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Purification and Immunological Reactivity of Commercial Microbial Milk Clotting Enzyme Preparations

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PURIFICATION AND IMMUNOLOGICAL REACTIVITY OF COMMERCIAL MICROBIAL MILK CLOTTING ENZYME PREPARATIONS

by

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DEDICATION

Dedicated to the memory of my father

Nze Thomas O. Osuala Anugwara

who gave so much to me.

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I extend my heart felt gratitude to my major professor Dr. R. J. Brown for his assistance and guidance in the completion of my study here at Utah State University. I also appreciate all the help he offered me in dealing with my father's death.

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Last but first, my mother, brothers and sisters, my wife Florenda and my children Eveonne and Augustine, I truly appreciate your sacrifices and endurance throughout the course of my education. I could not have done it without your support. Thank you for bearing with me.

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Chima I. Osuala

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Purification and Immunological Reactivity of Commercial Microbial Milk Clotting Enzyme Preparations

by

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Commercial microbial milk clotting enzyme preparations were purified by immunoaffinity chromatography using purified antibody covalently coupled to porous glass beads as the column matrix. Commercial enzyme preparation diluted in 1 m*M* sodium acetate buffer at pH 5.0 was then biospecifically adsorbed to the column matrix by end-over-end mixing of the glass-antibody complex in the enzyme solution for 12 h at 5°C. The antibody bound enzyme adsorbed glass beads were soaked in .2 *M* glycine or ethanolamine at pH 7.0 to block uncoupled reactive sites on the matrix. Following this, the column was washed with 1 m*M* sodium acetate buffer at pH 7.0, followed by additional wash with .5 *M* NaCl, until absorbance at 280 nm returned to baseline. Elution of adsorbed enzyme was achieved with .2 *M* sodium acetate at pH 3.0, .2 *M* acetate at pH 3.5, containing .15 *M* NaCl and .5 *M* acetate at pH 4.0 containing .5 *M* NaCl. At the same protein concentration, immunoaffinity chromatography purified enzymes had higher clotting activity than the commercial enzyme preparations. Amino acid analysis and OPA proteolysis tests of TCA soluble peptides liberated from casein hydrolysis showed purified enzymes to exhibit lower general proteolytic activity.

Immunological reactivity of *Mucor* enzymes with calf rennet was determined with antibodies produced by intramuscular injections of *Mucor miehei* protease, *Mucor pusillus* protease and calf rennet emulsified in Freund's adjuvant into three New Zealand White rabbits. Harvested antisera were heated at 56°C for 30 min to inactivate complement factors and contaminating proteins then centrifuged at 1700 × g for 30 min. Ouchterlony double immunodiffusion method was used to test for presence of antibodies in the antisera, and for cross immunoreactivity. Antibodies against *M. miehei* were cross reactive with *M. pusillus* antigen and *M. pusillus* antibodies cross reacted with *M. miehei* antigen. Immunodiffusion assay did not show cross reactivity of calf rennet antibodies with either *M. miehei* antigen or *M. pusillus* antigen. Antibodies against the *Mucor* enzymes did not show cross reactivity with calf rennet antigen.

Although their actions in milk differ, proteolytic enzyme preparations from *M. miehei* and *M. pusillus* are both used as calf rennet substitutes in cheese manufacture. Differences in the characteristics of the two *Mucor* enzyme preparations exist, even though they exhibit some immunological homology. From our results, at least one antigenic factor is common to both enzyme preparations.

(138 pages)

GENERAL INTRODUCTION

Owing to shortage in calf rennet supply, the cheese industry has been forced to develop calf rennet substitutes for cheese making (Alichanidis et al., 1984; Foda et al., 1978; Green, 1972; Green, 1977; Mickelsen and Fish, 1970; Rao and Mathur, 1979a; Richardson et al., 1967; Shaker and Brown, 1985a; Sponcet et al., 1985; Tam and Whitaker, 1973; Vanderpoorten and Weckx, 1972). Fungal rennets are accepted for use in the manufacture of cheese (Green, 1977; Richardson et al., 1967; Sardinas, 1968; Shaker and Brown, 1985a; Sponcet et al., 1985; Sternberg, 1972; Vanderpoorten and Weckx, 1972), but are found to produce bitter, soft and pasty aged cheese (Lobarzewski and VanHuystee, 1982; Mickelsen and Fish, 1970; Morvai-Racz, 1974; Sternberg, 1972). Defects in cheese made with fungal rennets are caused by the increased non-specific proteolytic action of these preparations after hydrolysis of κ-casein (Green, 1972; Green, 1976; Mickelsen and Fish, 1970; Vanderpoorten and Weckx, 1972).

Gel filtration patterns of *Mucor* rennets show several peaks, indicating multi-enzyme systems (Edelsten and Jensen, 1970,1973; Shaker and Brown, 1985a; Shehata et al., 1978). Proteolytic enzymes from *Mucor miehei* and *Mucor pusillus,* although used for cheese manufacture, are more proteolytic than calf rennet. Commercially available microbial rennets contain both milk clotting enzymes and other enzymes which are deleterious to the quality of cheese made with them. Separation of the contaminating enzymes should permit more uniformity in the quality of cheeses made from microbial rennets.

To determine the immunological relationship between *Mucor* enzymes and calf rennet, antibodies were produced against these enzymes and their reactivities compared. Antibodies are a class of immunoglobulins synthesized by animals in response to "foreign bodies" in their blood. Antibodies can be raised against almost all compounds foreign to a host if they are large enough to elicit antibody production or coupled to a suitable protein to render them immunogenic (Eisen and Siskind, 1964; Warr, 1982).

The objectives of this research were to:

1) evaluate the effectiveness of immunoaffinity chromatography in reduction of general proteolytic activities of microbial milk clotting preparations.

2) determine the immunological relationship between *Mucor* milk clotting enzymes and calf rennet.

CHAPTER I

PURIFICATION OF COMMERCIAL MICROBIAL MILK CLOTTING ENZYME PREPARATIONS BY IMMUNOAFFINITY CHROMATOGRAPHY

ABSTRACT

Purified antibodies against commercially available *Mucor miehei* and *Mucor pusillus* milk clotting enzyme preparations attached to porous glass beads were used to construct immunoaffinity columns. The columns were then used to isolate the milk clotting fractions from the commercial milk clotting enzyme preparations. Antibody in 1 m*M* sodium acetate buffer at pH 7.0 was covalently coupled to activated glass matrix, using a carbodiimide as catalyst . Commercial enzyme preparation diluted in 1 m*M* sodium acetate buffer at pH 5.0 was then adsorbed to the column, by recycling the enzyme solution through the column for 12 h at 5°C, . Unreacted sites on the matrix were blocked by recycling .2 *M* glycine or ethanolamine at pH 7.0 through the column. . The column was washed with coupling buffer followed with .5 *M* NaCl, until absorbance at 280 nm returned to baseline. Adsorbed enzyme was eluted from the column with different buffers. Effective elution was achieved with .2 *M* sodium acetate at pH 3.0, .2 *M* acetate at pH 3.5 containing .15 *M* NaCl, and .5 *M* acetate at pH 4.0 containing .5 *M* NaCl. Purification by immunoaffinity chromatography yielded enzymes with higher clotting activity as compared to the crude enzyme preparation when adjusted to the same absorbance at 280 nm. When adjusted to the same clotting activity, it also yielded fractions with lower general proteolytic activity as determined by peptides liberated from the proteolysis of casein.

INTRODUCTION

Shortage in calf rennet supply, has caused the cheese industry to seek substitutes for calf rennet in cheese making (Alichanidis et al., 1984; Foda et al., 1978; Green, 1972; Green, 1977; Mickelsen and Fish, 1970; Rao and Mathur, 1979b; Richardson et al., 1967; Shaker and Brown, 1985a; Sponcet et al., 1985; Tam and Whitaker, 1973; Vanderpoorten and Weckx, 1972). Fungal rennets are accepted as calf rennet substitutes and are used in the manufacture of cheese (Green, 1977; Richardson et al., 1967; Sardinas, 1968; Shaker

and Brown, 1985b; Sponcet et al., 1985; Sternberg, 1972; Vanderpoorten and Weckx, 1972), but they produce bitter, soft and pasty aged cheese (Lobarzewski and VanHuystee, 1982; Mickelsen and Fish, 1970; Morvai-Racz, 1974; Sternberg, 1972). Their increased non-specific proteolytic actions after the hydrolysis of κ-casein have been linked to defects in cheese made with fungal rennets (Green, 1972; Green, 1976; Mickelsen and Fish, 1970; Vanderpoorten and Weckx, 1972).

Although many enzymes of animal origin can be used in cheese making (Chow and Kassell, 1968), the active component in calf rennet, chymosin, is accepted as the most suitable enzyme for cheese manufacture (Fish, 1957; Fox, 1969; Green, 1977; Kobayashi et al., 1985a; Kobayashi et al., 1985b; Law, 1983; Shaker and Brown, 1985a,b; Visser, 1981). Chymosin exhibits specific, limited proteolytic action on casein and high milk clotting activity (Hubble and Mann, 1984; Kobayashi et al., 1985a; Law, 1983; Raymond et al., 1973; Shaker and Brown, 1985a,b; Tam and Whitaker, 1973; Visser and Slangen, 1977; Visser, 1981). It shows specific affinity for the milk protein κ-casein, by hydrolyzing the Phe_{105}-Met_{106} bond causing milk to coagulate (Creamer et al., 1971; Hubble and Mann, 1984; Kobayashi et al., 1985a; Law, 1983; Tam and Whitaker, 1973).

Calf rennet has been used in the manufacture of cheese for so many years that it is adopted as the standard for judging rennet substitutes (Shaker and Brown, 1985a,b; Sternberg, 1972). Generally, microbial rennets liberate more non-protein nitrogen (NPN) from α-, β- and whole-caseins than calf rennet, leading to increased losses of protein into whey when substitutes are used in cheese manufacture. This results in decreased cheese yield (Vamos-Vigyaso et al., 1980; Vanderpoorten and Weckx, 1972). Although microbial milk clotting preparations and calf rennet hydrolyze κ-casein at the same rates for the first 5 min of milk coagulation, microbial milk clotting preparations more rapidly hydrolyze κ-casein and whole casein with prolonged incubation time (Vanderpoorten and Weckx, 1972).

Gel filtration patterns show several protein peaks for commercial *Mucor* rennets (Edelsten and Jensen, 1973; Shaker and Brown, 1985a; Shehata et al., 1978). They contain

milk clotting enzymes and other enzymes which may be deleterious to the quality of cheese made with them. Separation of these accompanying enzymes should permit more uniformity in the quality of cheeses made from these rennets.

At the initial stages of milk coagulation, the clotting activities of *Mucor* enzymes follow a mechanism similar to that of calf rennet (Dennis and Wake, 1965; Sternberg, 1972; Tam and Whitaker, 1973), but their general proteolytic action increases beyond that of calf rennet with time (Tam and Whitaker, 1973).

To make the clotting activities of microbial milk clotting enzymes similar to that of calf rennet, researchers have tried different methods of modifying these enzymes (Colein and Delecourt, 1981; Cornelius, 1982; Cornelius et al, 1982; Havera and David, 1987). Methods such as heat treatment (Hamdy and Edelsten, 1970), acid treatment (Colein and Delecourt, 1981), oxidation treatment with peroxides or hypochlorates (Cornelius et al, 1982) and acylation (Havera and David, 1987; Higashi et al., 1983; Hubble and Mann, 1984) have been used. Milk clotting preparations suitable for cheese making must exhibit high specific cleavage towards κ-casein at the pH of milk and low general proteolytic activity under cheese ripening conditions (Visser, 1981).

Microbial Rennet Substitutes

The decline in the supply of calf rennet, attributed to the decreased demand for veal and to increased demands for calf rennet, has forced the cheese industry to consider microbial rennets as calf rennet substitutes in cheese making (Alichanidis et al, 1984; Green, 1977; Law, 1983; Miller et al., 1987; Rao and Mathur, 1979b; Richardson et al.,1967; Shaker and Brown, 1985b; Sponcet et al., 1985; Taylor et al., 1977; Visser and Slangen, 1977). Calf rennet substitutes have been obtained from bacterial and fungal sources (Otani et al., 1984; Fox, 1969; Kobayashi et al., 1985a; Larson and Whitaker, 1970; Lawrence and Creamer, 1969; Prins and Nielson, 1970; Puhan, 1969; Puhan and Irvine, 1973; Rao and Mathur, 1979a,b; Reps et al., 1970; Sponcet et al., 1985). These substitutes are more proteolytic

than calf rennet and yield bitter soft cheese (Foda et al., 1978; Green, 1977; Lawrence and Creamer, 1969; Lobarzewski and VanHuystee, 1982; Mickelsen and Fish, 1970; Rao and Mathur, 1979a; Sardinas, 1968; Shaker, 1983; Shaker , 1984; Sponcet et al., 1985; Sternberg, 1972; Reps et al., 1981).

Few bacterial enzymes are used for cheese making. Enzymes derived from *Bacillus* species can be used for Cheddar cheese manufacture (Rao and Mathur, 1979a; Sardinas, 1968). Increased proteolysis of milk proteins, faster acid production, higher fat and protein losses, bitterness and lower cheese yields result when *Bacillus* enzymes are used to make cheese (Law, 1983; Rao and Mathur, 1979a). Cheese produced with most *Bacillus* enzymes are hard and acid (Sardinas, 1968) although one enzyme of *Bacillus* origin, obtained from *Bacillus polymyxa* , is less proteolytic than calf rennet and is used in the manufacture of Cheddar and other cheeses (Poznanski et al., 1974).

Of rennet substitutes, those from fungal sources, obtained from *Mucor* and *Endothia* species, are acceptable replacements for calf rennet in cheese manufacture (Nelson, 1969; Itoh and Thomasow, 1971; Reps et al.,1978). Sternberg (1971) reported that cheese made from *Mucor miehei* enzyme is indistinguishable from that made from calf rennet. Gel filtration patterns of crude *M. miehei* rennet contain several protein peaks (Edelsten and Jensen, 1973; Shaker and Brown, 1985a; Shehata et al., 1978) with different optimum temperatures for milk clotting activity (Edelsten and Jensen, 1973; Sheheta et al., 1978). Shovers et al. (1972) developed an electrophoretic procedure to visualize the milk clotting fractions of milk clotting preparations. They showed a milk clotting fraction common to both *M. meihei* and crystalline calf rennin. Of the *Mucor* proteases, *Mucor pusillus* protease is more rennin-like in proteolytic activity (Larson and Whitaker, 1970; Law, 1983; Sternberg, 1971; Vanderpoorten and Weckx, 1972; Yu et al., 1969). *Endothia parasitica* protease is another enzyme of fungal origin used in cheese manufacturing (Hagemeyer et al., 1969; Itoh and Thomasow, 1971; Reps et al., 1978). It is commonly used in the manufacture of emmental and gruyere cheese (Law, 1983).

Milk curds formed by *Mucor*, bacterial and plant proteases take longer to firm than those formed by calf rennet, increasing the clot to cut time (Kowalchyk and Olson, 1979; Poznanski et al., 1974). This is due to their increased proteolytic activity (Kobayashi et al., 1985a; Kobayashi et al., 1985c; Law, 1983; Mickelsen and Fish, 1970; Shaker and Brown, 1985b; Sternberg, 1972) and their wide substrate specificity as compared to calf rennet (Hagemeyer et al, 1969). Protein loss to cheese whey is used as a test for increased proteolysis of milk clotting enzyme preparations (Reps et al., 1981; Shaker and Brown, 1985b). Microbial and plant rennets liberate more non-protein nitrogen (NPN) from caseins than calf rennet does, leading to increased losses of protein when substitutes are used in cheese manufacture (Edelsten and Jensen, 1973; Rao and Mathur, 1979a; Vamos-Vigyazo et al., 1980).

Flavor Defects in Cheese

Proteolysis plays a major role in the ripening of cheese and affects both the body and the flavor of cheese (Visser, 1976). Increased proteolysis can lead to flavor defects in finished cheese during storage (Hubble and Mann, 1984). The defects in cheese made with rennet substitutes are attributed to the increased non-specific proteolytic action of these preparations after the hydrolysis of κ-casein (Green, 1972; Green, 1976; Hubble and Mann, 1984; Martens and Naudts, 1973; Vanderpoorten and Weckx, 1972). The increase in NPN liberation during the action of milk clotting enzymes on caseins is used as a measure of non-specific proteolytic activity (Green, 1977; Vanderpoorten and Weckx, 1972; Visser, 1981). Both α_{s1}- and β-caseins are degraded more in cheeses made with calf rennet substitutes than in cheeses made with calf rennet (Rao and Mathur, 1979a).

Bitter flavor defect in cheese is considered a by-product of β-casein hydrolysis (Kobayashi et al., 1985a,b,c) and most rennet substitutes are more proteolytic against β-casein than calf rennet (Annibaldi et al., 1970; Green, 1977; Itoh and Thomasow, 1971; Kobayashi et al., 1985a; Rao and Mathur, 1979a; Vanderpoorten and Weckx, 1972).

Although chymosin degrades α_{s1}-casein during ripening of cheese, β-casein is untouched (Kobayashi et al., 1985a). Arg_{202}-Val_{209}, a peptide produced from the hydrolysis of β-caseins is 250 times more bitter than caffeine (Kobayashi et al., 1985b).

Microbial enzymes are generally more heat stable than calf rennet (Hubble and Mann, 1984; Law, 1983; Sternberg, 1971). This increased heat stability means that they persist longer in cheese, resulting in prolonged proteolysis and creating flavor defects in cheese during (Hubble and Mann, 1984; Law, 1983; Singh et al., 1967).

Fungal Rennets

Fungal rennets of concern in cheese manufacture are those enzymes produced by *Mucor* and *Endothia* species which have ability to clot milk. The clotting activities of *Mucor* and *Endothia* enzymes follow a mechanism similar to that of calf rennet (Kobayashi et al., 1985c; Sternberg, 1976; Tam and Whitaker, 1973). In the enzymic phase of milk coagulation, the κ-casein fraction of milk is attacked and the carboxyl terminal split off (Payens, 1976; Kobayashi et al., 1985c). This action yields a soluble macropeptide and an insoluble para-κ–casein, destabilizing the casein micelle (Kobayashi et al., 1985c; Tam and Whitaker, 1973). Presence of calcium ions causes the destabilized micelles to aggregate, leading to coagulation (Kobayashi et al., 1985c; McMahon and Brown, 1982; McMahon and Brown, 1985; McMahon et al. 1984a,c).

Calf rennet is the enzyme of choice for cheese manufacture (Shaker and Brown, 1985a,b; Green, 1977). It has been used so long for cheese making that it is accepted as the standard for judging rennet substitutes (Sternberg, 1972). Because of their excessive proteolytic activity, fungal proteases break down milk proteins more extensively than calf rennet does (Law, 1983; Vanderpoorten and Weckx, 1972). This results in reduced cheese yield and flavor defects (Law, 1983; Martens and Naudts, 1973; Mickelsen and Fish, 1970; Sternberg, 1972; Vanderpoorten and Weckx, 1972).

M. miehei, and *M. pusillus* proteases and chymosin, show some similarities in their amino acids composition (Sternberg, 1971; Sternberg, 1976). Arginine, tyrosine and histidine residues are present in the same amounts in chymosin and in the *Mucor* enzymes. The number of residues of phenylalanine, threonine and lysine are the same in hydrolyzed *M. miehei* protease and chymosin (Sternberg, 1971). Sternberg (1976) showed that *M. miehei*, *M. pusillus* and *E. parasitica* proteases as well as chymosin preferentially split bonds with aromatic and hydrophobic amino acid residues. Shovers et al. (1972) showed milk clotting fractions from both *M. miehei.* and crystalline chymosin to migrate the same distance on polyacrylamide gel during electrophoresis. These enzymes are all classified as acid proteases based on their pH optima for proteolytic activity on bovine serum albumin (Sternberg, 1971).

Properties of *Mucor miehei* Protease

M. miehei protease liberates more NPN from caseins than *M. pusillus* protease (Vanderpoorten and Weckx, 1972). It is stable to standard pasteurization temperatures and times (Sponcet et al., 1985). Prins and Nielson (1970) reported an optimum temperature of 60-65°C for its coagulation of milk while Reps and coworkers (1978) reported optimum milk clotting temperatures between 58-62°C compared to between 42-44°C for calf rennet. Temperatures exceeding 70°C cause inactivation of *M. miehei* protease milk clotting ability (Reps et al., 1978).

Reduction of milk pH from 6.7-6.3 reduces flocculation time by about 80-90% when the enzyme is used to coagulate milk (Reps et al., 1978). Amino acid profiles of the macropeptide liberated from the hydrolysis of κ-casein by chymosin and *M. miehei* enzyme are similar (Sternberg, 1972). Sternberg (1972) determined from peptides soluble in 12% TCA, obtained from hydrolysis of κ-casein, that fourteen of the eighteen amino acid residues checked occurred in equal concentrations. Both enzymes split κ-casein at the same points (Sternberg, 1972).

Based on gel filtration, *M. miehei* enzyme molecular weight is 34,000-39,000. It has about 255 amino acid residues (Sternberg, 1972). The pH optimums for *M. miehei* protease hydrolysis of casein are 5.0 (Reps et al., 1978) and 5.5-7.5 (Sternberg 1971). The enzyme is classified as an acid aspartyl proteinase (like chymosin), and the optimum pH for its proteolytic action on bovine serum albumin is pH 4.6 (Law, 1983; Ottesen and Rickert, 1970; Sternberg, 1971). *M. miehei* enzyme has one or two carboxylic groups present at its active site (Sternberg, 1972).

Reaction with 2,4-Dinitro-1-fluorobenzene blocks alanine and glycine amino acid residues at the N-terminal portion of *M. miehei* enzyme and reduces its enzymatic activity by about 60% (Sternberg, 1972). Sternberg (1972) and Law (1983) reported that *M. miehei* enzyme shows great specificity for hydrophobic chains and splits all bonds having aromatic amino acid at the carboxyl terminal. *M. miehei* enzyme hydrolysis of oxidized B chain of insulin shows similar specificity as chymosin. Oxidized B chain of insulin is hydrolyzed at seven points, and many of these points correspond to those obtained by hydrolysis with chymosin (Sternberg, 1972).

With reference to the ability to hydrolyze peptide bonds, *M. miehei* enzyme hydrolyzes the same bonds as chymosin except for one bond, Tyr_{26}-Thr_{27} (Sternberg, 1976). *M. miehei* hydrolyzes this bond while chymosin does not (Sternberg, 1976). The requirement for calcium ions for *M. miehei* enzyme coagulation of milk is similar to that of chymosin (Law, 1983).

Properties of *Mucor pusillus* Protease

M. pusillus enzyme liberates less NPN from α- and β-casein than *M. miehei* enzyme (Vanderpoorten and Weckx, 1972) but more than chymosin (Itoh and Thomasow, 1971). Its proteolytic activity resembles the proteolytic activity of calf rennet (Richardson et al., 1967; Yu et al., 1969). It is classified as an acid aspartyl protease (Law, 1983) with an optimum proteolytic pH of 4.5 for κ-casein, 3.5 for casein (Itoh and Thomasow, 1971) and

4.0 for hemoglobin (Shaker and Brown, 1985b). Its molecular weight is 29,000, and it contains 277-281 amino acid residues (Yu et al., 1969). The proteolytic activity to milk clotting activity ratio of the crude form of *M. pusillus* enzyme is 5-6 times higher than that of calf rennet (Morvai-Racz, 1974). Following a few purification steps, the proteolytic activity to clotting activity ratio of the enzyme is reduced to between 1.6-2 times that of calf rennet (Morvai-Racz, 1974).

The specific extinction coefficient of a 1% (w/w) *M. pusillus* enzyme solution at 280 nm is 10 and shows maximum absorbance at 276 nm (Arima et al. 1968). Presence of metallic ions, such as silver, zinc and mercury inhibits the proteolytic activity of this enzyme by about 30% while copper ions activates the proteolytic activity (Arima et al., 1968).

Milk clotting activity calcium ion requirement for *M. pusillus* enzyme is different from that of chymosin (Law, 1983). Higher concentrations of calcium ions are required for *M. pusillus* enzyme to achieve curd firmness similar to that of chymosin (Law, 1983).

Properties of *Endothia parasitica* Protease

E. parasitica protease is stable at pH 4-5 but unstable at pH 7 (Hagemeyer et al., 1969). Between pH 5.1 and 6.5, the milk clotting activity of *E. parasitica* is less sensitive to pH changes than chymosin. Based on hydrolysis of oxidized B chain of insulin and the synthetic substrate α-N-benzyloxycarbonyl-L-glutamyl-L-tyrosine, *E. parasitica* protease shows a broader specificity than chymosin or pepsin (Larson and Whitaker, 1970). The enzyme hydrolyzes acid denatured hemoglobin and casein optimally, at pH of 2.0 and 2.5.

Its isoelectric point is pH 5.5 and the molecular weight is between 34,000 and 39,000 (Sardinas, 1968). Hagemeyer et al. (1969) reported a molecular weight of 37,500 based on gel filtration on Sephadex G-100 and an isoelectric point of pH 4.6. Peruffo and coworkers (1984) reported a molecular weight of 37,000 based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Of the enzymes accepted for cheese manufacture, *E. parasitica* liberates more nitrogenous compounds in whey (Reps et al., 1981). It also shows the most extensive proteolysis on casein (Poznanski et al., 1974; Vanderpoorten and Weckx, 1972).

Milk Coagulation by Rennet Substitutes

Enzymically induced milk coagulation occurs in two stages (Garnot and Olson, 1982; McMahon et al, 1984a,b; Dagleish, 1979; Green and Crutchfield 1969; Payens, 1976). The first stage, the proteolysis of κ-casein yielding paracasein and macropeptide, is due to the cleavage of a sensitive bond of κ-casein (Payens, 1976; McMahon et al, 1984a,b). The second stage, is the aggregation of the para-κ-casein of the casein micelles in the presence of calcium chloride (McMahon et al., 1984c).

Microbial milk clotting preparations and calf rennet hydrolyze κ-casein at the same rates for the first minutes of milk coagulation reaction, but microbial milk clotting enzyme preparations more rapidly hydrolyze κ-casein and other caseins with (Edelsten and Jensen, 1973; Vanderpoorten and Weckx, 1972). Chymosin and *M. miehei* enzyme produce one degradation product upon hydrolysis of α_{s1}-casein solution at pH 6.0 while *E. parasitica* enzyme produces several degradation products (Kobayashi et al., 1985a).

MODIFICATION OF MICROBIAL RENNET PROTEOLYTIC ACTIVITY

Microbial enzymes' milk clotting activity can be made similar to that of calf rennet by different methods of modification (Colein and Delecourt, 1981; Cornelius, 1982; Cornelius et al., 1982; Havera and David, 1987). Several methods that have been used to achieve reduction in proteolytic activities of microbial enzymes include heat treatment, acid treatment (Colein and Delecourt, 1981), oxidation treatment with peroxides or hypochlorates (Cornelius, 1982; Cornelius et al., 1982) and acylation (Hubble and Mann, 1984; Havera and David, 1987; Higashi et al., 1983). These methods have no toxic effects

on humans and are effective in improving the properties of microbial rennets (Hubble and Mann, 1984). Treatment of *M. miehei* rennet with silicates causes reduction in proteolytic activity without reducing clotting ability (Sternberg, 1976).

Heat Treatment

Heat is used to partially inactivate microbial enzymes to minimize their proteolytic activities. It is effective in increasing the milk clotting/proteolytic activity ratio of treated enzymes (Hamdy and Edelsten, 1970).

Acid Treatment

Fungal rennets are more heat resistant than calf rennet and are, therefore, stable to the standard temperatures used for most cheese making. Heat stability of fungal rennets is the major reason why they persist longer in cheese leading to flavor problems in aged cheese.

Treatment with strong acids reduces proteolytic activity of *M. miehei* (Hubble and Mann, 1984). Treatment with high acid concentrations destabilizes fungal enzymes, resulting in decrease in heat stability and reduction in proteolytic activity. Enzymes treated in this manner become more readily heat labile at standard temperatures used in cheese manufacture (Hubble and Mann, 1984).

Oxidizing Treatment

Hydrogen peroxide, calcium peroxide and sodium hyperchlorate (1-5%) have been used to oxidize side chains of *Mucor* enzymes. Bovine liver catalase is used to remove excess hydrogen peroxide left over from such treatments (Cornelius, 1982).

Oxidation causes a reduction in the proteolytic activity of *Mucor* milk clotting enzymes and makes the enzymes more heat labile (Colein and Delecourt, 1981; Cornelius, 1982; Havera and David, 1987; Higashi et al., 1983). Methionine residues of the treated enzymes are oxidized to methionine sulfoxide. Treated enzymes retain 100% clotting activity, but

proteolytic activity is reduced by 80-90% when hydrogen peroxide is used as the oxidizing agent (Cornelius, 1982).

Acylation

Of the methods used for reduction of *Mucor* enzymes' proteolytic activity, acylation is most controllable (Havera and David, 1987). The milk clotting activity to proteolytic activity ratio of *M. pusillus* is increased after acylation with succinic anhydride (Higashi et al.1983). Succinylation increases the number of negative charges on fungal rennets by decreasing the number of amine groups (Higashi et al.1983). Oxidation with hydrogen peroxide followed by acylation with succinic anhydride is more effective in reducing proteolytic activity and increasing clotting activity than treatment with hydrogen peroxide or succinic anhydride alone (Azuma et al., 1986; Havera and David, 1987).

Although succinic anhydride is commonly used for acylation, increased milk clotting activity is achieved with maleic anhydride (Havera and David,1987). The effects of oxidation and acylation are additive (Azuma et al., 1986; Havera and David, 1987). Treated enzymes have higher milk clotting activity/proteolytic activity ratio and heat sensitivity similar to calf rennet (Azuma et al., 1986; Colein and Delecourt, 1981).

PURIFICATION TECHNIQUES

Affinity Chromatography

Affinity chromatography is a protein purification technique, relying on interactions between a protein component and a substance for which the protein exhibits bioselective affinity (Anon, 1986; Anon, 1979; Boyer, 1986; Sada et al., 1986; Scouten, 1981). Enzymes, substrates, inhibitors, analogs and cofactors have been employed in affinity chromatography (Sada et al., 1986; Scouten, 1981; Wankat, 1974; Woodward et al., 1986).

Protein purification by affinity chromatography yields high purity protein in one step (Aerts et al., 1986; Anon, 1986; Chase, 1984; Sada et al., 1986; Scouten, 1981). It is

employed in instances where great difficulty and much time are encountered if conventional procedures were employed in isolating the protein of interest (Chenais et al., 1977).

In affinity chromatography, the sample to be purified is run through a column to which a specific ligand (the adsorbent) for the protein of interest (the adsorbate) has been covalently immobilized (Scouten, 1981). The immobilized specific ligand exhibits affinity and biospecificity for the adsorbate. The ligand selectively binds the protein of interest while proteins with no affinity pass through the column (Cautrecasas et al., 1968; Robinson et al., 1972; Chase, 1984). The biospecifically bound protein is then eluted with an acid or a chaotropic agent.

Affinity chromatography has permitted the isolation and characterization of proteins from a wide variety of sources (Chenais et al., 1977; Scouten, 1981; Chase, 1984).

Immunoaffinity Chromatography

Immunoaffinity chromatography differs from affinity chromatography on one specific point. It employs, as adsorbent, an antibody or an antigen (Anon, 1986). It utilizes the specificity of an immobilized antibody to recognize and bind its antigen (Babashak and Phillips,1988; Phillips et al., 1985). Specific antibody against any antigen can be raised by immunization (Chase, 1984; Sada et al., 1986; Warr, 1982). The use of antibodies in immunoaffinity chromatography, enables antigens present in low amounts to be isolated and purified with good recovery of activity (Anon, 1986; Arai et al., 1986; Babashak and Phillips,1988; Boschetti et al., 1986; Frantz et al., 1988; Nielsen and Wilson, 1987; Sada et al., 1986; Scouten, 1981; Zoller and Matzku, 1976).

Protein purification by immunoaffinity chromatography results in higher fold purity when compared to purification by affinity chromatography (Oida et al., 1984). This is because of specific biological interactions between antibodies and antigens (Livingston, 1974; McConathy et al., 1985; Warr, 1982; Oida et al., 1984). Another possible explanation is that in affinity chromatography, the ligand or adsorbent employed is usually

smaller than the adsorbate. The ligand-adsorbate complex covers much of the reactive surface of the support matrix creating a shield that blocks out other ligand sites, making them unavailable for biospecific adsorption (Wankat, 1974).

Conflicting situations exist in immunoaffinity protein purification. While high affinity is required for antibody to effectively bind antigen (Bartholomew et al., 1982), low affinity is required, during elution, to dissociate the antigen-antibody (Ag-Ab) complex without causing irreversible denaturation of the adsorbed protein (Bartholomew et al., 1982; Chase, 1984; Sada et al., 1988; Vockley and Harris, 1984). Although antibodies exhibit high affinity for their antigens, use of antibodies in immunoaffinity chromatography does not mean that harsh conditions must be used for protein elution (Bartholomew et al., 1982; Bureau and Daussant, 1981; Oida et al., 1984; Sada et al., 1988). Bureau and Daussant (1981) reported a procedure in which water was employed as an effective eluent for barley β-amylase. In their experiment, elution was started with distilled water. The elution step was stopped for a few hours while the matrix was allowed to stay in contact with the water. After the delay period, elution was continued and the adsorbed proteins were effectively eluted from the column.

Dissociation constants range from 10^{-4} to 10^{-10} for antigen-antibody binding reactions (Anon, 1979; Chase, 1984; Anon, 1988). With such a wide range, conditions can be chosen to select for those antibodies with higher binding constants and lower affinities (Bartholomew et al., 1982; Bureau and Daussant, 1981). Switching of antibodies to lower affinities is reported to be achieved by titration with acids, under fairly mild conditions, resulting in release of bound antigen (Bartholomew et al., 1982; Sada et al., 1988). This might be why elution with acids is the most commonly used technique for release of bound antigens in immunoaffinity chromatography.

The ability to use high affinity antibodies permits efficient binding of low concentrations of antigens (Bartholomew et al., 1982; Sada et al., 1988) while the capacity to switch the antibodies to lower affinities permits release of biospecifically adsorbed

proteins (Sada et al., 1988). Antibodies retain their antigens in the form of antibody-antigen (Ab-Ag) complexes while materials not recognized by the antibodies are washed off the column with buffer.

Binding of an antibody to its antigen is brought about by formation of non-covalent bonds between antigen and antibody. Antigenic determinants on the surfaces of antigens allow antibodies to recognize antigens. Antigenic determinants and antigen binding sites on the antibody have complementary sequences which help bring the antigen and antibody close enough together for these bondings to take place (Warr, 1982). Other factors contributing to antigen recognition are presence of steric factors and the antigenic three-dimensional conformation (Warr, 1982). Non-covalent chemical bonds, such as hydrogen, electrostatic, hydrophobic and Van der Waals, hold the antibody-antigen complex together after biological recognition takes place (Anon, 1979; Chase, 1984; Warr, 1982).

The antibody-antigen complex can only be dissociated by breaking these bonds with the use of dissociation agents. Acids and chaotropic solutions are the most commonly employed dissociation agents (Anon, 1986; Bureau and Daussant, 1981). Introduction of acids or dissociating agents changes ionic parameters of the complex causing the bonds, by which immobilized antibodies hold their antigens, to break, with subsequent release of the bound antigens (Phillips et al., 1985). The released or eluted antigens are then carried down the column, resulting in purification of the antigens.

Many investigators have exploited the specificity of antibodies in immunoaffinity chromatography. Antibodies have been utilized in immunoaffinity chromatography for purification of proteins with similar physical characteristics (Aerts et al., 1986; Chase, 1984; Jenny et al., 1986; McConathy et al., 1985). Proteins found in very low concentrations in nature have received special attention. Immunoaffinity columns can be reused several times without regeneration (Aerts et al., 1986).

Immunoaffinity chromatography protein purification can be divided into five major steps:

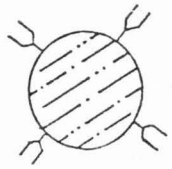
1. Derivatization of the support material.
2. Coupling of specific adsorbent to the support material.
3. Packing the column.
4. Contacting sample to the column to adsorb specific proteins.
5. Washing the column.
6. Dissociation or elution of adsorbed component with buffer.

Column matrix material can be purchased already derivatized and coupled to specific adsorbents. Therefore, steps one and two above can be by-passed. Proteins are biospecifically adsorbed to immunoaffinity column matrix materials under conditions of neutral pH and low ionic strength to retain reactivity (Comoglia et al., 1976). Protein elution is achieved by changing the pH, increasing the ionic strength or changing the polarity (Anon, 1986; Anon, 1979; Cautrecasas et al., 1968; Mauro et al., 1988).

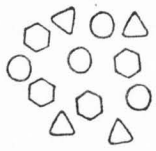
A major problem in both affinity and immunoaffinity chromatography is non-biospecific adsorption (Zoller and Matzku, 1976). The washing step is crucial because it eliminates non-biospecifically adsorbed materials (Anon, 1979; Anon, 1986). A schematic representation of the immunoaffinity process is shown in Figure 1. The same techniques involved in affinity chromatography also apply to immunoaffinity chromatography. The only difference is that in immunoaffinity chromatography, antibodies raised against specific antigens are used as ligands (Anon, 1979). The technique involves the covalent immobilization of antibodies to support matrix to create a biospecific matrix for protein purification (Anon, 1986; Chase, 1984). Because an antibody exhibits high affinity and specificity for a single compound, its antigen, antibodies are considered ideal biospecific adsorbents. They specifically bind their antigens and exhibit very little or no affinity for other compounds.Immunoaffinity chromatography as a technique for protein purification came into existence with the knowledge of effective coupling of antibodies to support

Figure 1. Stages of immunoaffinity chromatography protein purification (adapted from Chase, 1984)

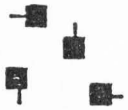
Key:



Matrix with covalently bound ligand

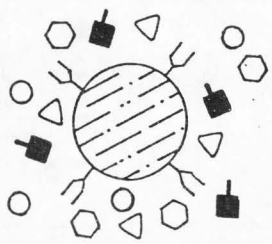


Contaminating proteins

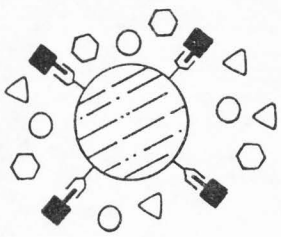


Protein of interest

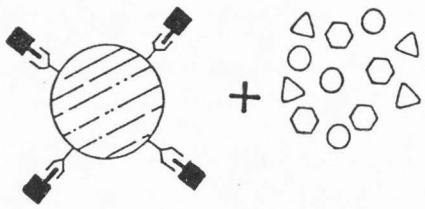
Contacting



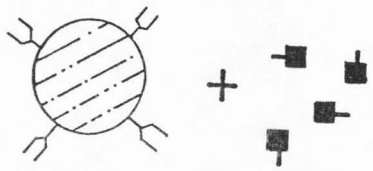
Biospecific adsorption



Washing



Elution



matrices and the demand for high purity proteins (Boyer, 1986). The motivation to use immunoaffinity chromatography for protein purification is due to the many draw backs of conventional methods. Conventional purification methods are time-consuming and give low yields (Anon, 1979; Boyer, 1986; Cautrecasas et al., 1968; Chase, 1984; Fido, 1987; Gershenwald et al., 1985; Lee et al., 1986; Livingston, 1974).

The biological reactivity of a protein is related to its three dimensional conformation, and severe changes in conformation affect reactivity. Proteins can be adversely affected by the chemical and physical changes in their environment leading to denaturation and loss of reactivity (Chase, 1984). Prudent judgement must be used during purification of proteins to insure that irreversible changes do not occur in the protein structure (Jenny et al., 1986), resulting in loss of biological reactivity. Extensive exposure of the protein to extremes of pH, high shearing forces, high temperatures and organic solvents are to be avoided during purification (Anon, 1986; Chase, 1984; McConathy et al., 1985).

Immunoaffinity chromatography is an effective means of protein purification and results in a high degree of purification in one step (Aerts et al., 1986; Anon, 1986; Arai et al., 1986; Gershenwald et al, 1985; Jenny et al., 1986; Pfeiffer et al., 1987). The technique eliminates the need for multiple separation steps employed in conventional protein purification. Proteins purified by one step in immunoaffinity chromatography have comparable purity to those purified by multiple step conventional techniques (Linhardt et al.,1985).

The amount of protein that can be purified by immunoaffinity chromatography depends on the concentration and purity of antibodies coupled to the support matrix, and on the concentration of antigen to be adsorbed to the column (Eveleigh and Levy, 1977; Lobarzewski and VanHuystee, 1982). Biological specificity is exploited in immunoaffinity chromatography (Gershenwald et al, 1985). A good example of this is the recognition of a specific antigen by a specific antibody.

Utilization of monoclonal antibodies for monospecific protein purifications has broadened the applications of immunoaffinity chromatography (Bazin and Malache, 1986; Boschetti et al., 1986; Dorer et al., 1984; Miyazaki et al., 1988). Bazin and Malache (1986) reported an automated technique for immunoaffinity chromatography using monoclonal antibodies. Monoclonal antibodies are essential in the purification of enzymes from a multigene family where similarities exist at the molecular level (Vockley et al., 1984). Monoclonal antibodies facilitate purification of proteins to well defined specificities (Dorer et al., 1984; Miller et al., 1987).

Immobilization of proteins to support matrix

Enzymes have been immobilized and used in the food industry to facilitate the study and recovery of the enzymes during and after processing (Olson and Richardson, 1974; Green and Crutchfield, 1969). Covalent immobilization of antibodies to a column matrix and their utilization in the purification of proteins is achieved due to weak binding and high specificity of interaction that occurs between antibodies and antigens (Anon, 1986; Babashak and Phillips, 1988). Antibodies have reactive amino and carboxyl groups suitable for covalent immobilization to an activated matrix. This makes the use of antibodies very desirable in purifying their corresponding antigens in immunoaffinity chromatography (Bartholomew et al., 1982; Chase, 1984). Both polyclonal and monoclonal antibodies have been employed (Sada et al,. 1986,1988).

The most commonly used method of immobilization utilizes cyanogen bromide activation (Fido, 1987; Jenny et al., 1986; Pfeiffer et al., 1987). Although this method has been used with great success in affinity chromatography, it decreases the antibody antigen binding capability when employed in immunoaffinity chromatography (Kennedy and Barnes, 1981; Pfeiffer et al., 1987).

Brown et al. (1979) reported a procedure which permits immobilization of enzymes that does not rely on the use of cyanogen bromide activation. In the procedure, porous glass

beads were silanized to activate the glass surface, and spacer arms interacted with the silanized beads. Enzyme was then connected to the spacer arms. The procedure, unlike cyanogen bromide activation, is nontoxic. Immobilized proteins retain their reactivity (Brown et al., 1979; Chase, 1984; Cheryan et al., 1975; Schell et al., 1979; Taylor et al., 1977; Yun et al., 1981) and optimum pH (Brown et al., 1979; Schell et al., 1979).

Adsorption

The adsorption stage in immunoaffinity chromatography is a crucial stage in the process (Chase, 1984). During adsorption, the sample to be purified is contacted with the adsorbent to biospecifically bind the protein of interest. Complications can occur due to non-specific adsorption by contaminating materials in the sample to be purified (Babashak and Phillips, 1988). Following adsorption, the column is washed to ensure that all contaminating materials are removed.

Non-specific adsorption is reduced or eliminated by washing with different ionic strength buffers, changing column pH, adding nonionic detergents or running organic solvents through the column after biospecific adsorption (Anon, 1986; Livingston, 1974; Zoller and Matzku, 1976). Washing following adsorption creates problems because some of the bonds responsible for non-specific adsorption are also involved in biospecific adsorption. Some loss of the protein of interest occurs during the washing procedure; so the objective is to remove as much contaminating material as possible without sacrificing too much of the protein of interest.

At saturation of adsorbent with adsorbate, only .1% of the adsorbent is involved in protein binding (Mauro et al., 1988). This is attributed to steric hindrance since the binding of protein by one ligand molecule may prevent the neighboring binding sites from participating in the binding reaction (Chase, 1984). The binding reaction rate between adsorbent and adsorbate, like any chemical reaction, depends on the chemical binding

constants and diffusion rate of adsorbate through the column matrix to active binding sites (Boschetti et al., 1986). Efficiency of adsorption is defined as (Chase, 1984):

$$\text{efficiency} = \frac{\text{amount of adsorbate retained by column} \times 100}{\text{amount of adsorbate applied to the column}}$$

Flow rate, concentration of adsorbate and column size affect the efficiency of adsorption.

Elution

Elution or desorption is the dissociation of the adsorbent/adsorbate complex to accomplish purification of the bound protein. Dissociation of antigen-antibody complexes requires treatments that cause changes in the charge density and conformation of the antibody-antigen complex (Chase, 1984). This can result in irreversible damage to the eluted protein. Eluted proteins are neutralized as soon as possible to minimize changes that can be caused by dissociating agents. Under ideal conditions, the adsorbate is eluted in small volume and high concentration. Eluted protein yield is increased if the loading of the immunoaffinity column is less than the maximum capacity of the column (Chase, 1984).

Several methods of elution have been discussed in the literature (Anon, 1986; Anon, 1979; Boyer, 1986; Chase, 1984; Gershenwald et al, 1985; Oida et al., 1984; Robinson et al., 1972). Because of the strong binding between antibody and antigen, a common factor to most elution steps is harsh condition (Kennedy and Barnes, 1981; McConathy et al., 1985; Oida et al., 1984; Sada et al., 1988; Wankat, 1974). Optimally, eluting agents are selected so that they are strong yet gentle enough not to cause irreversible damage to the three-dimensional structure of the proteins being released (Chase, 1984). The elution step, is carried out as quickly as possible with immediate neutralization following elution (Anon, 1986; Livingston, 1974). Trial and error is used to establish parameters for successful chromatographic elutions of unknown proteins in biologically active forms (Chase, 1984).

The most commonly used method for elution in immunoaffinity chromatography is nonspecific acid desorption (Anon, 1979; Anon, 1986; Boyer, 1986). The procedure involves alteration of both the physical and chemical properties of the binding between adsorbent and adsorbate. The conformation of the adsorbent and the adsorbate are temporarily altered during elution, changing the binding avidity between adsorbent and adsorbate and causing the adsorbate to be released (Boyer, 1986). The chemical nature of this change must be reversible to retain biological activity of the eluted protein (Chase, 1984). The best elution condition is selected, based on knowledge of the binding strength between adsorbent and adsorbate and the stability of the adsorbate against irreversible denaturation (Anon, 1986; Bartholomew et al., 1982; Kennedy and Barnes, 1981; Wankat, 1974; Chase, 1984).

SUPPORT MATERIALS AND COUPLING METHODS

Support Materials

Several techniques have been developed for attachment of antibodies to support matrices. The matrix comprises the bulk of the chromatographic medium. The type of matrix is a decisive factor in chromatography (Kennedy and Barnes, 1981). Some properties of an ideal support matrix (Anon, 1986; Chase, 1984; Scouten, 1981) are:

1. The support should be rigid enough to withstand hydrodynamic pressures.
2. It should be resistant to acidic and alkaline solutions.
3. It should have a porous structure to permit high flow rate.
4. The pores should be large enough to permit diffusion of large protein molecules to their binding sites within the pores.
5. Bonds formed between the matrix and proteins should be strong enough to withstand washing and elution.
6. It should exhibit no or low nonspecific adsorption of crude material.
7. It should be relatively easy to derivatize.

Support materials are usually porous spherical particles. Porosity increases available surface area, promotes rapid reaction, permits high flow rate and high rate of coupling per unit volume of matrix (Anon, 1986). Use of small spherical particles also decreases settling time. Chemical groups on an ideal matrix are easily derivatized so that proteins can be attached by stable covalent bonds that are strong enough to withstand washing, and elution of biospecifically bound proteins (Anon, 1986; Chase, 1984). Covalent attachment of an adsorbent to a matrix minimizes leakage and contamination of eluted products.

Properties of some support materials used in immunoaffinity chromatography are discussed below.

Agarose

The most popular and most commonly used support materials for chromatography are derivatives of agarose (Scouten, 1981) or Sepharose, a more highly purified agarose (Eveleigh and Levy, 1977). Agarose derivatives are made commercially by CNBr activation. CNBr activation changes the hydroxyl groups of agarose to form reactive esters (Cautrecasas, 1970). These derivatives are readily available and are the most utilized forms of agarose in both immunoaffinity and affinity chromatography (Scouten, 1981; Eveleigh and Levy, 1977; Chase, 1984).

Agarose has most of the desirable qualities required of an ideal support matrix. It is highly porous and hydrophilic (Anon, 1979; Anon, 1986; Scouten, 1981). Agarose beads are also commercially available in activated forms which can be directly coupled to enzymes, proteins, or other ligands (Scouten, 1981). Agarose beads have some limitations, however. They compact under mechanical pressure, are susceptible to shrinking and swelling with ionic strength changes, are soluble in denaturing and chaotropic solutions, and are adversely and irreversibly changed in organic solvents (Scouten, 1981).

Structural deformation of agarose beads occurs in the presence of urea, thiocyanate salts, and extreme pH (Chase, 1984; Kennedy and Barnes, 1981; Eveleigh and Levy,

1977). The tendency of agarose beads to compact under pressure with extended usage makes them unsuitable for use as matrix under conditions where high pressures are required (Scouten, 1981).

Porous glass beads

Controlled pore porous glass is the most widely used inorganic matrix in chromatography (Scouten, 1981). In immunoaffinity chromatography, harsh denaturing and chaotropic agents are generally employed in elution of biospecifically adsorbed proteins (Babashak and Phillips, 1988). Porous glass beads, because they are more compatible with these agents, are an alternative to agarose beads which cannot withstand harsh conditions (Phillips et al., 1985). Controlled pore glass beads offer the advantage of higher flow rates and larger surface areas (Eveleigh and Levy, 1977). These factors make porous glass beads more suitable than agarose beads for use in immunoaffinity chromatography and in high performance immunoaffinity chromatography where high pressures are required.

Porous glass matrix has been used mostly for the immobilization of enzymes (Line et al., 1971; Scouten, 1981; Weetall, 1969ab; Weetall and Hersh, 1969; Weetall, 1970; Weetall, 1974) for subsequent use as biocatalysts. Antibodies show a higher efficiency on porous glass than they do on Sepharose (Eveleigh and Levy, 1977).

Glass surface consists mostly of silanol (SiOH) groups which can cause non-specific ionic adsorption of proteins when underivatized glass is used in affinity chromatography (Chase, 1984; Scouten, 1981). Derivatization with organic chemicals to covalently block the SiOH groups eliminates ionic adsorption of proteins to derivatized glass. Silanization is the most commonly used method for the activation of glass. The chemical of choice is γ-aminopropyltriethoxysilane. It reacts covalently with the SiOH groups of glass and yields stable coupling arms (Brown et al., 1979; Chase, 1984). The activation and addition of coupling arms through the use of other techniques and chemicals are also reported in the literature (Scouten, 1981).

Nonporous glass beads

In the early stages of affinity chromatography, before the development of porous glass beads, nonporous glass beads were commonly used (Pfeiffer et al., 1987). Low flow rates and decreased surface area made it necessary to use large volumes of glass beads (Eveleigh and Levy, 1977; Kennedy and Barnes, 1981). The use of non-porous glass reduces the effects of diffusion within the pores of porous glass beads (Eveleigh and Levy, 1977). The rate of adsorption/desorption reactions is therefore dependent only on the mass transfer rate to the surfaces of non-porous beads and the chemical rate constant of the reaction (Eveleigh and Levy, 1977). The elimination of slow diffusion associated with pores allows for more rapid adsorption and desorption (Eveleigh and Levy, 1977).

The disadvantage of low capacity, which necessitates use of large volumes of matrix when non-porous glass beads are used in affinity chromatography, outweighs any of its advantages (Kennedy and Barnes, 1981; Eveleigh and Levy, 1977).

Other support materials

This group includes polyacrylamide and cellulose. Polyacrylamide and cellulose have been derivatized and used in affinity chromatography (Cautrecasas, 1970; Eveleigh and Levy, 1977). They are commercially available and exhibit good chemical stability (Scouten, 1981). However, they lack mechanical stability, have decreased porosity and penetration by large proteins is low (Scouten, 1981; Chase, 1984). The major disadvantage of polyacrylamide is that it adheres to glassware, making it difficult to use (Scouten, 1981).

Cellulose is a non-uniform support matrix. The main advantages of cellulose are, low cost, ease of derivatization, thermal and mechanical stability and compatibility with organic solvents (Chase, 1984; Scouten, 1981). Its disadvantages include, leakage of protein complexes and presence of small irregular pores (Scouten, 1981).

Coupling Methods

The procedure most often used and considered best for agarose activation is cyanogen bromide activation (Scouten, 1981; Pfeiffer et al., 1987). Although other coupling methods, such as epoxy-activation, silanization and succinylation have been used (Anon, 1986; Anon, 1979; Brown et al., 1979; Gershenwald et al., 1985; Jenny et al., 1986; Lee et al., 1986; Linhardt et al., 1985; Vretblad, 1976), CNBr activation remains the most popular method.

Coupling of proteins to an activated support matrix, involves the reaction of amino groups and other reactive side groups of the protein with the activated groups on the surfaces of the matrix (Anon,1979; Anon, 1986; Scouten, 1981; Chase, 1984). Proteins can be covalently attached to a matrix through multiple linkages because they contain several reactive groups. Although multiple linkage helps minimize leakage since an attached protein can only be detached if all its linkages are broken, it has been criticized in immunoaffinity chromatography (Cautrecasas 1970).

Cautrecasas (1970) attributed decreased interaction between immobilized antibody with antigen to be due to multiple linkage. He suggested antibody coupling be accomplished by the least possible number of linkages to a matrix. Multiple linkages severely distort antibody conformation and increase the chances of antigen binding site involvement in linkage formation (Cautrecasas 1970). Conflicting evidence that multiple binding of a protein to a column matrix helps increase stability can also be found in the literature (Chase, 1984; Gershenwald et al., 1985). Although stability of immobilized antibodies could be increased by multiple linkages, reactivity may be reduced (Chase, 1984).

Immobilization of antibodies or enzymes by covalent coupling to a solid matrix is reported to decrease immunoreactivity of antibodies or activity of enzymes (Comoglia et al., 1976; Olson and Richardson, 1974; Pfeiffer et al., 1987). This phenomenon is attributed to adverse effects of the coupling conditions employed during immobilization (Comoglia et al.,

1976; Olson and Richardson, 1974). The conditions for coupling, failure to use coupling arms, and the mass ratio of protein and matrix all affect reactivity of coupled proteins (Comoglia et al., 1976). Therefore, no one factor can be assigned the cause for loss of reactivity.

CNBr activation

Cyanogen bromide activation is the most commonly and widely used method of activation in affinity and immunoaffinity chromatography (Kennedy and Barnes, 1981; Scouten, 1981; Pfeiffer et al., 1987). CNBr activation is simple, and various cyanogen bromide activated derivatives are commercially available (Anon, 1979; Anon, 1986; Scouten, 1981). Coupling yields of about 90% are reported with cyanogen bromide activation (Comoglia et al., 1976; Lobarzewski and VanHuystee, 1982).

Coupling of proteins to a matrix by CNBr activation is achieved under high pH conditions (Anon, 1979). Hydrolysis of agarose active esters is increased at high pH reducing the amount of available active esters for coupling (Chase, 1984). High alkalinity of the CNBr coupling method affects immunoreactivity of antibodies (Comoglia et al., 1976). Comoglia and coworkers (1976) compared the effects of coupling at pH 6.4 and at pH 8.6. They reported significantly higher immunoreactivity retention at pH 6.4 than at pH 8.6. Their binding study showed a six-fold increase in concentration of residual binding sites at pH 6.4 as compared to that at pH 8.6 using the same matrix.

Cyanogen bromide reacts with hydroxyl groups of a support matrix to produce different reactive groups. Amino groups of proteins then react with the activated support resulting in covalent immobilization through isourea linkages (Anon, 1986; Eveleigh and Levy, 1977; Chase, 1984). Isourea linkage is criticized as a method for coupling. It introduces positively charged groups on the matrix, resulting in undesirable ionic properties (Eveleigh and Levy, 1977; Gershenwald et al., 1985). Proteins exhibit polyionic

characteristics because of ionizable side chains, so positive charges contributed by isourea linkages may be negligible (Chase, 1984).

Coupling active esters of CNBr activation are 85% cyanates and 15% imidocarbonates (Phillips et al.,1985). Cyanate esters are stable at low pH but are hydrolyzed at high pH while imidocarbonates are stable at high pH but are hydrolyzed at low pH (Phillips et al.,1985). A trade-off exists between the coupling esters and the coupling conditions employed. Coupling conditions are chosen so as to optimize stability of the protein of interest while minimizing loss of reactive coupling esters of the matrix. After covalent coupling of proteins, the remaining reactive esters are blocked or neutralized. This is accomplished, by reacting the matrix-protein complex with high concentrations of an amino molecule. Ethanolamine or glycine is frequently used for this purpose (Anon, 1979).

Silanization

Coupling of proteins to glass beads involves activation of the glass surface to introduce chemical groups which can then react with a protein molecule. Silanization is the method of choice for activating porous glass beads to which proteins can be coupled with little or no further modifications (Weetall, 1969a,b; Weetall and Hersh, 1969; Weetall, 1970; Weetall, 1974). Silanization activation offers some advantages over CNBr activation. It is non-toxic and coupling can be achieved under lower pH conditions (Brown et al., 1979; Weetall, 1970; Weetall, 1974). Up to 95% reduction in immunoreactivity is reported when antibodies are coupled by CNBr activation as compared to 32% for coupling through N-hydroxysuccinimide ester, after silanization of glass (Comoglia et al., 1976). Loss of immunoreactivity is attributed to shell formation during CNBr activation (Chase, 1984). Other procedures can be used in conjunction with silanization to increase the reactivity of an activated column matrix. Methods such as succinylation, acylation and thionylation have been used (Brown et al., 1979; Weetall, 1969ab; Weetall and Hersh, 1969; Weetall, 1970; Weetall, 1974). Following succinylation of silanized glass, carbodiimides are employed as

catalysts to achieve covalent coupling of proteins to glass (Brown et al., 1979; Weetall, 1970; Weetall, 1974). Carbodiimides react with carboxyl groups to form intermediates, which then react with amino groups to form stable covalent bonds between protein and matrix (Anon, 1979; Brown et al., 1979; Warr, 1982).

Spacer arms

Spacer arms consist of hydrocarbon chains (12 carbons or less) which are attached between the surfaces of a column matrix and a ligand (Aerts et al., 1986; Anon, 1986; Scouten, 1981) before protein adsorption. They are effective in optimizing the reactivity of adsorbed proteins by extending their reactive sites away from the matrix surfaces (Gershenwald et al., 1985). Spacer arms are commonly used in affinity chromatography but are not essential in immunoaffinity chromatography as antibodies are much larger than ligands used in affinity chromatography (Eveleigh and Levy, 1977; Chase, 1984). They help reduce steric hindrance caused by proximity of immobilized protein to the matrix. Incorporation of spacer arms helps to minimize loss of reactivity (Comoglia et al., 1976).

Spacer arms can be attached to ligands before coupling to a matrix or the matrix can be derivatized to incorporate spacer arms before coupling of the ligand. Succinyl groups enhance selective coupling of protein to the reactive sites on derivatized agarose by minimizing contributions of the matrix microenvironment (Linhardt et al., 1985; Comoglia et al., 1976).

Succinylation is an effective method of introducing a coupling arm (Comoglia et al., 1976; Linhardt et al., 1985), and coupling through a succinyl spacer arm is rapid under mild pH conditions (Linhardt et al., 1985). Silanization followed by succinylation has been used in porous glass derivatization for protein coupling (Brown et al., 1979; Weetall, 1974). It is possible that succinyl groups act in the same manner on activated glass as they do on agarose beads.

ORIENTATION AND REACTIVITY OF ANTIBODY

Orientation

Depending on their size, antibodies can be evenly coupled on a support matrix as long as the degree of matrix porosity and reactivity of activated coupling groups is optimal (Gershenwald et al., 1985). Antibodies have many groups capable of reacting with an activated support matrix. Most methods used for antibody coupling result in random orientation of the antibodies (Chase, 1984). Depending on the antibody groups involved in covalent coupling to a matrix, antibody antigen binding sites may be disoriented, minimizing or inactivating the ability to bind or interact with antigens (Chase, 1984).

Antibody Reactivity

Excessive immobilization of antibodies to a support matrix reduces interaction between antibody and antigen due to steric hindrance (Eveleigh and Levy, 1977; Chase, 1984). The movement of antigens to antibody binding sites may be restricted due to overlapping of the immobilized antibodies. These factors are possible reasons why the observed capacity of an immunoadsorbent may be less than that calculated based on the amount of antibody coupled to the matrix (Kennedy and Barnes, 1981). To achieve maximum interaction between antibody and antigen, antibodies should be coupled to a matrix at low protein density (Kennedy and Barnes, 1981).

MATERIALS AND METHODS

Microbial milk clotting enzyme preparations (Marzyme and Renzyme) obtained from *M. miehei* and *M. pusillus* were obtained from a commercial source (Marschalls Division of Miles Laboratories). Freund's adjuvants complete and incomplete, controlled pore size porous glass beads and Agarose were purchased from Sigma Chemical Co. (St. Louis, MO). Protein assay kit was purchased from Biorad (Cambridge, MA). Rabbits (New Zealand White) were obtained through the Laboratory Animal Research Center, Utah State

University (Logan, UT). Bactoagar was purchased from Difco Laboratories (Detroit, MI). N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide and 1 cyclohexyl-3 (2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate was purchased from Pierce (Rockford, IL). HPLC grade buffers were purchased from Beckman (Palo Alto, CA). Sephadex G-100, CM Sephadex, columns and fittings were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). All other chemicals were of reagent grade.

Antibody Production

Two Rabbits (New Zealand White) between 2 and 6 mo old were used as experimental animals in the production of antibodies. Each rabbit was assigned to an enzyme preparation. For the initial immunization, at least 150 μg of each enzyme preparation (Anon, 1984) contained in a total volume of 1 ml phosphate buffered saline (PBS), pH 7.0 was used as immunizing antigen. The antigen solution was combined with equal volumes of Freund's complete adjuvant and thoroughly mixed to form an emulsion.

Emulsification was achieved by repeated mixing of the enzyme/adjuvant mixture by passage through a No. 20 gauge needle to form a thick pasty consistency. The emulsion was injected intramuscularly into two sites in each hind area per animal with the use of plastic disposable syringes with a No. 25 gauge needle. The injected volume was limited to .5 ml at each site.

Subsequent immunizations or boosters utilized the same concentrations and volumes of enzymes as used for the initial immunization but were mixed with Freund's incomplete adjuvant. The emulsification and injection procedures followed were the same as for the initial immunization. The first boosters was given 3 wk after the initial immunization, and a second booster 2 wk thereafter. A total of three booster immunizations spread out over a six-week period was performed.

Blood was collected by bleeding from the external marginal vein of the ears into sterile plastic test tubes, 2 wk after the last immunization . Additional blood collections were

carried out every 2 wk until the antibody titer dropped enough to necessitate subsequent booster immunization. Blood collected from the same rabbit was pooled and allowed to clot. The clot was rimmed off the walls of the test tube and allowed to shrink. Antiserum was separated from the blood after clotting and decanted into sterile centrifugation tubes.

The antiserum was centrifuged at 1700 × g for 30 min in a 5°C coldroom to remove additional red blood cells. The antiserum was decanted into clean sterile test tubes and then purified by heating at 56°C for 30 min. It was allowed to cool in a 5°C coldroom and centrifuged at 2500 × g for 30 min to remove nonantibody proteins and fat. This treatment is cited to increase the concentration of antibody present in the antiserum because it denatures complement factors and contaminating proteins (Anon, 1986).

Antibody Assay

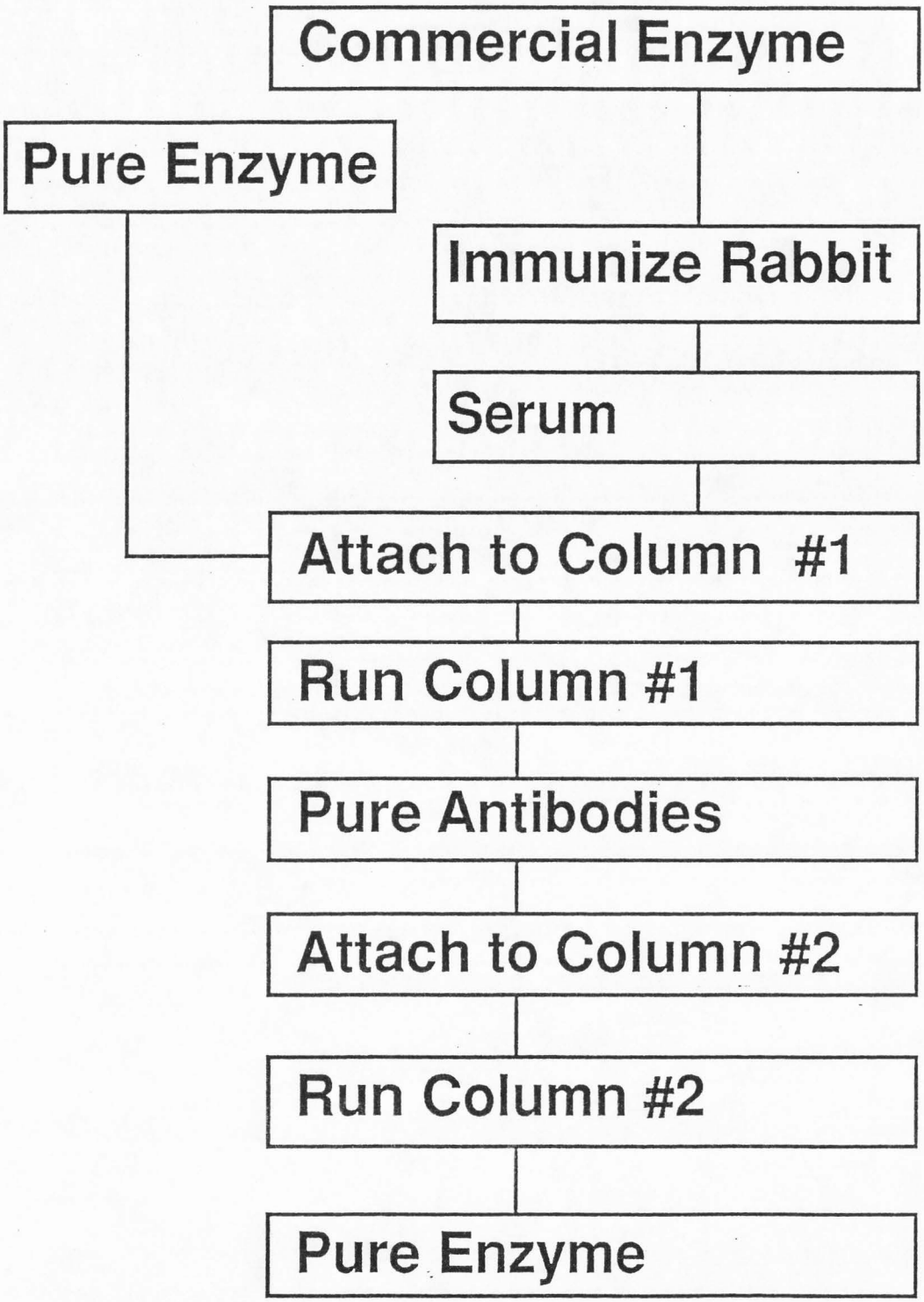
Agarose or Bactoagar (.5-1g) was suspended in 100 ml of PBS and placed in a boiling water bath until the solution became clear. The solution was then boiled for 3 min to melt the agar. The molten agarose or Bactoagar was poured into several plastic petri dishes to a thickness of 2 mm. The poured agarose or Bactoagar was allowed to solidify at room temperature. A central well and 2-6 peripheral wells were punched out of the gels.

Ouchterlony double immunodiffusion technique was used to assay for the presence of antibody in each antiserum (Ouchterlony and Nilsson, 1973; Warr, 1982). Antiserum (20 µl) was applied to the center wells, and 20 µl portions of each enzyme diluted in PBS were applied to the outer wells. The gel was incubated at room temperature for 1 h and then transferred to a 5°C coldroom to develop overnight after which the gel was visually examined for precipitin band formation.

Preliminary Purifications

All purification procedures were performed in a coldroom with the temperature maintained at 5°C. The schematic outline for the purification procedure is shown on Figure 2. Microbial enzyme preparations were first purified by gel filtration. The major

Figure 2. Schematic outline of the steps used for immunoaffinity purification of commercial microbial milk clotting enzyme preparations.


Commercial Enzyme
Pure Enzyme
Immunize Rabbit
Serum
Attach to Column #1
Run Column #1
Pure Antibodies
Attach to Column #2
Run Column #2
Pure Enzyme

milk clotting portion from each enzyme, obtained by gel filtration, was further purified by ion exchange chromatography.

Gel filtration

The gel filtration column matrix was prepared by making a slurry of Sephadex G-100 in deionized distilled water (1 g/125 ml). The slurry was swollen by placing it in a water bath for 2-6 h and allowed to cool at room temperature. The gel was degassed, then transferred to a 5°C coldroom to attain the temperature at which protein separations were performed. The gel was then gravity packed into a (2.5 × 125 cm) glass column connected to a peristaltic pump. The column was equilibrated with 1 m*M* sodium acetate buffer at pH 6.5. Fractionation of the crude enzymes was performed by application of 3-5 ml of each enzyme preparation to the Sephadex G-100 column and chromatographed at a flow rate of .6-1.0 ml/min, using the equilibration buffer as the eluent buffer. A fraction collector set to measure drops was used to collect eluting fractions in 4.0 ml portions. Each fraction was assayed for protein content at 280 nm using a Beckman DU 8B UV-VIS spectrophotometer, then, tested for clotting ability by the Formagraph method (McMahon and Brown, 1982).

Ion exchange chromatography

Further purification of the gel filtered milk clotting protein peak from each enzyme was performed by applying 15 ml from the clotting peak fraction adjusted to pH 3.5 to an ion exchange chromatography column (2.5 × 50 cm), using CM Sephadex as the column matrix.

The matrix was prepared by swelling the CM Sephadex in .15 N NaOH (1 g matrix/15 ml base). The mixture was allowed to stand for 30 min. The supernatant was decanted and the CM Sephadex washed several times with distilled water until the supernatant pH was 8. The CM Sephadex was then stirred into fifteen volumes of .15 N HCl and allowed to stand for 30 min. After 30 min of soaking in HCl, the supernatant was

decanted, and fifteen additional volumes of HCl were added. The mixture was allowed to stand an additional 30 min. The supernatant was again decanted and the matrix washed with distilled water until the pH of the filtrate was neutral.

The matrix was then stirred into .2 *M* sodium acetate buffer at pH 3.5, degassed and gravity packed into the column. The column was equilibrated by running a .05 *M* sodium acetate buffer at pH 3.5 through the column until the conductivity and pH of the effluent from the column were the same as that of the buffer. Sample was applied and allowed to penetrate the column matrix. The column was then washed with the equlibration buffer. Elution was started by running a continuous gradient of .05 *M* sodium acetate buffer at pH 3.5/.5 *M* sodium acetate buffer at pH 5.0 or a gradient of .05 *M* sodium acetate at pH 3.5/.25 *M* sodium acetate at pH 5.0. A fraction collector in the drop mode was used to collect eluting fractions in 4 ml portions. Absorbance at 280 nm was measured with a Beckman DU 8B UV-VIS spectrophotometer to assay protein content and fractions were neutralized and tested for clotting activity.

Immunoaffinity Chromatography

Column matrix construction

The procedure used for porous glass derivatization is as described by Brown et al., (1979). Porous glass beads were mixed with 10 ml 70% HNO_3 for each 1 g of glass and placed in a boiling water bath for 2 h, with three changes of acid. The supernatant was decanted, and the glass was washed extensively with deionized distilled water by sedimentation and decanting until the pH of the wash was 7. The glass was degassed by vacuum and dried to a moist cake under vacuum.

Silanization. The degassed and dried glass was silanized by mixing with a 10% solution (v/v) of γ-aminopropyltriethoxysilane in toluene or water (5 ml/g). The pH was adjusted to 4.0 with HCl. The reaction was monitored for 1 h at room temperature with continuous adjustment of the pH, then transferred to a 70°C water bath and incubated for

3 h in a rotary shaker. After incubation, the supernatant was decanted and the glass washed with acetone, drained and held overnight at 100°C in a vacuum oven.

Succinylation. Silanized beads were washed with acetone, then with distilled water (100 ml/g) and degassed under water. Succinic anhydride (1 g/g glass) was dissolved in enough water to cover the glass and the pH adjusted to 6.0 with NaOH. The mixture was held overnight at room temperature in a rotary shaker to complete the reaction. The supernatant was then decanted, and the succinylated glass was washed with water, then, acetone and dried in a vacuum oven at 125°C.

Thionylation. Thionyl chloride was reacted with the dried, cooled succinylated beads to form the acyl chloride derivative with a few drops of pyridine added as catalyst. The mixture was placed in a 70°C water bath and held for 1 h, after which the supernatant was drained and the glass washed several times with methylene chloride. The acyl chloride derivative was immediately reacted with either 3-mercaptopropionic acid or thioglycolic acid in a 70°C water bath for 1 h with occasional swirling of the flask. The derivatized glass was rinsed with methylene chloride then acetone and dried for 1 h at 125°C in a vacuum oven.

Preparation of antibody for coupling.

The major milk clotting protein peak obtained from the ion exchange chromatography purification of each enzyme preparation was covalently coupled to derivatized porous glass beads as described by Brown et al. (1979) as the final step in the construction of two separate immunoaffinity columns. The columns were then used in immunoaffinity chromatography purification of the antiserum from each enzyme preparation to isolate antibodies to the ion exchange chromatography purified milk clotting peaks.

Prior to covalent coupling of enzyme, the beads were washed with 1 m*M* sodium acetate buffer pH 6.0 and degassed in the same buffer under vacuum. The ion exchange chromatography milk clotting enzyme fraction was mixed with the derivatized glass and

carbodiimide was added (40 mg/g glass). N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide or 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate was added to the mixture in a 250 ml erlenmeyer flask, and the pH adjusted to 6. The mixture was transferred to a rotary shaker in a 5°C coldroom and the reaction was allowed to run overnight.

After covalent coupling of ion exchange chromatography purified enzyme, the solution was decanted and the glass washed with distilled water, coupling buffer and .5 *M* sodium chloride to eliminate non-covalently coupled materials. Unreacted sites were blocked with .2 *M* glycine or ethanolamine at pH 6. The enzyme-glass complex was gravity packed into a (.5 × 10 cm) column and washed with distilled water. The isolation of specific antibodies to the ion exchange chromatography purified enzyme clotting fraction, was achieved by recycling antiserum through the ion exchange chromatography purified enzyme-glass column for several hours. The column was then washed with distilled water followed by further washings with coupling buffer and .15 *M* sodium chloride to eliminate non-specifically adsorbed materials. Antibodies were then eluted from the column with buffers at different concentrations and pH.

Elutions were tried with .2 *M* sodium acetate at pH 3.0, .2 *M* sodium acetate at pH 3.5, containing .15 *M* NaCl , and .5 *M* sodium acetate at pH 4.0 containing, .5 *M* NaCl. The eluted fractions were immediately neutralized with NaOH, then dialyzed (5000 MW cut-off) in 1 m*M* phosphate buffer pH 7.0 and concentrated in an Amicon stirred cell with a Dialfo membrane. Immunoaffinity purified antibodies were accumulated by several runs of the columns.

Selecting the optimum elution buffer

To select for a buffer which could elute the proteins from the column, after biospecific adsorption in a biologically active form, enzyme was adsorbed to antibody-glass complex. Portions of the enzyme adsorbed antibody-glass complex were washed and incubated in

buffers of different concentrations and pH. The enzyme adsorbed glass beads were incubated in the above buffers for 10 min, with occasional mixing by turning the test tubes end over end. After the incubation period, the supernatant from each buffer was neutralized and assayed for clotting activity. Clotting activity was tested by the Formagraph method (McMahon and Brown, 1982)

Measuring effectiveness of coupling procedure

To determine how effective the enzymes can be covalently coupled to the derivatized porous glass beads, the milk clotting activity of the enzyme-glass complex was measured by the Formagraph method (McMahon and Brown, 1982). Clotting time was measured by determining the time in minutes for Berridge substrate (Berridge, 1952) to clot after addition of 200 µl of washed enzyme-glass complex. The enzyme glass complex was washed several times with buffert o eliminate contributions from residual enzymes. The supernatant from the final wash and the enzyme-glass complex were tested for clotting ability. The clotting assay was run in duplicate.

Coupling of purified antibody to the affinity matrix

The immunoaffinity purified antibodies from antiserum produced against each milk clotting preparation were covalently coupled to derivatized porous glass beads according to the procedure reported by Brown et al. (1979) to make two additional columns. The derivatized beads were washed with distilled water prior to covalent coupling of the purified antibodies. Purified antibodies were coupled by mixing with the derivatized glass, carbodiimide added as catalyst (40 mg/g glass) and the pH of the mixture adjusted to 6.5. The mixture was placed in a spiral stirrer and allowed to react for 12-16 h, to allow for optimum coupling of purified antibodies. After coupling, the supernatant was decanted, and unreacted sites on the matrix were blocked with .2 *M* glycine or ethanolamine at pH 7. The

antibody-coated beads were gravity packed into a (.5 × 10 cm) column and washed with 1m*M* sodium acetate buffer at pH 7.

Immunoaffinity purification of commercial enzymes

Each commercial microbial milk clotting preparation, diluted in 1 m*M* sodium acetate buffer at pH 6, was biospecifically adsorbed by recycling it through the appropriate glass antibody coupled matrix for several hours in a 5°C coldroom. The column was washed with distilled water, coupling buffer and .5 *M* sodium chloride to eliminate non-specifically adsorbed materials. Elution with .2 *M* sodium acetate buffer at pH 3.5 containing .15 *M* NaCl was started immediately after the wash readings approached baseline as measured with a spectrophotometer. Eluted fractions were collected in 500 µl portions, immediately neutralized, and absorbance at 280 nm was measured for protein content and assayed for milk clotting activity. Milk clotting fractions from several runs were pooled, diafiltered and concentrated by ultrafiltration in an Amicon stirred cell equipped with a PM 10 membrane filter and saved for proteolysis assays.

Alternative Purification of Antiserum

The major ion exchange chromatography purified milk clotting peak from each enzyme preparation was used to purify the antiserum produced against it. About 20 ml of enzyme from ion exchange chromatography clotting peak was added to 20 ml of rabbit antiserum and the mixture was allowed to stand for 12-24 h in a 5°C coldroom. The precipitate was removed by centrifugation at 2500 × g 30 min, washed with PBS buffer and solubilized in 5 ml of .2 *M* sodium acetate buffer at pH 3.5. It was then applied to a Sephadex G-100 gel filtration column (1 cm × 40 cm) to separate the antigen from the antibody using .2 *M* sodium acetate buffer at pH 3.5 containing .15 *M* NaCl as the separation buffer. Eluted peaks were neutralized and assayed for clotting ability.

Measurement of Clotting Activity

The Formagraph method as described by McMahon and Brown (1982) was used to determine clotting activities, with the temperature maintained at 37°C. Unless stated otherwise, fractions were adjusted to pH 6-6.7 prior to the clotting test. Berridge substrate (Berridge, 1952) made by reconstitution of 12 g low heat nonfat dry milk in 100 ml of .01 *M* $CaCl_2$ was used. Coagulation test for all pooled fractions was run in duplicate. 10 ml of Berridge substrate was added to each well of the sample block, and 200 µl of each enzyme was added to assigned wells and quickly stirred to thoroughly mix in the enzyme. The Formagraph instrument photographic recorder was turned on simultaneously upon addition of enzyme to the wells. After stirring, the pendulums were immersed into the milk samples.

Measurement of Proteolytic Activity

OPA method

The OPA method as described by Church and coworkers (1983) was used to measure peptides liberated from the proteolysis of casein by the commercial enzyme preparations and their immunoaffinity purified fractions. Commercial enzyme preparations and their fractions purified by immunoaffinity chromatography were adjusted to the same clotting activity as measured by the Formagraph method (McMahon and Brown, 1982), using purified chymosin as the standard. Casein solution (2%) in .1 *M* phosphate buffer at pH 6.7 was used as substrate for the assay of the proteolytic activities of these enzymes. About 500 ml of casein was made and then divided into five aliquots. One aliquot was assigned to the control and each of the remaining aliquots was assigned to a different enzyme.

To each 100 ml portion in a 250 flask, 2 ml of each enzyme was added to the appropriate flask. For time zero, 80 µl of each enzyme was inactivated in 4 ml TCA and then added to 4 ml of the casein solution. The flasks were then incubated in a 37°C water

bath. Samples of 4 ml were pipetted out of each flask into test tubes following addition and mixing of enzymes every 15 min for the first 30 min, and, subsequently, 4 ml samples were removed every 30 min. To each 4 ml sample removed, 4 ml of 8% TCA was immediately added and mixed by agitation to stop the enzyme action and to precipitate unhydrolyzed casein. The mixture was then filtered through a Whatman No. 5 filter paper and the filtrate saved for analysis. The OPA assay was run in duplicate.

The extent of proteolysis was measured by the determination of TCA soluble peptides in the filtrate liberated from the casein/enzyme mixture. OPA reagent was prepared as described by Church and coworkers (1983) by addition of 25 ml 100 m*M* sodium borate, 2.5 ml 20% (wt/wt) SDS, 1 ml solution of OPA (40 mg dissolved in 1 ml methanol) and 100 µl β-mercaptoethanol. Reagent volume was then made up to 50 ml with distilled water. A 200 µl sample of the TCA filtrate was mixed with 1 ml of OPA reagent solution end over end at room temperature for 10 sec in a microcuvette. Absorbance at 340 nm was measured with a Beckman DU 8B UV-VIS spectrophotometer.

Amino acid analysis method

Commercial enzyme preparations and their fractions purified by immunoaffinity chromatography were adjusted to the same clotting activity as measured by the Formagraph method (McMahon and Brown, 1982), using purified chymosin as the standard. Whole casein solution (2%) in .1 *M* phosphate buffer at pH 6.7 was used as substrate. About 500 ml of the solution was made and then divided into five aliquots. One of the flasks was assigned to the control, and each of the four remaining flasks was assigned to a different enzyme. Except for the control, to each 100 ml substrate was added 2 ml of the appropriate enzyme. For time zero, 80 µl of each enzyme was inactivated in 4 ml TCA and then added to 4 ml casein solution. The flasks were then incubated in a 37°C water bath.

Samples of 4 ml were pipetted out of each flask after the addition and mixing of enzymes every 15 min for the first 30 min, and subsequently, 4 ml samples were removed

every 30 min. 4 ml of 8% TCA was immediately added to each sample and mixed to stop the enzyme action and to precipitate unhydrolyzed casein. The mixture was then filtered through a Whatman No. 5 filter paper. Duplicate samples of 2 ml of the filtrate from the casein-enzyme-TCA mixture were transferred into ampoules and freeze-dried. The freeze-dried material was resolubilized by addition of .6 ml of 6 N HCl, degassed under vacuum, flushed with nitrogen and sealed by flame. The samples were hydrolyzed at 110°C for 20 h in a heating block.

After hydrolysis, 20 µl portions of each hydrolyzate were transferred to two test tubes and dried with nitrogen gas. 250 µl of buffer (Na-S HPLC grade)was added to each test tube to solubilize the material. The content of each tube was thoroughly mixed with a vortex mixer, filter sterilized through a .2 υm syringe filter and aspirated into a Beckman amino acid analyzer sample cartridge. The cartridges were then loaded into the amino acid analyzer refrigerated chamber. Amino acid analysis was performed with a Beckman Amino Acid Analyzer System 6300. The extent of proteolysis was determined by measuring the amino acid content of the TCA soluble peptides in the filtrate liberated from the proteolysis of casein.

RESULTS AND DISCUSSION

Gel Filtration

Gel filtration separated *M. miehei* and *M. pusillus* milk clotting preparation into two protein peaks. A major peak and a minor peak for *M. miehei* and two major peaks for *M. pusillus*. Milk clotting activity test performed by the Formagraph method, showed the minor peak of *M. miehei* to exhibit milk clotting properties while the major peak did not. Clotting test showed the first major peak of *M. pusillus* to have clotting properties while the second major peak did not. The results are shown in Figures 3 and 4. The results provide evidence that commercial microbial preparations are mixtures of enzymes which contain

Figure 3. Gel filtration chromatogram of *Mucor miehei* milk clotting enzyme preparation using Sephadex G-100 as the column matrix and .05 *M* sodium acetate buffer at pH 6.5 as eluting buffer.

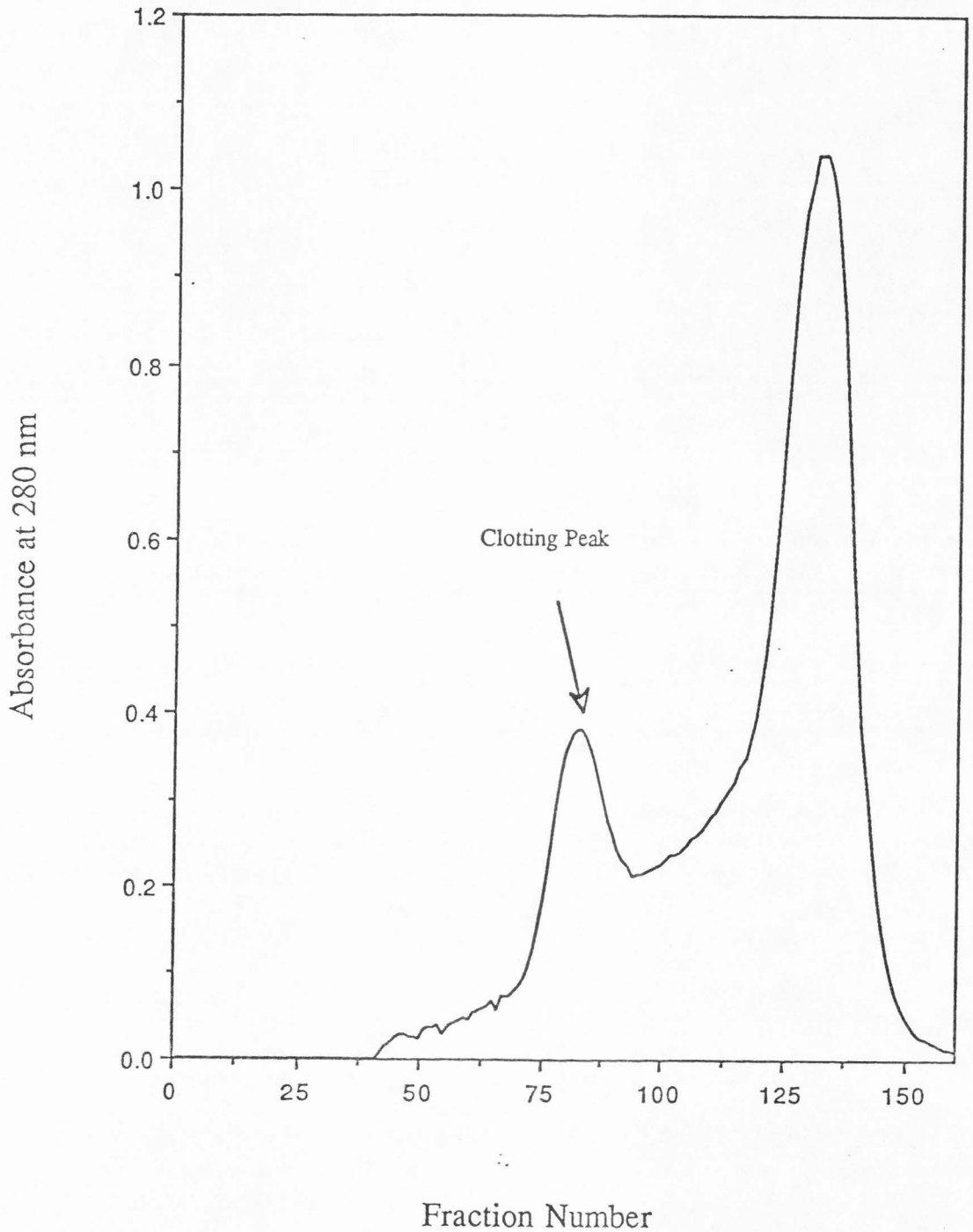
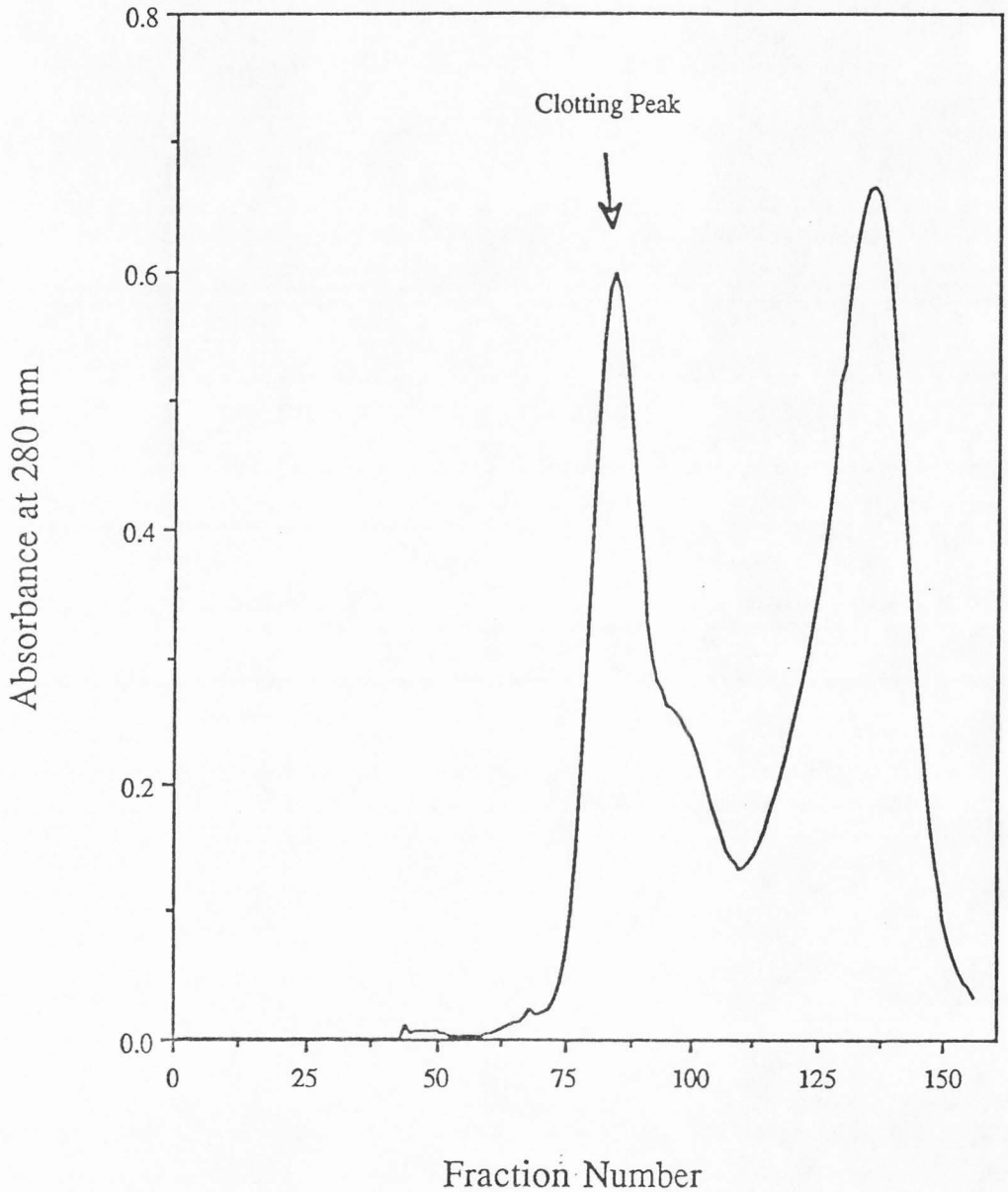

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 280 nm
Clotting Peak
0
25
50
75
100
125
150
Fraction Number

Figure 4. Gel filtration chromatogram of *Mucor pusillus* milk clotting enzyme preparation using Sephadex G-100 as the column matrix and .05 *M* sodium acetate buffer at pH 6.5 as eluting buffer.


Clotting Peak
Absorbance at 280 nm
0.8
0.6
0.4
0.2
0.0
0
25
50
75
100
125
150
Fraction Number

milk clotting enzymes and other proteins or enzymes which could be deleterious to the quality of cheeses made from them.

Gel filtration separates proteins based on their molecular sizes. The gel filtration chromatograms show that the commercial milk clotting enzyme preparations can be resolved to two fractions, a clotting fraction and a nonclotting fraction. The results show that the commercial milk clotting enzyme preparations used in this study contain at least two proteins based on molecular size.

Ion Exchange Chromatography

Ion exchange chromatography purification was used to further purify the milk clotting protein peak obtained by gel filtration from each preparation. *M. miehei* milk clotting peak yielded three protein peaks, two major peaks and one minor peak, while *M. pusillus* milk clotting peak yielded four protein peaks, two major and two minor peaks. Milk clotting test showed two milk clotting protein peaks for both enzymes. While one major and one minor peak showed milk clotting activity for *M. pusillus* , one of the major peaks and the minor peak showed clotting activity for *M. miehei* . For both enzymes, the biggest protein peak did not show milk clotting activity. The results are shown in Figures 5 and 6.

Ion exchange chromatography separates proteins based on their charges. From the results, the milk clotting protein peak obtained from gel filtration of both enzyme preparations can be further purified to yield three or more protein peaks based on charge. Purification by ion exchange chromatography worked better for the purification of *M. miehei* milk clotting preparation than it did for *M. pusillus* milk clotting preparation.

The results indicate that there are at least two major proteins in the gel filtration milk clotting protein peak obtained from each milk clotting preparation. Ion exchange chromatography purification showed better resolution for *M. miehei* preparation milk clotting protein peak from gel filtration than did *M. pusillus* preparation milk clotting protein peak.

Figure 5. Ion exchange chromatogram of the gel filtration milk clotting protein peak of *Mucor miehei* milk clotting enzyme preparation using CM Sephadex as the column matrix and .05 *M* sodium acetate buffer at pH 3.5/.5 *M* sodium acetate buffer at pH 5.0 for gradient elution.

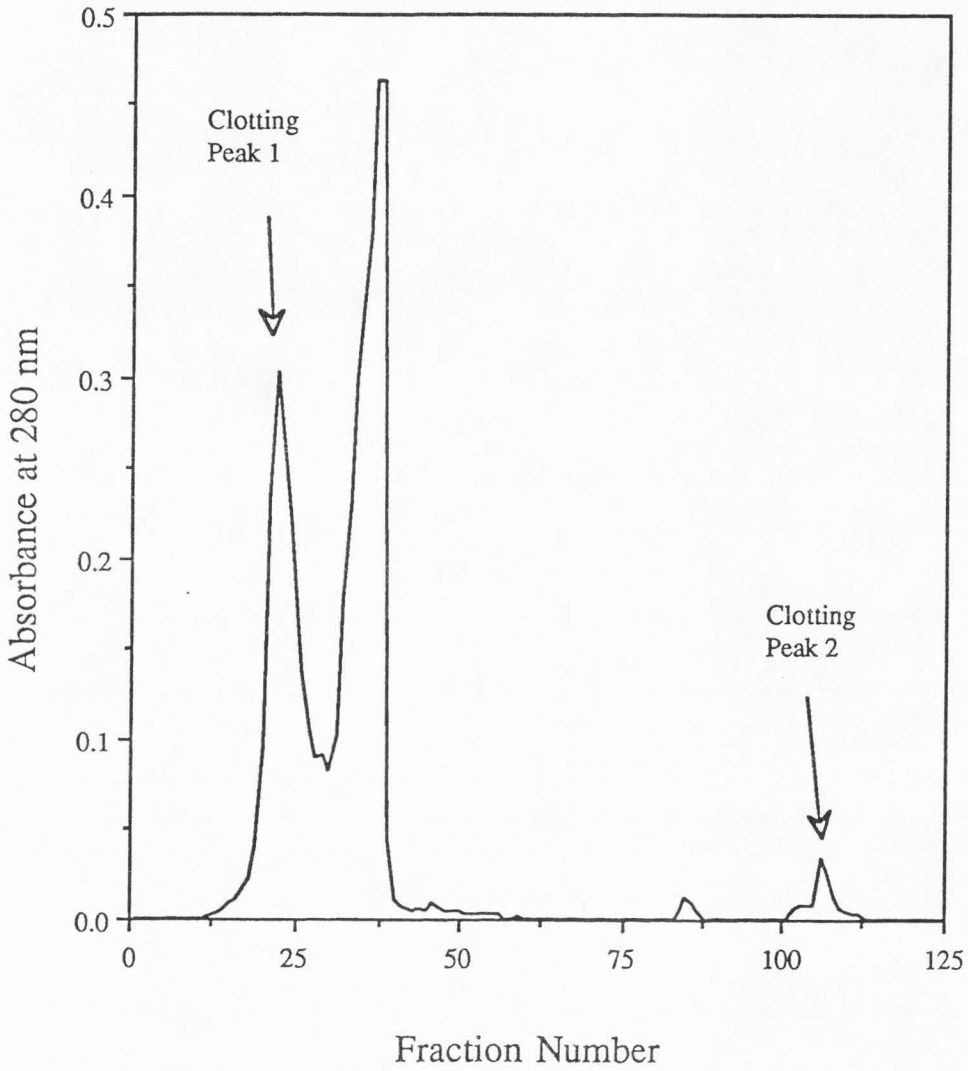
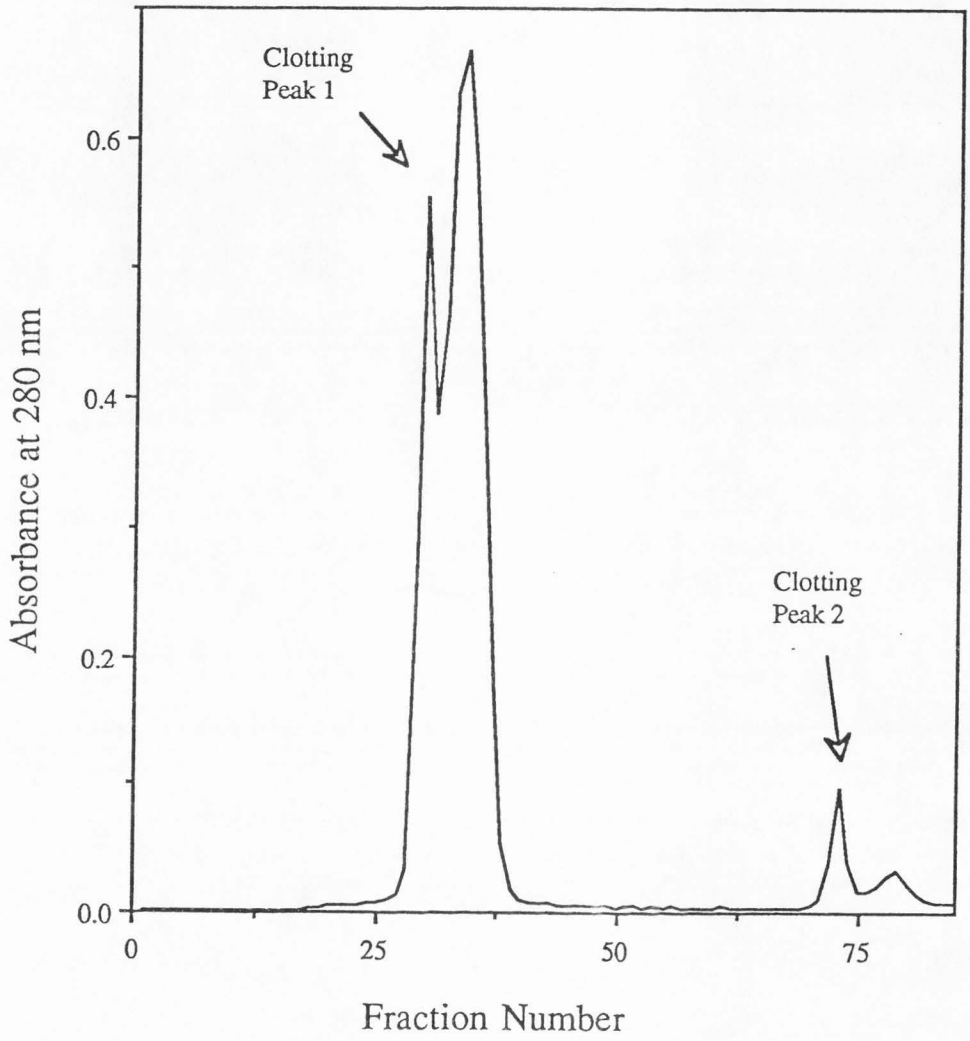

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 280 nm
Clotting
Peak 1
Clotting
Peak 2
0
25
50
75
100
125
Fraction Number

Figure 6. Ion exchange chromatogram of the gel filtration milk clotting protein peak of *Mucor pusillus* milk clotting enzyme preparation using CM Sephadex as the column matrix and .05 *M* sodium acetate buffer at pH 3.5/.5 *M* sodium acetate buffer at pH 5.0 for gradient elution.


Clotting
Peak 1
Clotting
Peak 2
Absorbance at 280 nm
0.0
0.2
0.4
0.6
0
25
50
75
Fraction Number

Immunoaffinity Chromatography

Enzyme purification by immunoaffinity chromatography was achieved by first purifying the antiserum for each milk clotting preparation to obtain specific antibodies. The major ion exchange chromatography milk clotting protein peak from each milk clotting preparation was covalently coupled to derivatized porous glass beads and used as the immunoadsorbent for the purification of the appropriate antiserum. A representation of several runs of antisera immunoaffinity purification chromatogram is shown on Figure 7.

Purified antibodies were then covalently coupled to a new batch of derivatized porous glass beads and used for the purification of the appropriate commercial milk clotting enzyme preparation. A representation of several runs of the milk clotting enzyme preparations immunoaffinity purification chromatogram is shown on Figure 8. Enzymes purified by immunoaffinity were compared with their parent enzyme preparations for proteolytic activity. The OPA method (Church et al., 1983) and amino acid analysis of TCA soluble peptides liberated from the hydrolysis of casein were used as a measure of the extent of proteolytic activity of the enzymes.

Column matrix derivatization

Each step in the porous glass derivatization was tested by covalently coupling enzyme to the derivatized matrix and checking aliquot of the glass-enzyme complex for clotting activity before the next derivatization step was performed. The results show that once the glass beads are silanized, enzymes can be coupled without the need for further derivatization. Succinylation of the silanized glass beads, does however, seem to stabilize and to improve the clotting ability of the enzyme-glass complex. The thionylation step after succinylation of the silanized glass did not make any difference in the clotting ability of the enzyme-glass complex.

Few papers have been published on the use of porous glass with covalently immobilized enzymes for affinity and immunoaffinity chromatography. Reasons for the

Figure 7. Immunoaffinity purification of milk clotting enzyme preparation antisera using ion exchange purified enzymes covalently coupled to derivatized porous glass as the column matrix and .2 *M* sodium acetate buffer at pH 3.5 as the elution buffer.

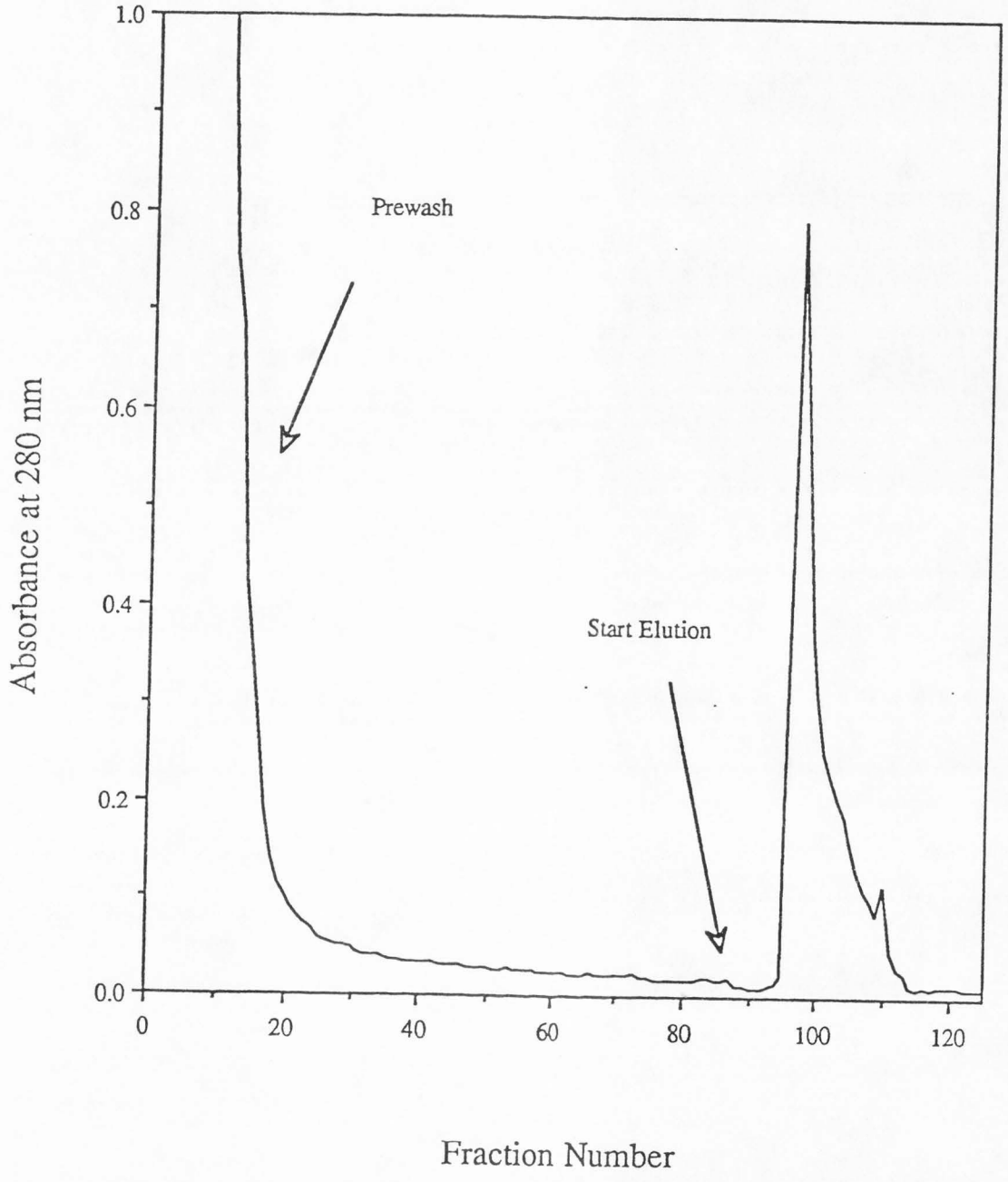
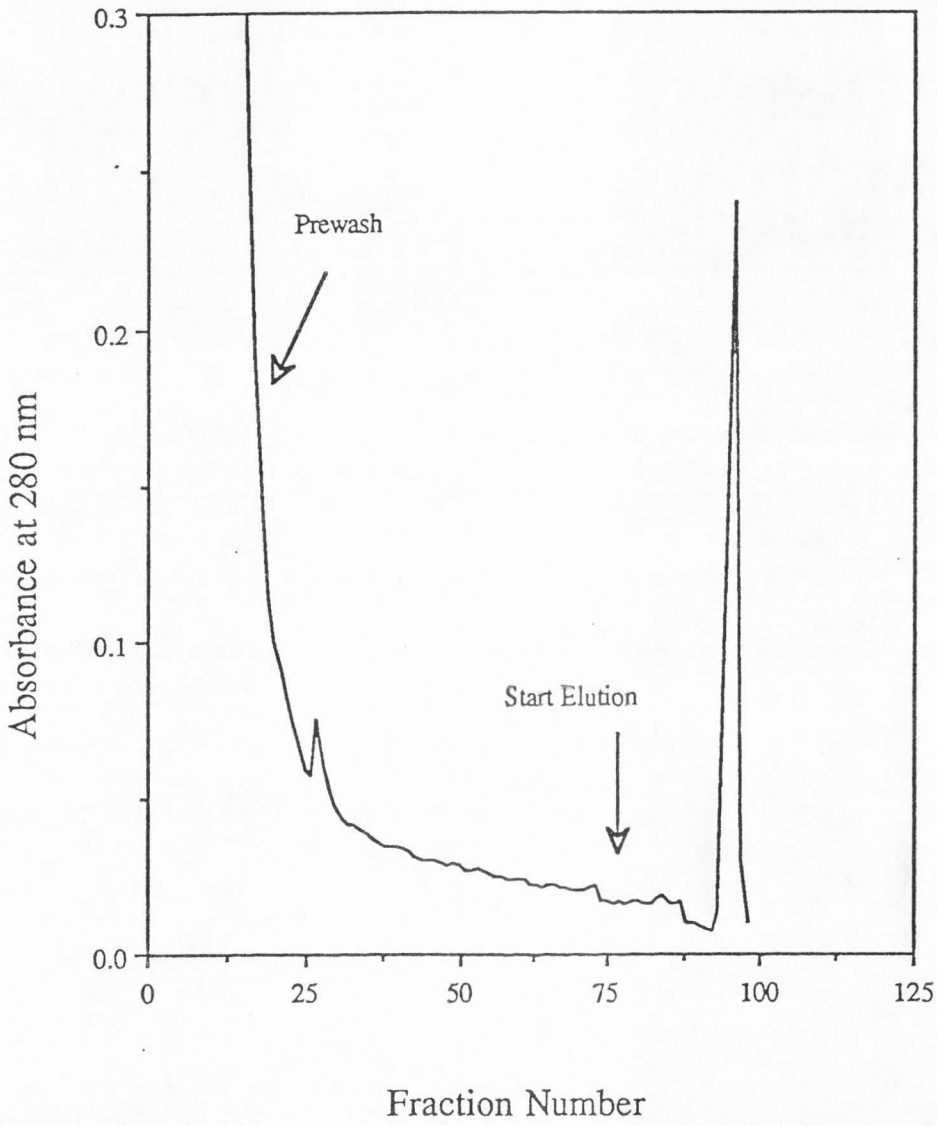

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 280 nm
Prewash
Start Elution
0
20
40
60
80
100
120
Fraction Number

Figure 8. Immunoaffinity purification of microbial milk clotting enzyme preparations using purified antibodies covalently coupled to derivatized porous glass as the column matrix and .2 *M* sodium acetate buffer at pH 3.5 as the elution buffer.


0.3
0.2
0.1
0.0
Absorbance at 280 nm
Prewash
Start Elution
0
25
50
75
100
125
Fraction Number

limited use of porous glass are attributed to the fact that derivatized forms are not readily available commercially (Scouten, 1981; Aerts, 1986; Chase, 1984) and underivatized glass beads exhibit high surface charges which result in non-specific adsorption of enzymes (Scouten, 1981). These problems are easily eliminated by laboratory scale porous glass derivatization, and the structural advantages that porous glass beads afford make the problems encountered in derivatization worth the effort (Scouten, 1981).

Enzymes, immobilized on inorganic supports such as glass, are reported to show increased storage and operational stability over those enzymes immobilized on organic supports (Line et al., 1971; Weetall, 1969a,b; Weetall and Hersh, 1969; Weetall, 1970; Weetall, 1974). Glass is more resistant to mechanical pressure than agarose and cannot be degraded by bacteria. It is thermally stable and chemically compatible with most solvents employed in chromatographic techniques (Scouten, 1981).

Measuring effectiveness of coupling procedure

The effectiveness of the coupling procedure was measured by testing the coupled enzyme-glass complex for ability to clot Berridge substrate. The activity of a milk clotting enzyme is measured by the time (min) it takes the enzyme to coagulate milk under standardized conditions after addition of the enzyme to milk. Clotting time is inversely proportional to enzymic activity (McMahon and Brown, 1982; McMahon and Brown, 1985). All milk clotting assays in this study used Berridge substrate as the media and the Formagraph recorder to measure milk coagulation time. The Formagraph is equipped with a sample block with ten wells, pendulums, photographic paper and strobe light. To measure milk clotting activity, milk samples are filled into the sample wells and a pendulum is immersed into each well. The movement of each pendulum immersed in each well is traced on the photographic paper. The point at which the line begins to diverge to two curving lines is the clotting point (McMahon and Brown, 1982). The photographic paper advances at 2 mm/min and this rate is used as a conversion factor to change the length of the traced

line to minutes in obtaining the clotting time. An illustration of the Formagraph milk clotting plot is shown on Figure 9. The *M. miehei* enzyme-glass complex and the *M. pusillus* enzyme-glass were both able to clot milk. This was taken as an indication of effective coupling of the enzymes to the derivatized glass matrix. A schematic representation of the ability of the enzyme-glass complex to clot Berridge substrate is shown in Figure 10. McMahon and Brown (1982) have a detailed discussion on the workings of the Formagraph instrument.

Selection of optimum elution buffer

Several buffers at different pH were used to determine which buffer could be most effective in eluting the enzymes in biologically active forms. Since it was easier to measure clotting activity of eluted enzymes, derivatized glass to which purified antibodies had been covalently coupled was reacted with enzyme. The biospecifically adsorbed enzyme was then eluted with different buffers as described in the materials and methods section. Although elutions were achieved with most of the buffers, based on absorbance measurement at 280 nm, elutions with .2 *M* sodium acetate at pH 2.0 and pH 2.5 leached antibody from the column matrix. The neutralized eluted fractions were also unable to clot milk. It is possible that this is due to complete irreversible denaturation of the eluted enzymes. Effective elutions were achieved with .2 *M* sodium acetate pH 3.0, .2 *M* acetate pH 3.5 containing .15 *M* NaCl and .5 *M* acetate pH 4.0 containing .5 *M* NaCl.

Proteolysis Test

Commercial enzyme preparations and their fractions purified by immunoaffinity chromatography were adjusted to the same clotting activity as measured by the Formagraph method, using purified chymosin as the clotting standard. Liberation of peptides from casein is used as a measure of general proteolysis of milk clotting enzymes (Vanderpoorten and Weckx, 1972). Peptides liberated from casein hydrolysis by commercial milk clotting

Figure 9. Schematic of the Formagraph recording of milk clotting activity of an enzyme. Clotting time is represented by (r) (adapted from McMahon and Brown, 1982).

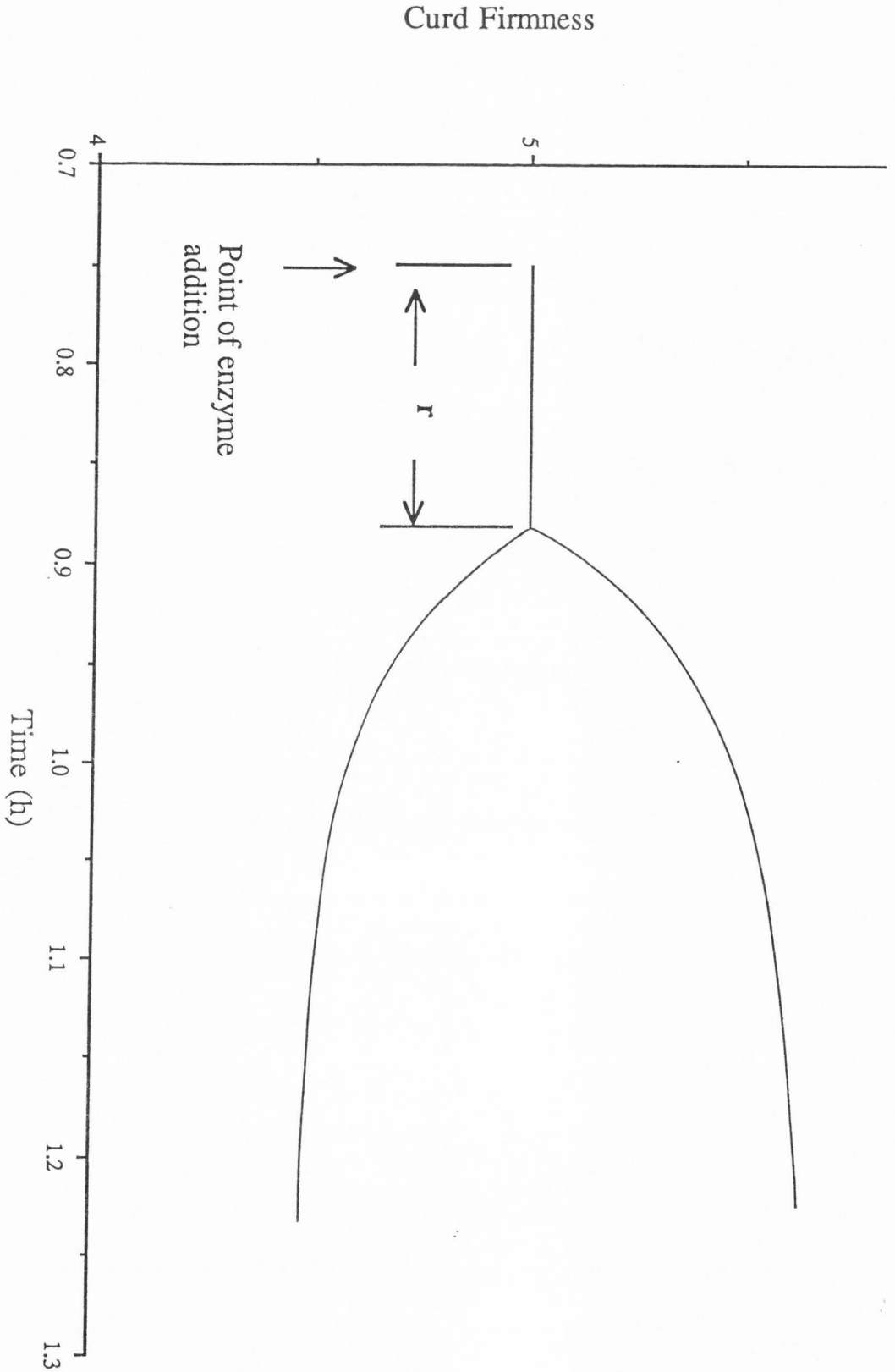
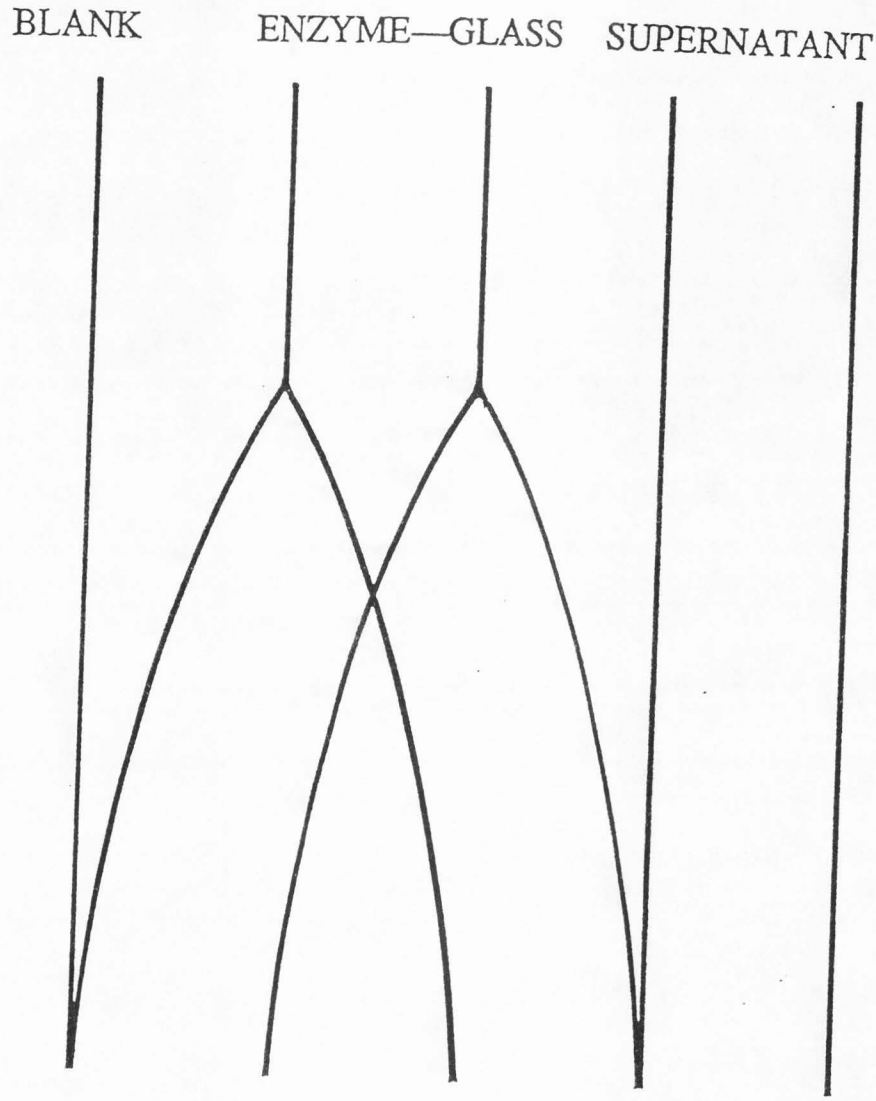

Curd Firmness
4
5
0.7
0.8
0.9
1.0
1.1
1.2
1.3
Time (h)
Point of enzyme addition
r

Figure 10. Schematic of the Formagraph recording of milk clotting activity of derivatized enzyme-glass complex. Clotting test of the complex was done after several washings of the complex with buffer. Complex and supernatant were then assayed for ability to clot Berridge substrate.


BLANK
ENZYME—GLASS
SUPERNATANT

preparations and their immunoaffinity purified fractions were measured by the OPA method (Church et al., 1978) and by amino acid analysis.

The immunoaffinity purified fractions showed slightly less proteolysis than their parent milk clotting enzyme preparations. The results are shown in Figures 11 and 12, and in Tables 1 and 2. This is in agreement with results reported in the literature which show that the elimination of contaminating enzymes from some milk clotting preparations contributed to decreased proteolysis (Morvai-Racz, 1974; Shaker and Brown, 1985b). However, the decrease in proteolysis was not pronounced for either of the microbial milk clotting preparations. The immunoaffinity purified fraction of *M. miehei* enzyme preparation showed less proteolytic activity than did the *M. pusillus* fraction when they were compared with their parent milk clotting preparations as shown in Figures 11 and 12.

CONCLUSIONS

Although much time is involved in preparing biospecific adsorbent for use in immunoaffinity chromatography purifications, a rapid purification with good yields and high purity makes the trouble worthwhile. Immunoaffinity chromatography represents a possible tool for the isolation of higher clotting and lower general proteolytic fractions from commercially available microbial milk clotting enzyme preparations.

Results from several chromatographic enzyme purification steps show that commercial microbial milk clotting preparations are mixtures of enzymes. Gel filtration chromatography showed that the commercial microbial milk clotting preparations are mixtures of enzymes which contain, apart from milk clotting enzymes, other proteins or enzymes which could affect the quality of cheeses made from them.

Gel filtration separates proteins based on their molecular sizes. The gel filtration chromatograms show that the commercial milk clotting enzyme preparations can be resolved to two fractions, a clotting fraction and a nonclotting fraction. The results show that the commercial milk clotting enzyme preparations used in this study contain at least two

Figure 11. OPA proteolysis test of liberation of TCA soluble peptides by commercial *Mucor miehei* milk clotting enzyme preparation and its immunoaffinity purified fraction. Whole casein (2%) in .1 *M* phosphate buffer at pH 6.7 was used as substrate. Equal volume of 8% TCA was used to stop enzyme activity.

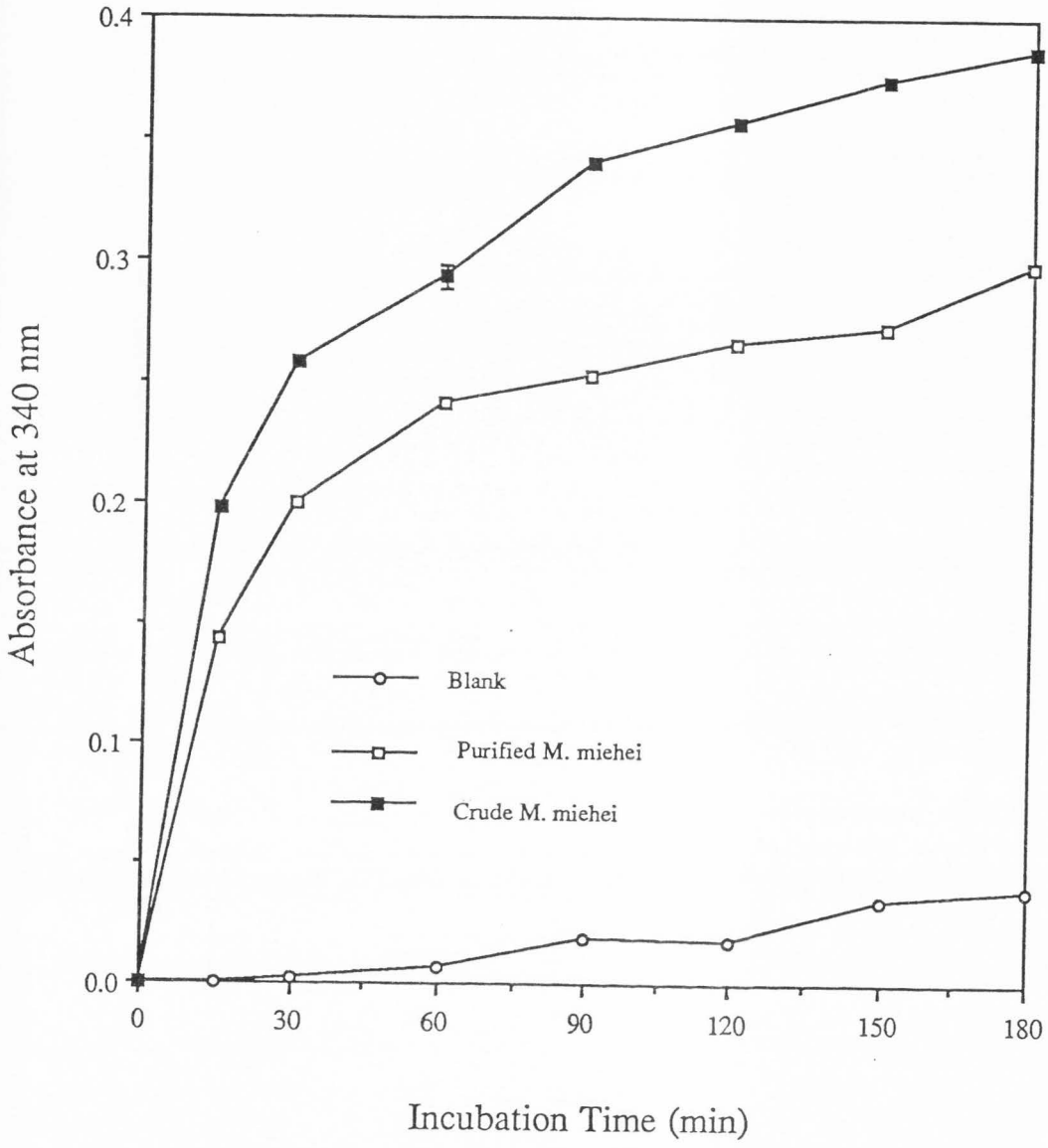

0.4
0.3
0.2
0.1
0.0
Absorbance at 340 nm
0
30
60
90
120
150
180
Incubation Time (min)
Blank
Purified M. miehei
Crude M. miehei

Figure 12. OPA proteolysis test of liberation of TCA soluble peptides by commercial *Mucor pusillus* milk clotting enzyme preparation and its immunoaffinity purified fraction. Whole casein (2%) in .1 *M* phosphate buffer at pH 6.7 was used as substrate. Equal volume of 8% TCA was used to stop enzyme activity.

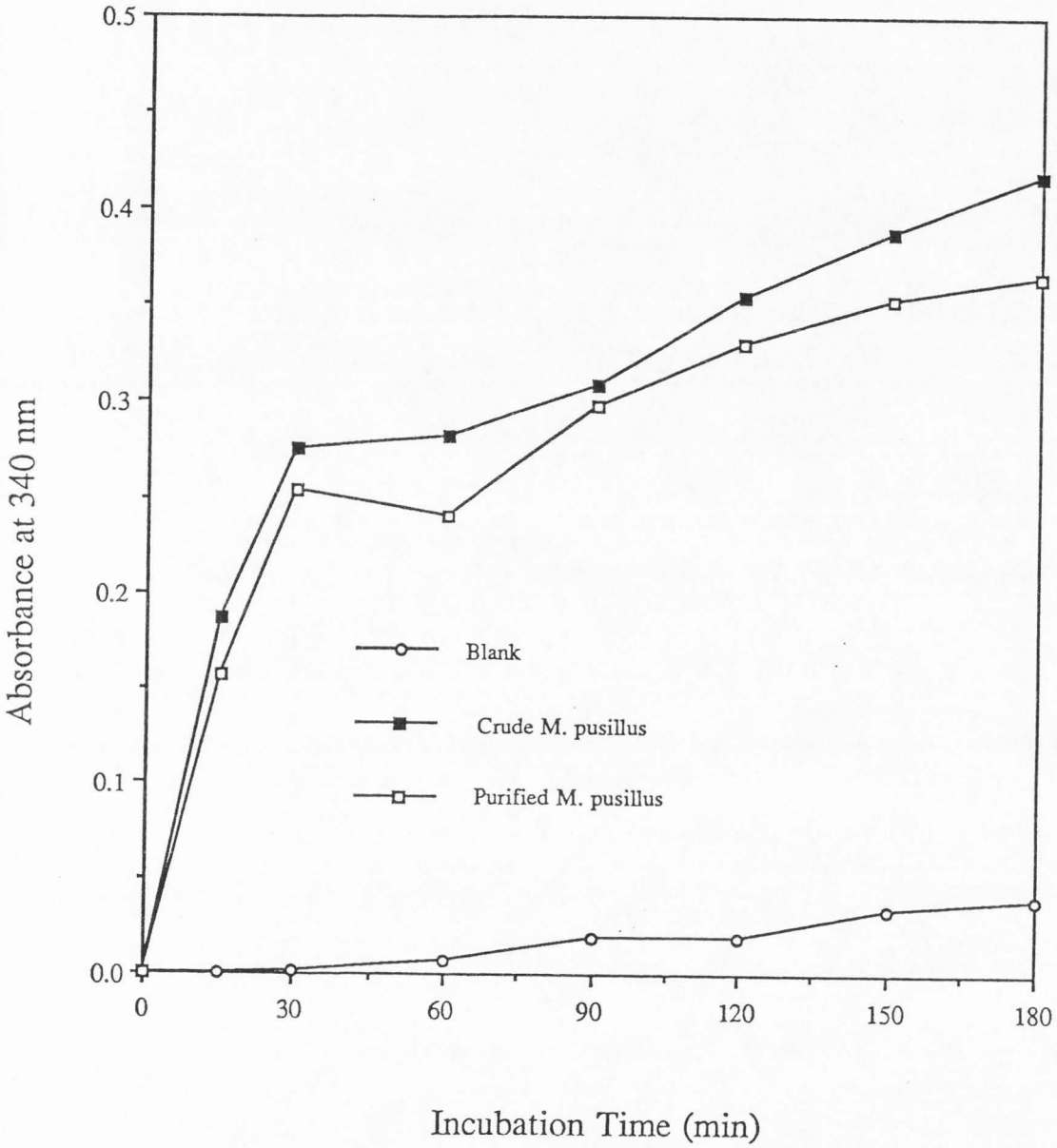

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 340 nm
0
30
60
90
120
150
180
Incubation Time (min)
Blank
Crude M. pusillus
Purified M. pusillus

Table 1. Comparison of milk clotting and proteolytic activities of commercial *Mucor michei* milk clotting enzyme preparation with its purified milk clotting fractions.

Sample	Absorbance (A_{280})	Activity (RU/ml)	Specific Activity (RU/(A_{280}.ml)	Proteolytic Activity (A_{340})	Specific Activity/Proteolytic Activity (RU/(A_{280}.A_{340}.ml)
Crude	39.64	144.98	3.66	1.37	2.67
Gel filtration	.45	4.32	9.6	—	—
Ion exchange	.12	4.08	34.0	—	—
Immuno-affinity	.11	4.12	37.45	1.14	32.85

Table 2. Comparison of milk clotting and proteolytic activities of commercial *Mucor pusillus* milk clotting enzyme preparation with its purified milk clotting fractions.

Sample	Absorbance (A_{280})	Activity (RU/ml)	Specific Activity (RU/(A_{280}.ml)	Proteolytic Activity (A_{340})	Specific Activity/Proteolytic Activity (RU/(A_{280}.A_{340}.ml)
Crude	40.54	187.2	4.62	1.28	3.61
Gel filtration	.57	5.61	9.80	—	—
Ion exchange	.25	5.30	21.22	—	—
Immuno-affinity	.16	4.73	29.56	1.13	26.16

proteins based on molecular size. The ion exchange chromatogram showed that the milk clotting protein peak obtained from gel filtration of both enzyme preparations can be further purified to yield three or more protein peaks based on charge. Ion exchange chromatography worked better for the purification of *M. miehei* milk clotting preparation than it did for *M. pusillus* milk clotting preparation. Resolution of the enzymes by ion exchange chromatography was better for *M. miehei* preparation than for *M. pusillus* preparation.

Separation of these accompanying enzymes could lead to more uniformity in the quality of cheeses made with microbial rennets due to decreased proteolysis. Purification by immunoaffinity chromatography yielded enzymes with higher clotting activity as compared to the crude enzyme preparation when adjusted to the same absorbance at 280 nm. Proteolysis tests showed that when adjusted to the same clotting activity, immunoaffinity chromatography purified fractions yielded fractions with lower general proteolytic activity as determined by peptides liberated from the proteolysis of casein. The elimination of contaminating enzymes improved the milk clotting/proteolytic activity ratio of the commercial milk clotting enzyme preparations.

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CHAPTER II

CROSS REACTIVITY OF MICROBIAL MILK CLOTTING ENZYME ANTIBODIES

ABSTRACT

Although their actions in milk differ, proteolytic enzymes from *Mucor miehei* and *Mucor pusillus* have both been used as calf rennet substitutes in cheese manufacture. Antibodies were produced by intramuscular injection of *M. miehei* and *M. pusillus* commercial rennets emulsified in Freund's complete adjuvant into New Zealand White rabbits. Harvested antiserum was heated at 56°C for 30 min to inactivate complement factors and contaminating proteins then centrifuged at 1700 × g for 30 min. Ouchterlony double immunodiffusion assay was used to test for presence of antibody in the antiserum and for cross reactivity. Antibodies against *M. miehei* milk clotting enzyme cross-reacted with *M. pusillus* milk clotting enzyme and antibodies against M. *pusillus* milk clotting enzyme cross-reacted with *M. miehei* milk clotting enzyme. Immunodiffusion assay did not show cross reactivity between calf rennet antibodies with either *M. miehei* or *M. pusillus* enzymes. Antibodies against the *Mucor* enzymes did not show cross reactivity with calf rennet. Differences in the characteristics of the two *Mucor* milk clotting enzymes exist, even though they are immunologically cross-reactive. From our results, at least one antigenic factor is common to both enzyme preparations.

INTRODUCTION

Antibodies are proteins synthesized by animals in response to antigens (foreign bodies) in their blood. Antibodies can be raised against almost all compounds foreign to a host if they are large enough to elicit antibody production or coupled to a suitable protein to render them immunogenic (Eisen and Siskind, 1964; Warr, 1982). Binding of antigen to antibody is due to formation of noncovalent bonds between the antigen and antibody. Antigenic determinants on the surfaces of antigens allow antibodies to recognize their respective antigens. Presence of steric factors and the antigenic conformation also act as means of identifying antigens (Warr, 1982).

Chemical bonds such as, hydrogen bonds, electrostatic, hydrophobic and Van der Waals (Chase, 1984; Warr, 1982) hold the antibody-antigen complex together, once biological recognition takes place. Complementary sequences on the antigenic determinants of the antigen and the antigen binding sites of the antibody aid in bringing antigens and antibodies close enough together for bonding to take place (Warr, 1982).

Cross reactivity is the ability of an antibody produced for one antigen to recognize and react with a different antigen. This can be caused by the presence of shared antigenic determinants on the antigens, due to antigenic similarities or the presence of polyfunctional binding sites on the antibodies. Cross reactivity between antibodies and similar antigens occurs through competitive binding of the antigens (Garvey et al., 1977).

Enzymes of fungal origin obtained from *M. miehei* and *M. pusillus* are accepted for use in cheese making but are more proteolytic on milk caseins than calf rennet, resulting in cheese defects (Annibaldi et al., 1970; Shovers et al., 1972; Alichanidis et al., 1984).

The objectives of this study were: 1) to determine the immunological reactivities of antibodies produced against *M. miehei* and *M. pusillus* milk clotting enzymes and 2) to check for immunological similarities between the *Mucor* enzymes and chymosin.

Fungal Proteases

Fungal proteases of concern in cheese making are those which exhibit ability to clot milk. Proteases of *Mucor* species are acceptable for cheese manufacture (Alichanidis et al., 1984; Green, 1977; Richardson et al., 1967). The clotting activities of *Mucor* enzymes follow a mechanism similar to that of chymosin during coagulation of milk (Dennis and Wake, 1965; Sternberg, 1976; Tam and Whitaker, 1973), but their general proteolytic activities increase more rapidly with time.

All suitable milk clotting enzymes split off the carboxyl terminal of κ-casein of milk at the sensitive Phe_{105}-Met_{106} bond during the enzymic phase of milk coagulation. This action yields a soluble macropeptide and an insoluble para-κ-casein, destabilizing the casein

micelle (Tam and Whitaker, 1973). The presence of calcium ions, causes the destabilized micelles to coagulate (McMahon and Brown, 1985). Calf rennet has been used in cheese manufacture for so long that it has become the standard for judging chymosin substitutes (Sternberg, 1972; Shaker and Brown, 1985a). Assays developed for chymosin are usually applied without modification to evaluations of microbial rennets.

The excessive proteolytic activities of fungal proteases tend to break down milk proteins more extensively, resulting in reduced cheese yield and bitter flavor (Law, 1983; Martens and Naudts, 1973; Mickelsen and Fish, 1970; Sternberg, 1976; Vanderpoorten and Weckx, 1972).

Similarities in amino acid composition exist between *M. miehei* and *M. pusillus* proteases. The enzymes have the same number of residues of arginine, tyrosine and histidine and preferentially hydrolyze bonds containing aromatic and hydrophobic amino acid residues at the carboxyl end (Sternberg, 1972). Fungal proteases are stable at standard temperatures used in cheese manufacture (Sponcet et al., 1985). The enzymes are classified as acid proteases based on their optimum pH for proteolytic activity on bovine serum albumin (Sternberg, 1971).

The milk clotting fractions of *M. miehei* and *M. pusillus* migrate to different distances on polyacrylamide gel during electrophoresis. *M. pusillus* milk clotting fraction migrates further down the anode (Shovers et. al., 1972).

Properties of *Mucor miehei* Protease.

M. miehei protease liberates more nonprotein nitrogen (NPN) than *M. pusillus* protease or chymosin upon hydrolysis of α, β and whole casein (Vanderpoorten and Weckx, 1972). A molecular weight of 34,000-39,000 is reported for *M. miehei* protease based on gel filtration and it consists of about 255 amino acid residues (Sternberg, 1971). Its optimum temperature for milk coagulation is between 58-62°C, (Reps et al., 1970) and 60-65°C (Prins and Nielson, 1970) as compared to that for calf rennet of 42-44°C (Reps et al.,

1970). Its pH optimum for hydrolysis of casein is pH 5-7.5 (Reps et al., 1970; Sternberg, 1971).

Based on its pH optimum of 4.6 for the hydrolysis of bovine serum albumin, *M. miehei* protease is classified as an acid protease (Sternberg, 1971). Reaction with 2,4 Dinitro-1-fluorobenzene blocks alanine and glycine at its N-terminal end and reduces the enzymic activity by about 60% (Sternberg, 1972). All bonds split by *M. miehei* protease have an aromatic amino acid at the carboxyl terminal (Sternberg, 1972).

Properties of *Mucor pusillus* Protease

M. pusillus protease liberates less NPN from α-, β- and whole-caseins than *M. miehei* protease (Vanderpoorten and Weckx, 1972) but more than rennin (Itoh and Thomasow, 1971). Its proteolytic activity on milk caseins resembles more closely the proteolytic activity of chymosin than any of the other milk clotting enzymes (Richardson et al., 1967; Yu et al., 1969). It is an acid protease with a pH optimum of 3.5, 4.0 and 4.5 for the hydrolysis of casein, hemoglobin and κ-casein respectively (Itoh and Thomasow, 1971; Shaker and Brown, 1985b). Its has a molecular weight of 29,000, and contains 277-281 amino acid residues per mole (Yu et al., 1969).

The proteolytic activity to milk clotting activity ratio for the crude enzyme is about 3.5 times that of calf rennet but can be decreased to between 1.5-2 with purification (Morvai-Racz, 1974). The specific extinction coefficient of a 1% (w/w) solution of *M. pusillus* enzyme measured at 280 nm is 10, with the solution showing maximum absorbance at 276 nm (Arima et al., 1968). Metal ions, such as silver, zinc and mercury decrease while copper ions increase the proteolytic action of this enzyme (Arima et al., 1968).

Comparisons Between Calf Rennet and *Mucor* Proteases

Similarities in amino acid composition exist between *M. pusillus*, *M. miehei* proteases and calf rennin. *M. miehei* and calf rennin have the same number of residues of

phenylalanine, threonine and lysine (Sternberg, 1971). The clotting activities of *Mucor* proteases and rennin follow similar mechanisms (Sternberg, 1971).

In the enzymic phase of milk coagulation, they attack the κ-casein fraction of milk and hydrolyze the sensitive Phe_{105}-Met_{106} bond. This action yields a soluble macropeptide and an insoluble para-κ-casein, destabilizing the casein micelle (Sternberg, 1972). Calcium ions aid in causing the micelles to aggregate (McMahon and Brown, 1985).

The proteolytic activity to milk clotting activity ratio is higher for *Mucor* enzymes than for calf rennet (Morvai-Racz, 1974). Calf rennet and the *Mucor* enzymes are classified as acid proteases based on their pH optima for proteolytic activities. They preferentially hydrolyze bonds with hydrophobic amino acid in the carboxyl terminal (Sternberg, 1972).

The optimum temperature for milk coagulation is between 58-62°C, (Reps et al., 1970) and 60-65°C (Prins and Nielson, 1970) for the *Mucor* enzymes as compared to 42-44°C (Reps et al., 1970) for calf rennet. *Mucor* proteases are more heat stable than calf rennet (Sponcet et al., 1985).

MATERIAL AND METHODS

M. miehei and *M. pusillus* milk clotting enzyme preparations were obtained from a commercial source (Marschalls Division of Miles Laboratories) and chymosin was purchased from New Zealand Coop. (Petuluma, CA). Freund's complete and incomplete adjuvants and Agarose were purchased from Sigma Chemical Co. (St. Louis, MO). New Zealand White rabbits were obtained through the Laboratory Animal Research Center, Utah State University (Logan, UT). Bactoagar and agarose were purchased from Difco (Detroit, MI). All other chemicals were of reagent grade.

Antibody Production

Three Rabbits (New Zealand White) between 2 and 6 mo old were used as experimental animals in the production of antibodies. Each rabbit was assigned to an enzyme

preparation. For the initial immunization, at least 150 υg protein (Anon, 1984) of each enzyme preparation contained in a total volume of 1 ml phosphate buffered saline (PBS) at pH 7 was used as immunizing antigen. The antigen solution was mixed with 1 ml of Freund's complete adjuvant to form an emulsion. Emulsification was achieved by repeated mixing and passage through a No. 20 gauge needle to form a thick pasty consistency. The emulsion was injected intramuscularly into two sites in each hind area per animal with the use of plastic disposable syringes with a No. 25 gauge needle. The injected volumes did not exceed .5 ml at each site.

Subsequent immunizations or boosters utilized the same concentrations and volumes of enzymes as used for the initial immunization but were mixed with Freund's incomplete adjuvant. The emulsification and injection procedures followed were the same as for the initial immunization. Boosters were given every 2 wk thereafter, with a total of three booster immunizations spread out over a six-week period.

Blood was collected by bleeding from the external marginal veins of the ears into sterile plastic test tubes 2 wk after the last immunization. Additional blood collections were carried out every 2 wk until the antibody titer dropped enough to necessitate subsequent booster immunizations. Blood collected from the same rabbit was pooled and allowed to clot. The clot was rimmed off the walls of the test tube and allowed to shrink; then antiserum was separated from the clot. Antiserum was decanted from the tubes into sterile centrifugation tubes.

The antiserum was centrifuged at 1700 × g 30 min was performed in a 5°C coldroom to remove additional red blood cells. The antiserum was decanted into test tubes and then heated at 56°C for 30 min. It was allowed to cool in a 5°C coldroom and centrifuged at 2500 × g for 30 min to remove the bulk of nonantibody proteins and fat from the antiserum. This treatment increases the concentration of antibody present in the antiserum because it denatures complement factors and contaminating proteins (Anon, 1986).

Antibody Assay

Agarose or Bactoagar (.5-1g) was suspended in 100 ml of PBS and placed in a boiling water bath until the solution became clear. The solution was then boiled for 3 min to completely melt the agar. The molten agarose or Bactoagar was poured into several plastic petri dishes to a thickness of 2 mm. The poured agarose or Bactoagar was allowed to solidify at room temperature. A central well and 2-6 equidistant peripheral wells were punched out of the gels.

Ouchterlony double immunodiffusion technique was used to assay for the presence of antibody in each antiserum (Ouchterlony and Nilsson 1973; Warr, 1982). The antiserum (20 µl) of the appropriate enzyme was applied to the center wells, and 20 µl portions of the enzyme diluted in PBS were applied to the outer wells. The gel was incubated at room temperature for 1 h and then transferred to a 5°C coldroom to develop overnight after which the gel was visually examined for precipitin band formation.

Partial Purification of Antibodies

The ion exchange chromatography purified enzyme fractions from the microbial milk clotting prparations were used to purify the respective antserum. Ion exchange chromatography purified enzyme was added to about 20 ml of rabbit antiserum and the mixture was allowed to stand for 4 h in a 5°C coldroom. The precipitate formed was recovered by centrifugation at 2500 × g for 30 min, washed with phosphate buffered saline and solubilized in 5 ml of .2 *M* acetate buffer at pH 3.5. It was then applied to a Sephadex G-100 gel filtration column (1 cm × 40 cm) to separate the antigen from the antibody using .2 *M* sodium acetate buffer at pH 3.5, containing .15 *M* NaCl as the separation buffer. Eluted peaks were pooled and immediately neutralized. Pooled peaks from several runs were diafiltered and concentrated in an Amicon ultrafiltration unit and assayed for enzyme and antibody activity. Alternatively, the eluted fractions were exhaustively dialyzed and then concentrated.

Cross Reactivity Test

Agarose or BactoAgar plates were prepared as described above. Ouchterlony double immunodiffusion technique was used to test for cross reactivity. Antiserum (20 µl) to *M. pusillus, M. miehei* or calf chymosin was applied to the center wells, and M. *miehei, M. pusillus* enzyme or calf chymosin dilutions in phosphate buffered saline pH 7 was applied to the peripheral wells. The gel was incubated at room temperature for 1 h and then transferred to a 5°C cold room to develop overnight after which the gel was visually examined for the presence of precipitin bands.

Purified antibodies and enzymes were also tested for cross reactivity using the same procedures as described above. Purified antibody (20 µl) to *M. pusillus* or *M. miehei* was applied to the center wells, and purified *M. miehei* and *M. pusillus* enzymes were applied to the peripheral wells. The gel was incubated at room temperature for 1 h and then transferred to 5°C coldroom to develop overnight after which the gel was visually examined for the presence of precipitin bands.

RESULTS AND DISCUSSIONS

Antibody Production and Assay

The width and intensity of the precipitin band produced by the reaction of antibody with antigen is used as an indication of the antibody titer present in the antiserum (Warr, 1982). Significant antibody titers were obtained 6 wk after the initial immunization doses. Booster immunizations increased and maintained the antibody titers throughout the antibody production time.

Ouchterlony double immunodiffusion test (Ouchterlony and Nilsson 1973) showed presence of antibodies in the antisera produced against *M. miehei, M. pusillus* milk clotting enzymes and chymosin. Visualization of the bands was better in Bactoagar than in agarose

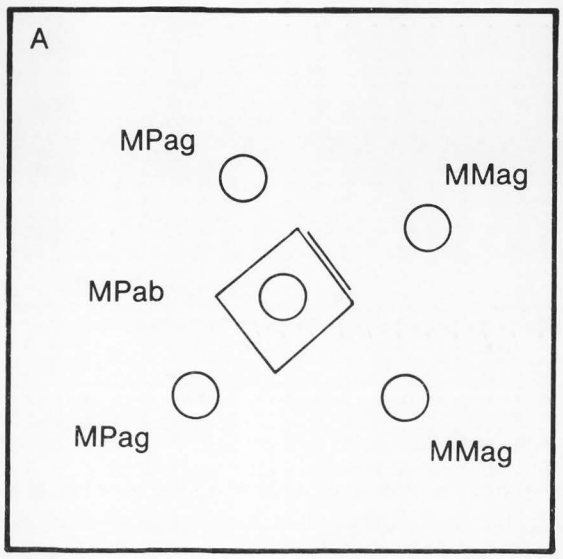
gels. Staining with comassie blue was used to better visualize the precipitin bands in the gels.

Cross Reactivity Test

Ouchterlony double immunodiffusion test (Ouchterlony and Nilsson 1973) was used to determine the existence of cross reactivity between antibodies produced against *M. miehei* and *M. pusillus* milk clotting enzymes as shown in Figure 13. Precipitin band formation in the assay with *M. miehei* and *M. pusillus* enzymes and their antisera demonstrated that there is an immunological relationship between these enzymes. The antibody raised against *M. miehei* reacted with *M. pusillus* antigen, and the antibody against *M. pusillus* reacted with *M. miehei* antigen. Precipitin bands were observed for both tests, indicating that the antibody raised against one enzyme was capable of recognizing and reacting with one or more antigenic determinants on the other enzyme.

Ouchterlony double immunodiffusion assay was also used to show lack of cross reactivity between the antibodies produced against the *Mucor* enzymes and calf rennet. Cross reactivity provides evidence for the existence of homology between different antigens (Kobayashi et al., 1973). The three dimensional conformations of immunogenic macromolecules play crucial roles in determining the specificities of the antibodies they produce (Maoz et al., 1973). Evidence of this is provided from the finding that antibodies against native proteins show little or no cross reactivity with denatured proteins (Maoz et al., 1973). Cross reactivity among these antibodies could be explained by the presence of shared antigenic determinants on the molecular structures of the milk clotting enzymes or similarities in the structural conformation of the enzymes. *M. miehei* and *M. pusillus* antibodies might also be exhibiting polyfunctional binding sites. The sharing of one or more germ-line DNA-encoded hypervariable regions present in the antibody combining sites by antibodies has been cited as the reason for cross reactivity (Hoffmann et al., 1985).

Figure 13. Ouchterlony double immunodiffusion assay showing cross reactivity between antibodies raised against *Mucor miehei* and *Mucor pusillus* milk clotting enzyme preparations.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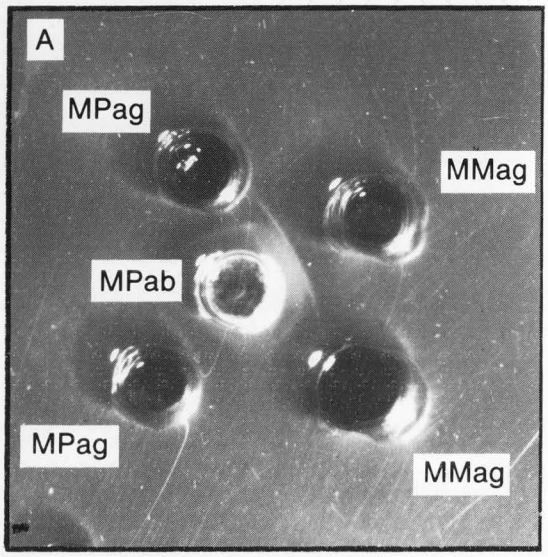
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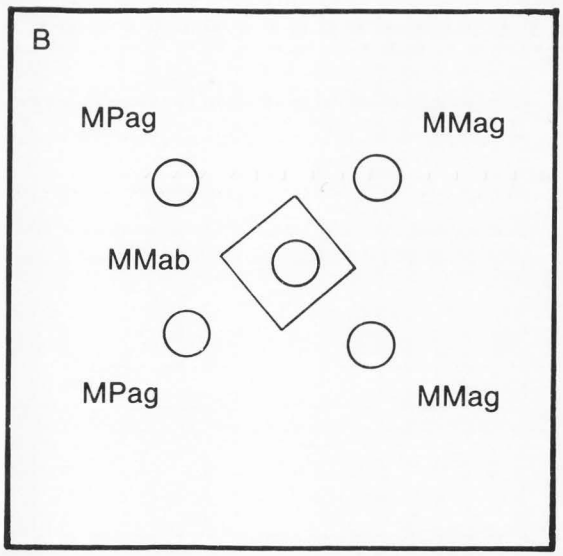
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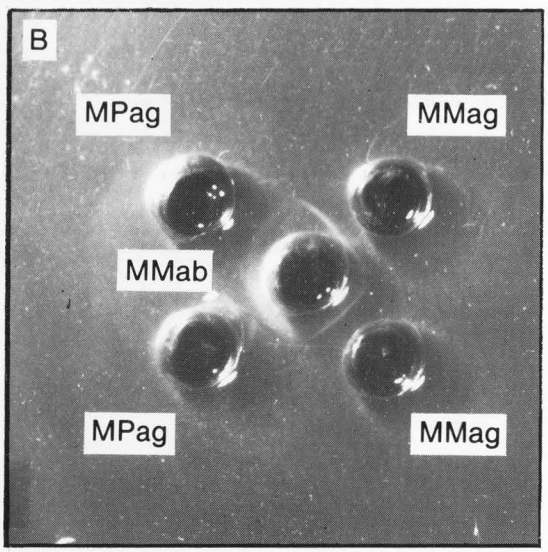
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CONCLUSIONS

Despite the differences in their action on milk proteins, we have shown in this study, that the enzymes from *M. miehei* and *M. pusillus* milk clotting preparations exhibit immunological cross reactivity. At least, one antigenic factor is common to both *Mucor* enzymes. Cross reactivity among these antibodies could be because of presence of shared antigenic determinants on the molecular structures of the milk clotting enzymes or similarities in structural conformation of the enzymes. *M. miehei* and *M. pusillus* antibodies might also be exhibiting polyfunctional binding sites.

It is reported in the literature that the *Mucor* enzymes and chymosin contain the same number of residues of phenylalanine, threonine and lysine (Sternberg, 1971). The lack of cross reactivity between *M. miehei* and *M. pusillus* enzymes with chymosin is an indication that these shared amino acid residues must not be present at the antigenic sites of these enzymes.

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GENERAL SUMMARY

Conventional and immunoaffinity chromatography protein purification worked better for *M. miehei* milk clotting preparation than for *M. pusillus* milk clotting preparation. The proteolysis assay indicates that the proteolytic activity and the milk clotting activity either copurify or must be contained on the same enzyme. Results of double immunodiffusion assays with *M. miehei* and *M. pusillus* enzymes and antisera directed against them demonstrated immunological cross reactivity between the *Mucor* enzymes. The antibody raised against one enzyme was capable of recognizing and reacting with one or more antigenic determinants on the other enzyme Figure 13. This can be explained by the presence of polyfunctional binding sites on the *M. miehei* and *M. pusillus* antibodies. Shared antigenic epitopes on the surfaces of the enzyme molecules may also contribute to the cross reactivity.

It is reported in the literature that the *Mucor* enzymes and chymosin contain the same number of residues of arginine, tyrosine and histidine (Sternberg, 1971), and that *M. miehei* enzyme and chymosin contain the same number of residues of phenylalanine, threonine and lysine (Sternberg, 1971). We did not find cross reactivity between the *Mucor* enzymes and calf chymosin. It must mean that the antigenic determinants do not include these shared amino acid residues.

Despite the differences in their action on milk proteins, the enzymes from *M. miehei* and *M. pusillus* exhibit some immunological homology. At least one antigenic factor is common to both of the *Mucor* enzymes.

Use of purified enzymes in the production of antibodies should increase the specificity of the antibodies. The use of purified proteins for immunization, should also eliminate the need to purify the antiserum before using it for immunoaffinity chromatography.

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1983-1986, member American Meat Science Association (AMSA)

Honors:

1983 Max Factor Certificate
1986 Martin Luther King Fellowship
1988 Martin Luther King Fellowship
1989 Summer Institute for African Agricultural Research Fellowship Rockefeller Foundation

Abstracts and Publications:

Bartholomew, D.T., Woodbury, B.L. and Osuala, C.I. 1984. Mutton in summer sausage: good taste and good cents. Utah Science. 45(4):122.

Bartholomew, D.T. and Osuala,C.I. 1986. Modifying lamb/ mutton flavors in processed meat products. J. Food Sci. 51(6):1560.

Bartholomew, D.T. and Osuala,C.I. 1988. Use of the InfraAlyzer in the proximate analysis of mutton. J. Food Sci. 53(2):379.

Osuala, C.I. and Brown, R.J. 1988. Cross reactivity of antibodies produced against Mucor miehei and Mucor pusilus milk clotting enzymes. J. Dairy Sci. (Suppl. 1) 71:182 (Abstract).

Osuala, C.I. and Brown, R.J. 1989. Purification of Mucor miehei and Mucor pusillus milk clotting enzymes by immunoaffinity chromatography. J. Dairy Sci. (Suppl. 1) 72:92 (Abstract).

Personal Data:

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