cellulose at pH 5.0 and 6.0. This indicates that a major portion of the enzyme was still negatively charged at each of these pH levels. Consequently, the isoionic point for the enzyme can be restricted to a pH range of 3.0 to 5.0 rather than 3.0 to 7.0. Binding experiments were not conducted with the remaining three cation exchangers.

Each type of ion exchanger was used under varying conditions of pH and/or ionic strength. The results tabulated in Table 10 depict the problems associated with trying to further purify the BP-13 protease by ion exchange chromatography. The enzyme was tenaciously bound to the ion exchanger and as a result little or no activity or protein was recovered regardless of the method of elution. The best results were obtained when the enzyme was placed on a SE cellulose column equilibrated with 0.02 M citrate buffer (pH 4.44, μ = 0.02) and eluted with an ionic strength

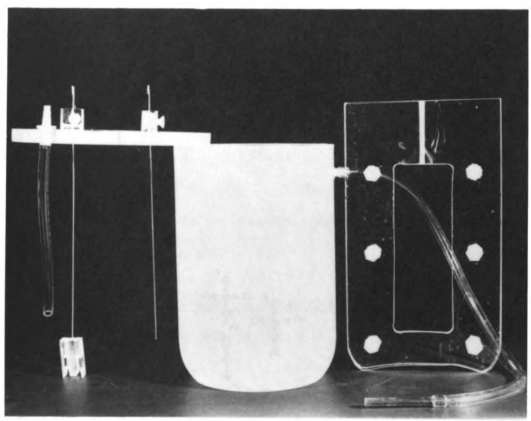


Figure 25. Electrodialysis equipment used in removing peptides and amino acids from BP-13 protease.

TABLE 8.--Characteristics and method of regeneration of ion exchangers used in the purification of BP-13 protease.

Type of Ion Exchanger	Functional Group	pK	
Anion - Diethyl Amino Ethyl (DEAE) Cellulose	$R\text{-}O\text{-}CH_2\text{-}CH_2\overset{+}{N}H(CH_2H_5)_2$	9.5	2 ℓ equilibrium buffer
Cation - Sulfoethyl (SE) Cellulose	$R\text{-}O\text{-}CH_2\text{-}O\text{-}\overset{O}{\overset{\|}{\underset{O}{\|}{S}}}\text{-}O^-$	<1.0	2 ℓ equilibrium buffer
Cation - Phosphoric Acid (P) Cellulose	$R\text{-}O\text{-}\overset{O}{\overset{\|}{\underset{O^-}{\|}{P}}}\text{-}O^-$	1.0-2.0 and 6.5	2 ℓ equilibrium buffer
Cation - Amberlite IRC-50	$R\text{-}COO^-$	3.0	a) 200 ml 2N HCl b) 2 ℓ equilibrium buffer
Cation - Amberlite IR-120	$R\text{-}O\text{-}\overset{O}{\overset{\|}{\underset{O}{\|}{S}}}\text{-}O^-$	<1.0	a) 200 ml 2 M NaCl b) 2 ℓ equilibrium buffer

TABLE 9.--Residual activity in supernatant after mixing of BP-13 protease with DEAE and SE cellulose at various pH levels.

Ion Exchanger	Residual Activity in Supernatant (%)				
	pH 3	pH 4	pH 5	pH 6	pH 7
DEAE Cellulose	29.1	11.6	2.9	2.9	n.d.[a]
SE Cellulose	8.5	16.8	24.1	31.6	. .

[a]Not detectable.

gradient. Under these conditions, 18.2% activity and 26.5% protein were recovered. This was totally unacceptable as a purification step due to the high loss of protein and activity. All attempts to further purify the BP-13 protease were abandoned at this point.

Determination of Molecular Weight

A molecular weight of 49,000 was obtained for the BP-13 protease, using gel filtration (Figure 26). When SDS acrylamide gel electrophoresis was used, a molecular weight of 45,000 was obtained. This value was based on the BP-13 protease having the same electrophoretic mobility as ovalbumin. Attempts to establish a standard curve using aldolase, ovalbumin, chymotrypsinogen A, pepsin and ribonuclease A were unsuccessful: aldolase would not enter the acrylamide gel; chymotrypsinogen was impure and ribonuclease could not be located on the stained gel regardless of how much protein was applied.

TABLE 10.--Recovery of BP-13 protease activity and protein from cellulose ion exchangers when eluting with citrate buffer at various pH levels and ionic strengths.

Type of Ion Exchanger	Equilibrium Buffer	Type of Elution	pH or Ionic Strength of Limiting Buffer	% Recovery of Activity	% Recovery of Protein
DEAE-Cellulose	0.01M citrate pH 6.5 μ = 0.0528	Stepwise ionic strength elution	30 ml of pH 6.5	n.d.[a]	n.d.
			a) μ = 0.1	n.d.	n.d.
			b) μ = 0.2	n.d.	n.d.
			c) μ = 0.4	n.d.	n.d.
			d) μ = 0.8	0.1	n.d.
			e) μ = 1.6	n.d.	n.d.
			f) μ = 3.2	n.d.	n.d.
		Stepwise pH elution	30 ml of μ = .1		
			a) pH 6.0	n.d.	n.d.
			b) pH 5.0	n.d.	n.d.
			c) pH 4.0	n.d.	n.d.
			d) pH 3.0	n.d.	n.d.
SE Cellulose	0.02M citrate pH 3.2 μ = 0.015	Ionic Strength gradient	μ = 2.0	n.d.	n.d.
	0.005 citrate pH 3.5 μ = 0.0033	Ionic Strength gradient	μ = 0.5	4.2	9.3
	0.02M citrate pH 4.44 μ = 0.02	Ionic Strength gradient	μ = 2.0	18.2	26.5

P Cellulose	0.005M citrate pH 3.6 μ = 0.006	Ionic Strength gradient	μ = 0.8	n.d.	n.d.
		pH gradient	0.03M citrate pH 8.1 μ = 0.3	n.d.	n.d.
Amberlite IRC-50	0.01M citrate pH 3.6 μ = 0.0125	pH/Ionic Strength gradient	0.03M citrate pH 8.1 μ = 0.3	n.d.	n.d.
Amberlite IR-120	0.01M citrate pH 3.6 μ = 0.0125	pH/Ionic strength gradient	0.1M citrate pH 8.2 μ = 0.8	5.2	24.9

[a]n.d. not detectable.

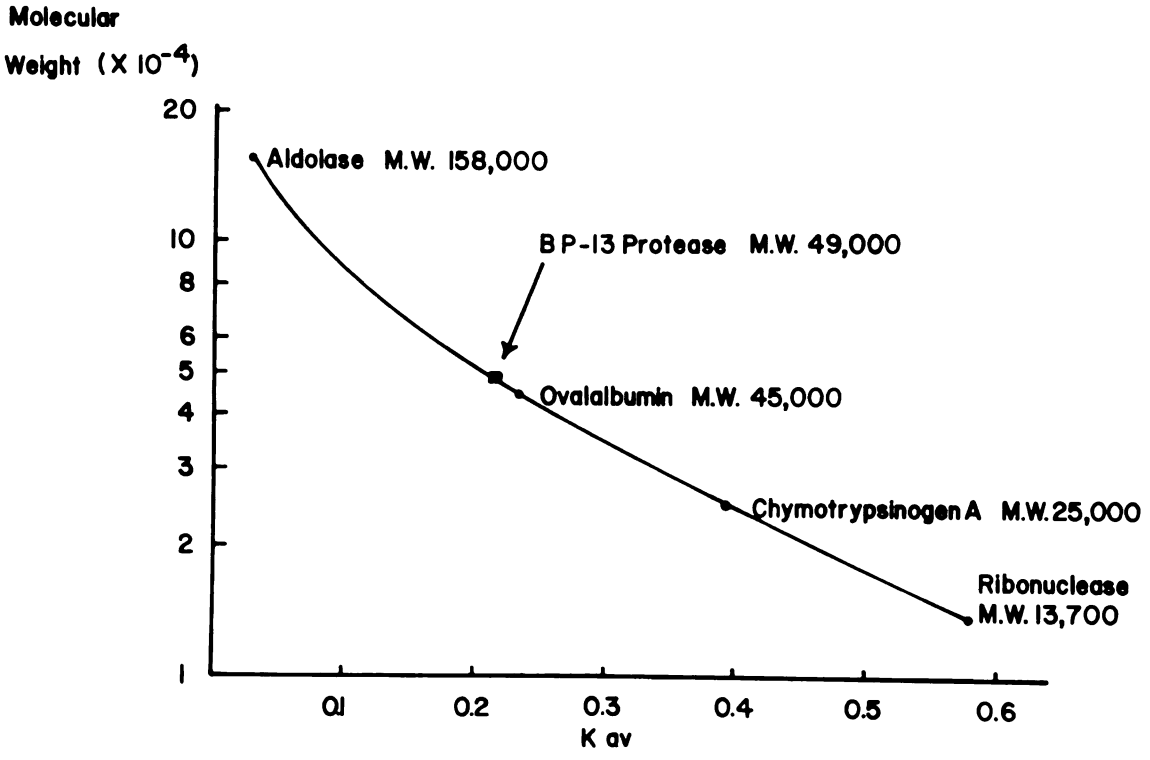
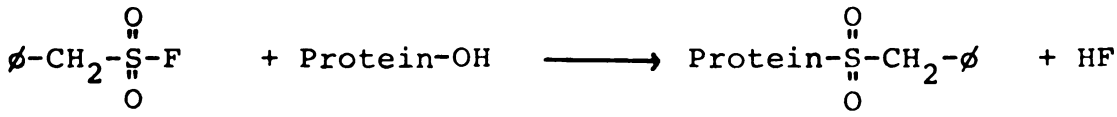


Figure 26. Standard curve for determining molecular weight by gel filtration using G-100 Sephadex (1.6 x 100 cm column). Kav = Ve - Vo/Vt - Vo.

Inhibition Studies

Serine Protease Inhibitor

The electrophilic sulfur atom of phenylmethyl sulfonylfluoride reacts with the nucleophilic hydroxyl group of serine, eliminating a fluoride ion and forming a stable derivate of the enzyme:



This derivate is similar in some respects to the acyl intermediates formed in the hydrolytic reactions of serine proteases (Means and Feeney, 1970a).

Phenylmethyl sulfonylfluoride appeared to have a stimulatory affect on both pepsin and the BP-13 protease (Figure 27). Ryle (1970) indicates that no activators of pepsin have been reported to-date. Therefore, the apparent increase in activity is most likely due to modification of the substrate (1% BSA) rather than activation of either pepsin or the BP-13 protease. The phenylmethyl sulfonyl-fluoride made have denaturated the BSA and exposed additional cleavage sites. This would then result in larger amounts of TCA soluble nitrogen being released and produce an apparent increase in activity. Apparently the BSA should have been denatured by heating.

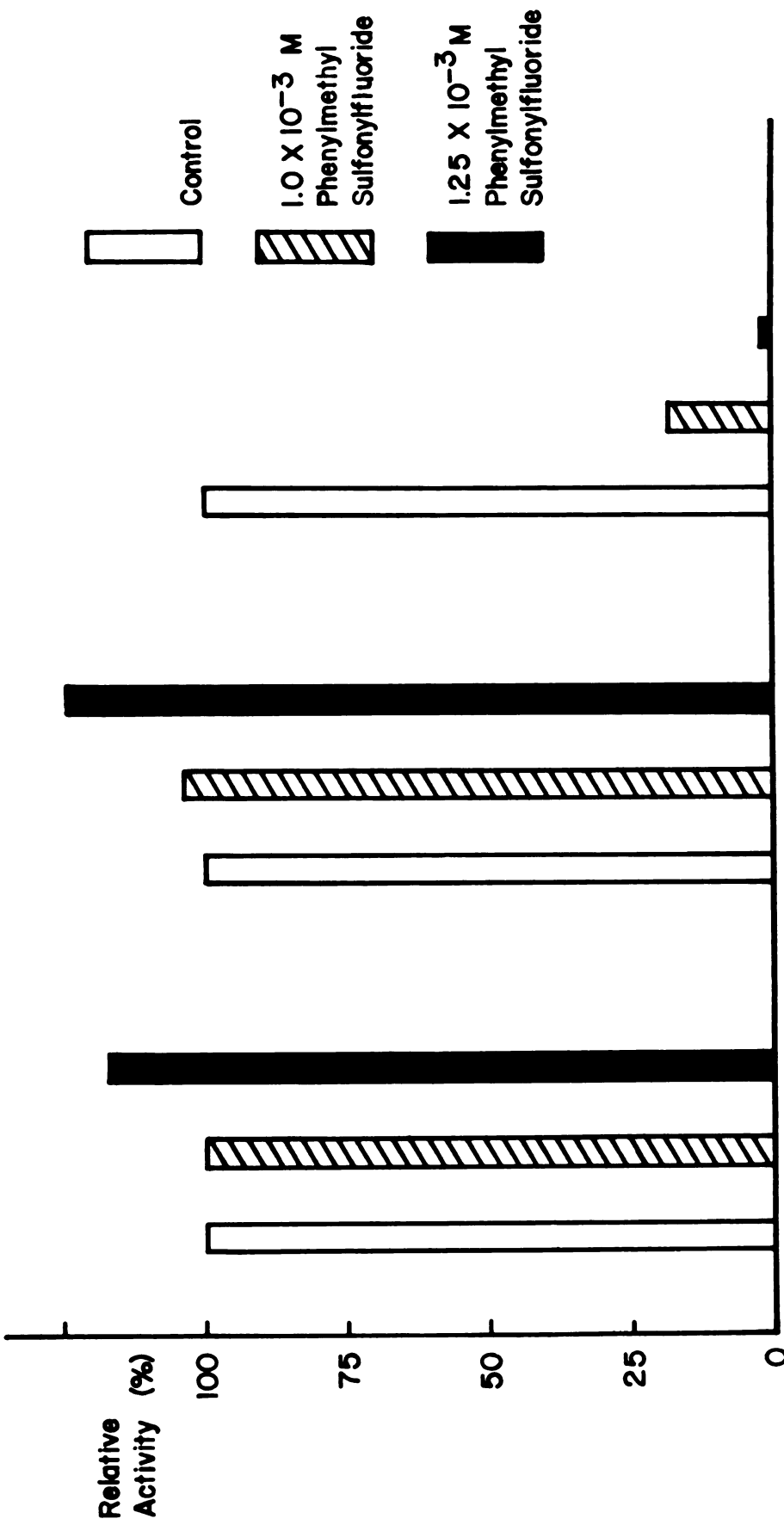
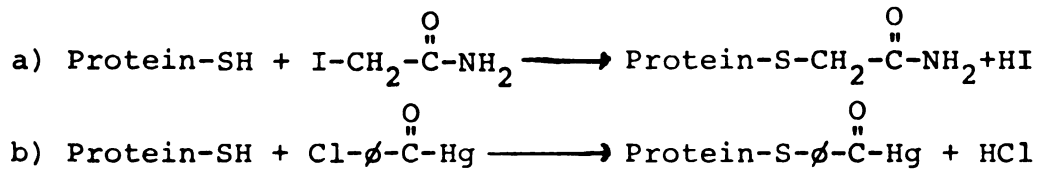


Figure 27. Affect of phenylmethyl sulfonylfluoride on the activity of pepsin, BP-13 protease and trypsin.

Trypsin, a serine protease, was almost totally inhibited (97%) when exposed to phenylmethyl sulfonyl-fluoride for 20 min at 25 C (Figure 27). These results then indicate that the BP-13 protease is not a serine protease.

Sulfhydryl Inhibitors

Iodoacetamide and p-chloromercuribenzoate combine with the free SH groups according to the equations given below:



The derivatives formed have an inhibitory action on sulfhydryl proteases such as papain (Figure 28) but the activity of an acid protease like pepsin is reduced by only 20% with iodoacetamide and 25% by p-chloromercuribenzoate. The activity of the BP-13 protease was reduced 14% by iodoacetamide and 28% by p-chloromercuribenzoate (Figure 28). The free sulfhydryl groups of the BP-13 protease do not appear to play an essential role in the active site of this protease even though the activity was reduced to some extent. Derivatizing the SH groups undoubtedly produces conformational changes in the enzyme. This may in turn reduce the hydrolytic activity of the protease.

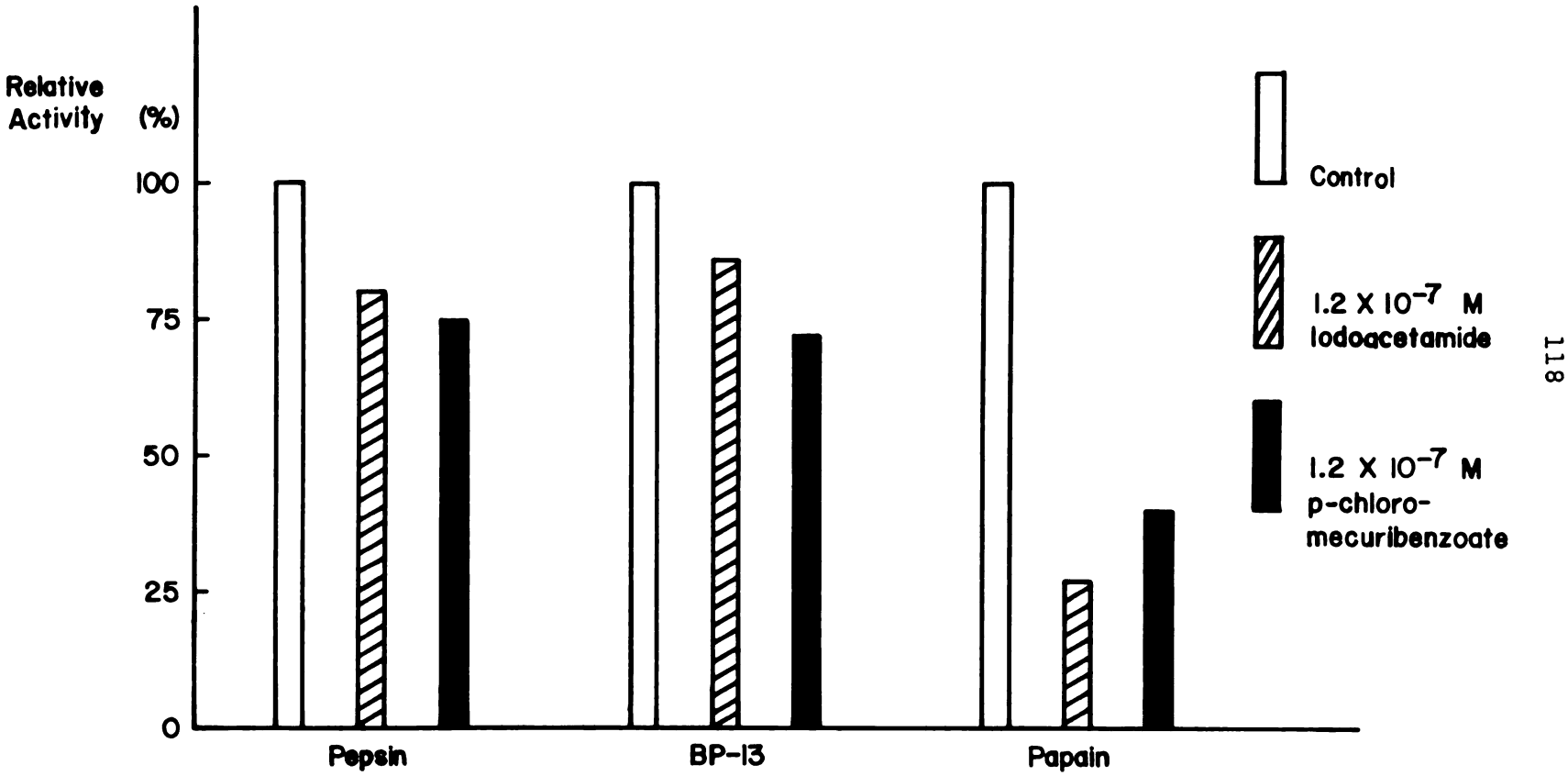
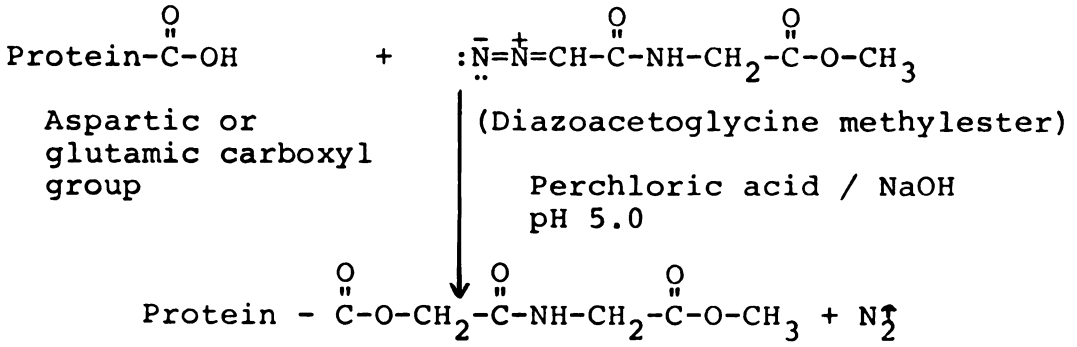


Figure 28. Affect of iodoacetamide and p-chloromercuribenzoate on the activity of pepsin, BP-13 protease and papain.

Carboxyl Modification

Means and Feeney (1970b) indicate that diazoacetates are very specific for carboxyl groups of proteins. The reaction takes place under mild conditions:



Doscher and Wilcox (1961) found the unionized carboxyl group to be the reactive species. The optimum pH for reaction with carboxyl groups is near pH 5.0 according to Riehm and Scheraga (1965). At lower pH levels, diazoacetates hydrolyze easily and limits the extent of modification (Means and Feeney, 1971b). Due to the highly reactive nature of diazoacetates in aqueous solution, excess reagent must be added. Perchloric acid and its salts do not react with diazoacetates and can be used to adjust the pH or ionic strength of the reaction solutions. The decomposition of diazoacetoglycine methylester can be followed easily: the solution will turn from yellow to colorless and nitrogen evolution will cease.

The proteolytic activity fo both pepsin and the BP-13 protease was inhibited to the extent of 91.5 and 91.9% respectively by the diazoacetoglycine methylester

(Figure 29). These results strongly suggest the presence of at least one carboxylic amino acid residue at the active site of the BP-13 protease. Trypsin was unaffected by the diazoacetoglycine methylester (Figure 29).

Effect of EDTA and Calcium

Enzyme sols, 10 mM with respect to EDTA and calcium chloride, exhibited the same proteolytic activity as the controls. The BP-13 protease then does not have a dependency on divalent cations for activity, nor does Ca stimulate protease activity.

Hydrolysis of Synthetic Peptides and Amino Acids Esters by BP-13 Protease

Many enzymes have K_m values as low as 1×10^{-3} M. For this reason each substrate was used at a concentration of 10×10^{-3} M (10 mM) to insure that zero order kinetics would be attained in the event of hydrolysis. Normally substrate concentrations of 10 to 100 Km are employed in most assay procedures.

Of the 15 synthetic substrates listed in Table 11, only L-leucyl-L-tyrosine was hydrolyzed. In this case, 1.9% hydrolysis occurred after incubation for 1 hr at 30 C. This was considered too slow to be of any value in an assay procedure. Failure of the BP-13 protease to hydrolyze any of the remaining 14 substrates may indicate:

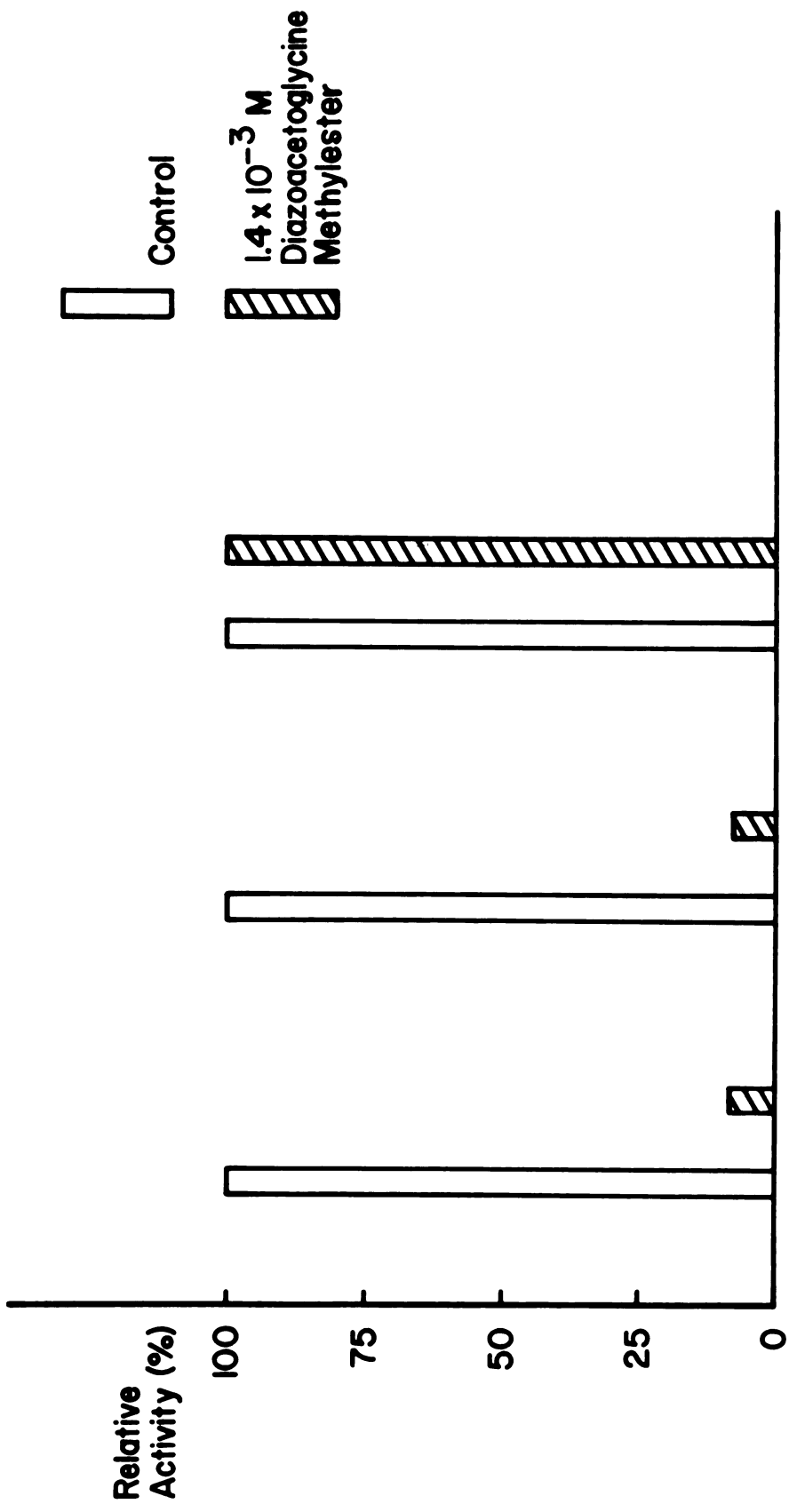


Figure 29. Affect of diazoacetoglycine methylester on the activity of pepsin, BP-13 protease and trypsin.

TABLE 11.--Hydrolysis of synthetic peptides and amino acid esters by BP-13 protease.

Substrate	% Hydrolysis		
	Assay pH		
	3	4	5
N-CBZ[f]-alpha Glutamyl-L-Tyrosine[b]	n.d.[e]	n.d.	n.d.
N-CBZ-alpha-L-Glutamyl-L-Phenylalanine[ac]	n.d.	n.d.	n.d.
N-CBZ-Glycyl-L-Tyrosine[b]	n.d.	n.d.	n.d.
N-CBZ-Glycyl-L-Glutamic acid[a]	n.d.	n.d.	n.d.
N-CBZ-Glycyl-L-Serine[a]	n.d.	n.d.	n.d.
N-CBZ-Glycyl-L-Tryptophan[bc]	n.d.	n.d.	n.d.
N-CBZ-Glycyl-L-Phenylalanine[a]	n.d.	n.d.	n.d.
N-CBZ-Glycyl-L-Leucine[a]	n.d.	n.d.	n.d.
N-Acetyl-L-Phenylalanyl-L-Diiodiotyrosine[acd]	n.d.	n.d.	n.d.
L-Leucyl-L-Tyrosine[b]	n.d.	n.d.	1.9%
L-Leucyl-L-Phenylalanine[b]	n.d.	n.d.	n.d.
L-Methionyl-L-Phenylalanyl-Glycine[b]	n.d.	n.d.	n.d.
L-Methionyl-L-Phenylalanine[b]	n.d.	n.d.	n.d.
Para Tosyl-L-Agrinine Methyl Ester	n.d.	n.d.	n.d.
N-Benzoyl-L-Arginine Ethyl Ester	n.d.	n.d.	n.d.

[a]Dissolved in 0.03M NaOH at 35 C.

[b]Dissolved in 0.01M NaOH at 35 C.

[c]Only slightly soluble at pH 3.

[d]Moderately soluble at pH 4.

[e]n.d. = not detectable.

[f]CBZ = Carbobenzoxy.

a. The enzyme was not specific for any of the amino acid combinations present and/or the peptides were too short for the enzyme to bind and hydrolyze.

b. The N terminal carbobenzoxy group present on seven of the peptides may have prevented binding of substrate to the enzyme.

The methyl and ethyl esters of arginine are hydrolyzed by trypsin, which is not only a peptide peptidohydrolyase but also an esterase. The BP-13 protease does not possess esterase activity when p-tosyl-L-arginine methyl ester and N-benzoyl-L-arginine ethyl ester are used as substrates.

Hydrolysis of the Oxidized B Chain of Insulin

Two dimensional separation of the insulin hydrolysates (B chain) was achieved by high voltage electrophoresis and descending chromatography (Figure 30). A total of 14 ninhydrin positive areas were identified on the peptide map. Twelve of these spots could be clearly identified while two areas were less distinct but still discernible. Free proline was identified by the color reaction to ninhydrin. The release of this amino acid would require hydrolysis of the peptide bonds between Thr-Pro (residues 27 and 28) and Pro-Lys (residues 28-29).

The presence of 14 hydrolystate products indicates cleavage of insulin at 13 sites. The peptide map in Figure 30 is representative of the proteolytic nature of

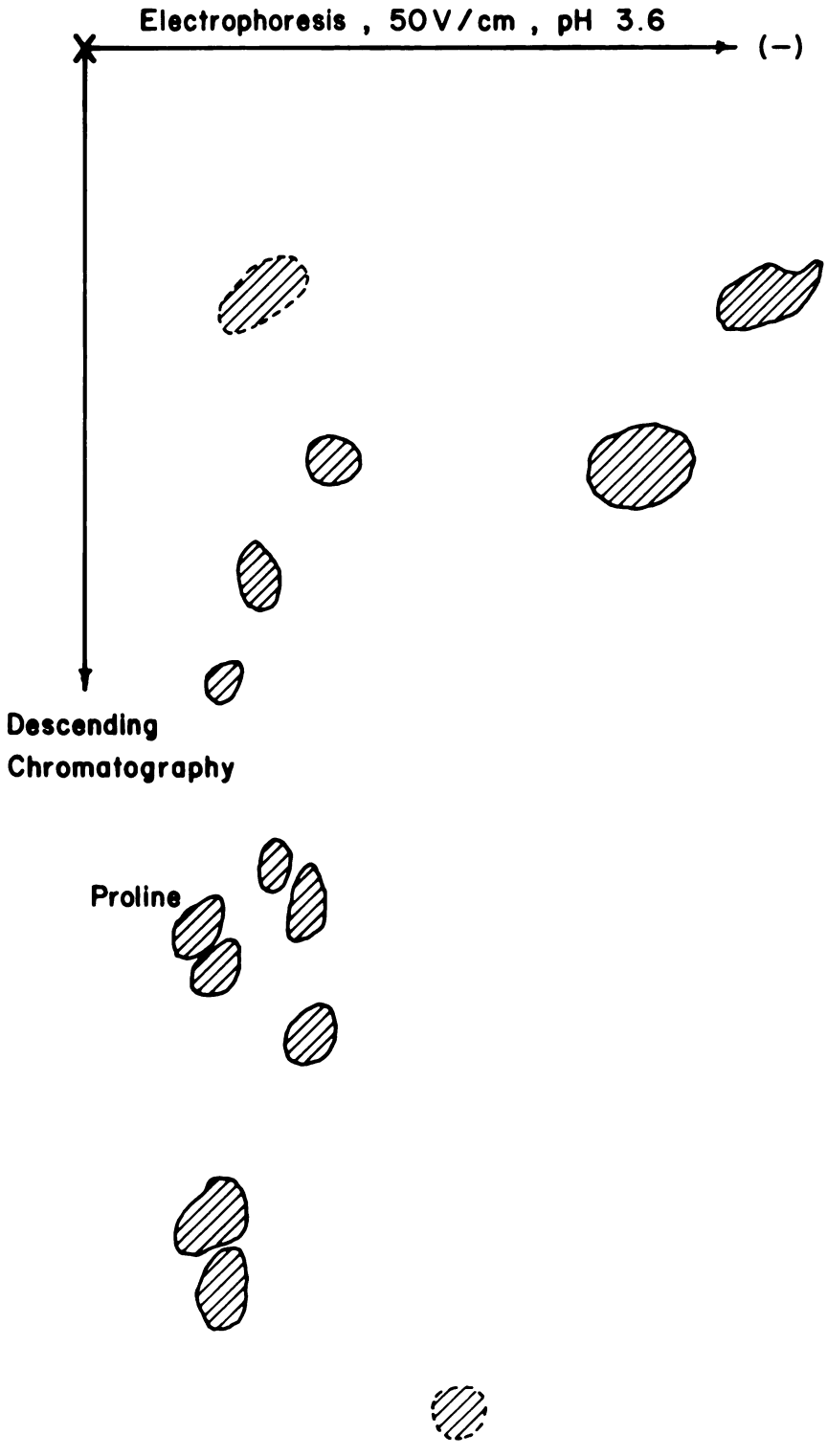


Figure 30. Separation of insulin (oxidized B chain) hydrolysates using high voltage electrophoresis followed by descending chromatography. (Dotted areas indicate low color intensity.)

the BP-13 protease only if all bonds that are susceptible to cleavage are hydrolyzed to the extent of 100%. Incomplete hydrolysis would yield a peptide map which over emphasizes the true proteolytic nature of the enzyme.

Cleavage of the oxidized B chain of insulin at 13 sites would seem to indicate that the BP-13 protease is very proteolytic; however, rennin has the ability to cleave this same substrate in nine places while pepsin can hydrolyze 10 peptide bonds. Despite the fact that both rennin and pepsin cleave several places, only two bonds are hydrolyzed beyond 20% by rennin when incubated for 20 hr under optimum conditions (Rickert, 1970). In this same paper, Rickert also points out that pepsin hydrolyzes four bonds between 10 and 20% with the remaining bonds being cleaved less than 10%. Mucor miehei only cleaves 6 linkages of the B chain of insulin but hydrolysis at five of these sites is in excess of 20%. The most susceptible site of attack was between Try (16) and Leu (17). Sixty-three percent of the B chain was cleaved between these two amino acid residues (Rickert, 1970). In a related experiment, McCullough and Whitaker (1971) obtained 9 spots on a peptide map of Mucor pusillus when incubated for 20 hr with the B chain of insulin. Analysis showed cleavage at 8 peptide bonds. In this instance, the peptide map was almost a quantitative reflection of the number of cleavage sites. In the case of rennin and pepsin, each of the peptides has

to be sequenced to accurately determine which peptide bonds are hydrolyzed.

The BP-13 protease is considerably more proteolytic than pepsin, calf rennet or any of the major commercial fungal rennets. Consequently, the peptide map obtained in Figure 30 should be an accurate reflection of the number of peptide bonds cleaved by the extracellar protease of P. roqueforti.

Comparison of the Proteolytic Action of the BP-13 Protease and Calf Rennet

Hydrolysis of a 1% casein solution (pH 5.75) by the BP-13 protease and calf rennet are compared in Figure 31. After 1 min and 5 min both enzymes had released approximately the same quantity of TCA soluble nitrogen but between 5 and 60 min the BP-13 protease produced a ΔA of 0.685 while calf rennet reflected a ΔA of 0.063. The high initial velocity by the calf rennet is due to rapid hydrolysis of the methionyl-phenylalanine linkage of κ-casein. After the cleavage of this bond, hydrolysis is slow and non-specific. Hydrolysis by the BP-13 protease appears also to be non-specific but very rapid compared to calf rennet. The portion of the curve between 20 and 60 min for hydrolysis of casein by the BP-13 protease (Figure 31) is nearly linear even though the reaction is proceeding at a rapid rate. Again this indicates generalized proteolysis rather than specificity for a particular peptide bond.

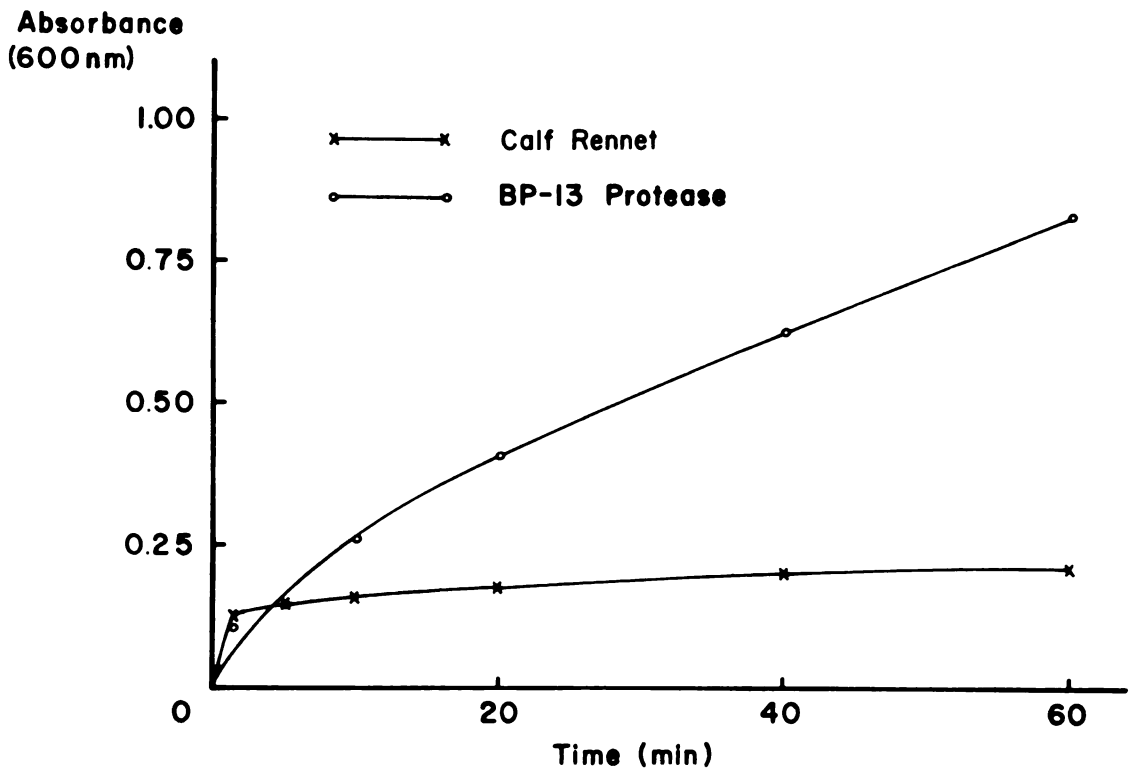


Figure 31. Hydrolysis of 1% casein (pH 5.75) with calf rennet and BP-13 protease.

Figure 32 represents the gel patterns obtained when the BP-13 protease is incubated with 5% casein at pH 5.75. Samples were taken at 1 min, 5 min, 10 min, 30 min, 3 hr and 20 hr. After only 1 min both the α_s- and β-casein had changed slightly with a number of additional light staining bands moving ahead of the α_s-casein. The β-casein appears to be hydrolyzed slightly more rapidly by the BP-13 protease but after 20 hr both the α_s- and β-casein are no longer identifiable on the alkaline gel pattern. Kappa casein remains virtually unchanged throughout the entire hydrolysis procedure. One major peptide, with high electrophoretic mobility, appears to be preferentially released after 1 min of hydrolysis. This band has the highest electrophoretic mobility and continues to increase in size up to 3 hr but after 20 hr this component also disappears.

The gel pattern in Figure 32 confirms the experimental results obtained in Figures 30 and 31 in which the BP-13 protease was characterized as being very proteolytic, yet non-specific in its action on protein.

The BP-13 protease would not be an acceptable calf rennet substitute on the basis of the gel pattern obtained in Figure 32 because both the α_s- and β-casein are extensively hydrolyzed while κ-casein is virtually unaffected. An ideal substitute is one which acts preferentially on κ-casein but has little affect on either α_s- or β-casein.

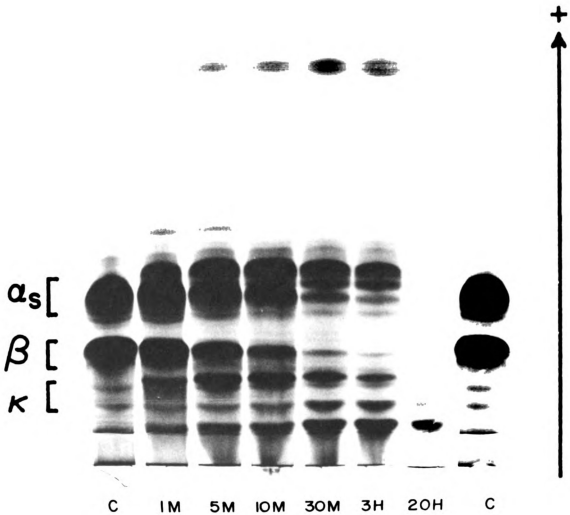


Figure 32. Alkaline acrylamide gel electrophoresis of casein (C) and BP-13 hydrolysates of casein at various time intervals (M-min; H-hr).

Evaluation of Milk Clotting Ability

When the BP-13 protease was used at a concentration comparable to veal rennet, coagulation was very slow and a consequence of acid formation rather than a result of hydrolytic action by the fungal protease. The clotting time could be shortened to 1 hr by adding 10 times as much of the enzyme but this was still unacceptable:

a. Clotting time was still too long.

b. The milk never formed a smooth firm gel but tended to coagulate.

This was followed by syneresis and precipitation of the casein.

The BP-13 protease had a milk clotting activity/ proteolytic activity ratio of 0.005 while the ratio for calf rennet was 0.073 (Table 12). The fungal rennet is than 0.073/0.005 or 14.6 times as proteolytic as the calf rennet for the same milk clotting activity.

TABLE 12.--Comparison of milk clotting activity and proteolytic activity of calf rennet and BP-13 protease.

Enzyme	Milk Clotting[a] Activity (MCA)	Proteolytic Activity[b] (per 0.5ml)	MCA/ Proteolytic Activity
Calf Rennet	10.6	145	0.073
BP-13 Protease	4.57	880	0.005

[a] $\text{MCA} = \frac{2400}{t(\text{sec})}$ x dilution factor.

[b] Mg of TCA soluble nitrogen released at time of clotting.

SUMMARY AND CONCLUSIONS

P. roqueforti (strain BP-13) was shake cultured in Czapek-Dox broth containing 0.5% Proteose-Peptone No. 3. The extracellular protease was isolated from a concentrated CFE by fractionation on a series of Sephadex columns. A 140 fold purification was obtained in the final enzyme preparation.

The protease isolated was thought to be truly extracellular for three reasons:

a. Culturing conditions were adjusted to minimize mycelial breakdown.

b. The vegetative growth was centrifuged out and never subjected to induced cellular disruption.

c. The enzyme contains carbohydrate, which is characteristic of most extracellular proteins.

The pH optimum was 3.0, 5.5 and 3.0 to 4.0 for BSA, casein and the oxidized B chain of insulin respectively. Based on the pH optimum for BSA and insulin, the enzyme would appear to be an acid protease. This was confirmed in an experiment where the BP-13 enzyme was reacted with an

acid protease inhibitor (Diazoacetoglycine methylester) and found to be almost totally inactivated. Either aspartic and/or glutamic acid would appear to be involved in the active site of this enzyme. Serine and sulfhydryl protease inhibitors did not significantly reduce the action of the BP-13 protease when the activity of this enzyme was compared to pepsin. Ethylenediaminetetraacetate, a chelating agent, had no affect on enzymatic activity. Therefore, the enzyme does not appear to have a dependency on divalent cations for activity.

The enzyme exhibited maximum stability to pH in the range of 3 to 6. Inactivation on either side of this range was due to denaturation of the enzyme.

An optimum temperature of 45 to 46 C was obtained when the enzyme was assayed with 1% casein at pH 5.75. Inactivation, due to heat denaturation, was evident at 48 C when using the standard 9 min end point assay. Increasing the temperature beyond 48 C resulted in first order irreversible thermal inactivation of the enzyme. An activation energy of 8000 cal/mole was calculated for the hydrolysis of casein under standard assay conditions where the only variable was temperature.

There was some doubt as to whether total purity had been obtained in the final enzyme preparation. Small molecular weight components, identified as amino acids and/or peptides, were present in the final enzyme

preparation. Extensive dialysis, electrodialysis ion exchange and precipitation of the BP-13 protease with ammonium sulfate failed to remove this fraction. Whether the small molecular weight components were present as impurities (from the Proteose-Peptone added initially) or were formed as a result of autolysis, was not resolved. Amino acids and peptides will not bind dyes such as Amido Black or Coomossie Blue. This may account for obtaining one band when the final enzyme preparation was subjected to acid, alkaline or SDS gel electrophoresis. The addition of mercaptoethanol to the protease resulted in the formation of 14 bands on an alkaline acrylamide gel. Either the enzyme is composed of a number of polypeptide chains linked to each other by disulfide bonds, or the large number of bands in the gel pattern are an artifact caused by the carbohydrate moiety associated with the protease.

Molecular weights of 49,000 and 45,000 were obtained using gel filtration and SDS gel electrophoresis respectively.

The action of the BP-13 protease on 15 synthetic peptides and amino acid esters was very limited. Only L-leucyl-L-tyrosine was hydrolyzed to a detectable extent. The hydrolysis of this substrate was too slow to be of any value as an assay method for proteolysis. Failure to hydrolyze any of the remaining 12 peptides may have been a function of chain length and/or a lack of specificity for the amino acid residues present. Also the N terminal

carbobenzoxy group, present on 7 of the 13 peptides, may have blocked hydrolysis if these substrates by the enzyme under investigation. In addition, the methyl and ethyl esters of arginine were not hydrolyzed by the BP-13 protease.

Incubation of the oxidized B chain of insulin with the BP-13 protease resulted in the formation of 14 ninhydrin positive areas on the peptide map of the hydrolysate. These results indicated the protease was capable of hydrolyzing a large number of peptide bonds. The peptide map was thought to be an accurate reflection of the number of bonds hydrolyzed. The accuracy of this map is totally dependent on the degree of hydrolysis of each cleavage site.

The initial rates of hydrolysis of a 1% solution of casein by calf rennet and the BP-13 protease were nearly equal but between the time period of 5 and 60 min, hydrolysis of casein by the fungal protease proceeded at approximately 11 times the velocity of veal rennet. After hydrolysis of the methionyl-phenylalanine linkage of κ-casein, the rate of hydrolysis of substrate by calf rennet fell off rapidly while the velocity of the BP-13 protease reaction remained almost linear with time.

The affect of the Penicillium protease on the major casein components was clearly shown by acrylamide gel electrophoresis of the casein hydrolysates. After 20 hr of enzymatic action, both the α_s- and β-casein were no

longer evident on the gel pattern, but κ-casein remained virtually unchanged. These results alone indicated the BP-13 protease was far more proteolytic than pepsin, chymosin or any of the commercially acceptable fungal rennets.

The BP-13 protease was found to have over 14 times the proteolytic activity of calf rennet for the same milk clotting ability. Calf rennet formed a smooth firm gel when the milk clotted. When the Penicillium protease was substituted for calf rennet, the milk tended to coagulate into distinct particles. This was followed by syneresis and precipitation of the casein from suspension. These results are a consequence of the proteolytic nature of the enzyme:

a. κ-casein appeared to be resistant to hydrolysis by the BP-13 protease while α_s- and β-casein were rapidly hydrolyzed.

b. The enzyme acted in a very non-specific manner on a large number of peptide bonds.

c. The protease was unable to hydrolyze the methionyl-phenyalanine linkage of a synthetic peptide containing these two amino acids. However, this may be a function of peptide chain length rather than specificity.

The action of the BP-13 protease on insulin and casein indicate the enzyme is an endopeptidase. An Enzyme Commission number (E.C. no.) of 3.4.4.99 can be applied to the enzyme with the information gained in this study. The numbers from left to right represent class (Hydrolase), sub class (Peptide hydrolase) sub-sub class (Peptide peptidohydrolase) and serial number. A serial number of 99 was assigned to the BP-13 protease because the nature of the reactions catalyzed have not been fully investigated to determine which peptide bonds are hydrolyzed.

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APPENDIX

APPENDIX

Type I Medium

Martley et al. (1970) formulated an improved medium for the detection of proteolytic organisms in total bacterial counts. The composition was as follows:

	g/ℓ
Standard Methods Agar (SMA)	23.5
Sodium caseinate	10.0
Trisodium citrate	4.41
Calcium chloride ($6H_2O$)	4.38

Type III Medium

Czapek-Dox broth was obtained from Difco or formulated as follows:

	g/ℓ
Sucrose	30.0
Sodium nitrite	3.0
Dipotassium phosphate	1.0
Magnesium phosphate	0.5
Potassium chloride	0.5
Ferrous sulfate	0.01

Type IV Medium

Meyers and Knight (1958) formulated a medium for the submerged growth of P. roqueforti. The medium consisted of:

	g/ℓ
Sucrose	30.0
Ammonium sulfate	9.43
Dipotassium phosphate	1.00
Magnesium sulfate ($7H_2O$)	0.5
Potassium chloride	0.5
Ferrous sulfate ($7H_2O$)	0.05
Sodium acetate	2.0
Sodium lactate	5.2
Oleic acid	0.2

The oleic acid was eliminated from the above formulation in shake culturing experiments with P. roqueforti. To one ℓ of the above solution, 1.0 ml of mineral solution was added. On a 100 ml basis, this solution contained:

	mg
Cupric chloride	15.5
Zinc sulfate ($7H_2O$)	175.6
Magnesium chloride ($4H_2O$)	36.0
Calcium chloride ($2H_2O$)	183.4
Ammonium molybdate ($4H_2O$)	10.2

Universal Buffer

A stock buffer, 0.4 M with respect to phosphate, acetate and borate, was prepared by adding 39.2 g of phosphoric acid, 24.0 g of acetic acid and 24.8 g boric acid to a 1 ℓ volumetric flask (Coch Frugoni, 1957). After solvation of the acidic components, the solution was diluted to volume.

A 100 ml of this stock solution was diluted to 1 ℓ prior to adjustment of pH (0.2 N sodium hydroxide) and ionic strength (sodium or potassium chloride). The

quantity of alkali and salt added to obtained a specific pH and ionic strength given in Table A-1 on following page.

Alkaline Copper Sulfate-Sodium Tartrate Solution

a. Protease activity

Two ml of 1.0% copper sulfate (w/v) were mixed with 2 ml of 2.7% sodium tartrate (w/v). To this, 96 ml of 0.35 N sodium hydroxide in 2.0% sodium bicarbonate (w/v) were added.

b. Protein determination

The alkaline copper sulfate-sodium tartrate was prepared as in "a" except the N of the sodium hydroxide was reduced from 0.35 to 0.20.

Ninhydrin Solution

Four hundred mg of stannous chloride ($2H_2O$) were dissolved in 250 ml of 0.2 M acetate buffer at pH 5.0. This solution was mixed with 250 ml of methyl cellosolve (Ethylene glycol monomethyl ether) containing 10 g of dissolved ninhydrin (Calibiochem, A grade). The solution was flushed with nitrogen and stored in a brown glass bottle at 4 C.

TABLE A-1.--Protocol for preparation of 0.04 M Universal buffer.

pH Desired	n[a]	μ[b]	g Salt to Attain μ = 0.3[c]	
			NaCl	KCl
2	10	0.020	17.35	22.13
3	175	0.036	18.62	23.75
4	235	0.040	18.92	24.13
5	345	0.050	19.71	25.14
5.75	410	0.056	20.03	25.60
6.0	425	0.060	19.92	25.41
7.0	535	0.073	20.23	25.80
8.0	620	0.090	20.00	25.50
9.0	705	0.098	19.83	25.30
10.0	805	0.110	19.76	25.21
11.0	890	0.130	18.23	23.26
12.0	10.75	0.150	17.79	22.70

[a]ml 0.2N NaOH required to bring the pH of 1 ℓ of 0.04 Universal buffer (pH 1.95) up to the level desired.

[b]This is the ionic strength of the buffer after "n" ml of 0.2 N NaOH have been added.

[c]The g of salt is based on the total volume, i.e., 1000 ml + n ml of 0.2 NaOH.

Ninhydrin Spray

Preparation of this reagent involved solvation of 0.35 g of ninhydrin (Calbiochem, B grade) in 350 ml of absolute ethanol, 135 ml of glacial acetic acid and 140 ml of collodin (2, 4, 6 Trimethyl pyridine).

Kjeldahl Digestion Mixture

Five g of copper sulfate ($5H_2O$) and 5.0 g of selenium dioxide were dispersed in 500 ml of concentrated sulfuric acid.

TABLE A-2.--Summary of BP-13 protease properties.

Property	Value or Comments
Optimum pH	3.0-BSA; 5.5-casein
Stability to pH (maximum)	pH 3.0 to 6.0
Optimum temperature	45 C (9 min endpoint assay)
Stability to temperature	stable up to 45-46 C for 9 min
Energy of activation for hydrolysis of casein	8000 cal/mole
Molecular weight	45,000 Daltons (Acrylamide gel electrophoresis with SDS) 49,000 Daltons (Gel filtration-G100 Sephadex)
Enzyme type	Acid protease
E.C. no.	3.4.4.99

List of MSU photo lab negatives of figures, graphs and photographs in thesis.

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9	724140-6
10	724140-11
11	724140-5
12	724140-12
13	724140-10
14	724290-3
15	724140-21
16	723625-3
17	723625-6
19	723625-4
20	724290-2
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22	724290-6
23	724140-2
24	724140-9
25	724290-5
26	724140-7
27	724140-14
28	724140-13
29	724140-44
30	724140-15
31	724140-8
32	724290-1