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Enzyme-Hydrolyzed Retentate for the Development of a Whipped topping Mix

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ENZYME-HYDROLYZED RETENTATE FOR THE DEVELOPMENT OF A WHIPPED TOPPING MIX

by

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Shirley Bond

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ABSTRACT

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by

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Ultrafiltered skim milk concentrated to 20-22% solids was hydrolyzed with a mixture of proteases from *Aspergillus oryzae* and an acid protease from *Aspergillus niger*. The enzyme preparation from *A. oryzae* did not produce bitterness, so the effects of its hydrolyzing action on the whipping properties of the retentate in liquid and powdered forms were examined. Its effect on the amount of soluble nitrogen in the powder was also determined. After inoculation of 250 ml retentate with .05% w/v enzyme, the samples were held in a 45°C water bath. The samples thickened with increasing hydrolysis until syneresis occurred. They also took on a slightly ropy or slimy appearance after thickening and before syneresis. Hydrolysis was stopped by removing from the 45°C bath and holding in an 80°C bath for 35 minutes.

When cooled to refrigerator temperature, retentate samples produced a stable whip with fine bubbles and firm but soft peaks. Air capacity of whipped samples decreased with increasing hydrolysis time and retentate solids. Stability increased with increasing solids and longer hydrolysis times.

Skim milk retentate to be dried was concentrated to 20-24% solids and reconstituted to 20% before whipping. Hydrolysis time up to 20 minutes before enzyme denaturation did not have an effect on the air capacity of the whipped reconstituted powder. Air capacity decreased as the retentate solids increased. Whip stability increased slightly then decreased with increasing hydrolysis. Whip stability was impaired by protein denaturation in the powder during the drying process.

Soluble nitrogen in the powder, as determined by the Harland-Ashworth procedure, increased with increasing hydrolysis and decreased with increasing solids.

(93 pages)

INTRODUCTION AND STATEMENT OF THE PROBLEM

Current market trends show a demand for the use of dairy proteins in foods for their nutritional and functional value. However, there are some population groups that lack the β-galactosidase enzyme and cannot tolerate the lactose in milk, regardless of its nutritional value.

Researchers of new food product formulations are trying to satisfy the consumer demands for less fat and fewer additives. Industrial demand calls for more proteinaceous products with specific and practical functional properties such as whipping or emulsion capabilities.

Purpose and Objectives

1. To determine an appropriate set of time, enzyme, and substrate concentration parameters for hydrolysis of ultrafiltered retentate to obtain acceptable foaming characteristics from the retentate powder. This would identify a treatment producing the best combination of air capacity and whip stability.
2. To evaluate the effects of enzyme hydrolysis on the soluble protein nitrogen of the retentate powder as determined by the undenatured whey protein nitrogen index (WPN).
3. To examine the samples for bitterness from enzyme hydrolysis.

Hypotheses

Enzyme treatment of milk proteins produces hydrolysates with improved whipping properties. When skim milk proteins are obtained from ultrafiltered retentate, the resulting product contains less lactose and more protein than a product concentrated through evaporation.

Enzyme-hydrolyzed retentate, both in liquid and powder form, might be able to perform well, with its functional and nutritional properties, as a base in whipped topping formulations and possibly in other dairy products as well. The general objective of this work is to investigate the whipping properties of enzyme-hydrolyzed retentate.

LITERATURE REVIEW

Protein Functionality

Protein functionality has been defined as "the physiochemical behavior which proteins exhibit while interacting with other constituents of multicomponent food systems." (60, pg. 275). This is based on the chemistry of proteins, which are composed of long chains of amino acid residues covalently bonded together. The chains are arranged in many different configurations and may associate with each other or with other chains through covalent or noncovalent bonding (60).

The attributes of the final product reflect the interactions between the properties of the protein, other food

components and their environment in the food system (30). Functionality varies with the source and concentration of the proteins, methods of isolation, processing, and modification. The functionality of a mixture may only reflect one or two components and not the total mixture (29).

There are no standardized methods for the evaluation of functional properties in foods. Researchers have used model systems that fit their own needs and equipment (56).

Foaming. Both air capacity and foam stability have been used to evaluate the foaming properties of food components. Air capacity is considered to be the volume increase from the incorporation of air into a protein solution (29). One of the methods used to indicate air capacity in foaming and whipping experiments has been to measure the expansion in volume of 100 ml after whipping (42,62). Gunther (21) measures air capacity as density of the whip, with decreasing density indicating more air held. Hansen and Black (23) and Kuehler and Stine (32) report air capacity as percent overrun.

Foam stability indicates the ability of a foam to maintain its volume over time and is indicated by the leakage rate of fluid from the foam and the accompanying volume decrease (29). There have been a number of different methods used to evaluate foam stability. Puski (62) has reported it to be the residual foam volume remaining after standing for thirty minutes with larger volume indicating greater stability. Another method has been to measure the amount of

liquid drained in a given amount of time from a foam placed in a funnel in a graduated cylinder (23,42).

Foams are made of gas (usually air) bubbles within a liquid that contains a surfactant which lowers the surface tension in the liquid, allowing expansion of its surface area. The surfactant should be soluble in the liquid phase and able to quickly diffuse to the gas-liquid interface (29,31).

Protein foams are formed in three stages. The first stage involves protein migration to the air-water interface where they concentrate and reduce surface tension. A protein must diffuse rapidly to the surface, reorient itself, and form a viscous film without aggregation and coagulation (31). During foam formation, surface-denatured protein is adsorbed at the air-water interface (11). A surface-denatured protein's conformation has changed from its native state by unfolding to conform to the interface.

Secondly, polypeptides unfold and reorient so the hydrophilic portions are in the liquid phase (31), and the hydrophobic portions are towards each other as shown in Figure 1. Air is trapped forming bubbles in the interface between hydrophobic portions of the surfactant (11).

Finally, protein-protein interactions form a stable film. Surface denaturation and coagulation may occur (15,31) as the film is stretched at the interface. There, more protein is adsorbed to help stabilize the film (11).

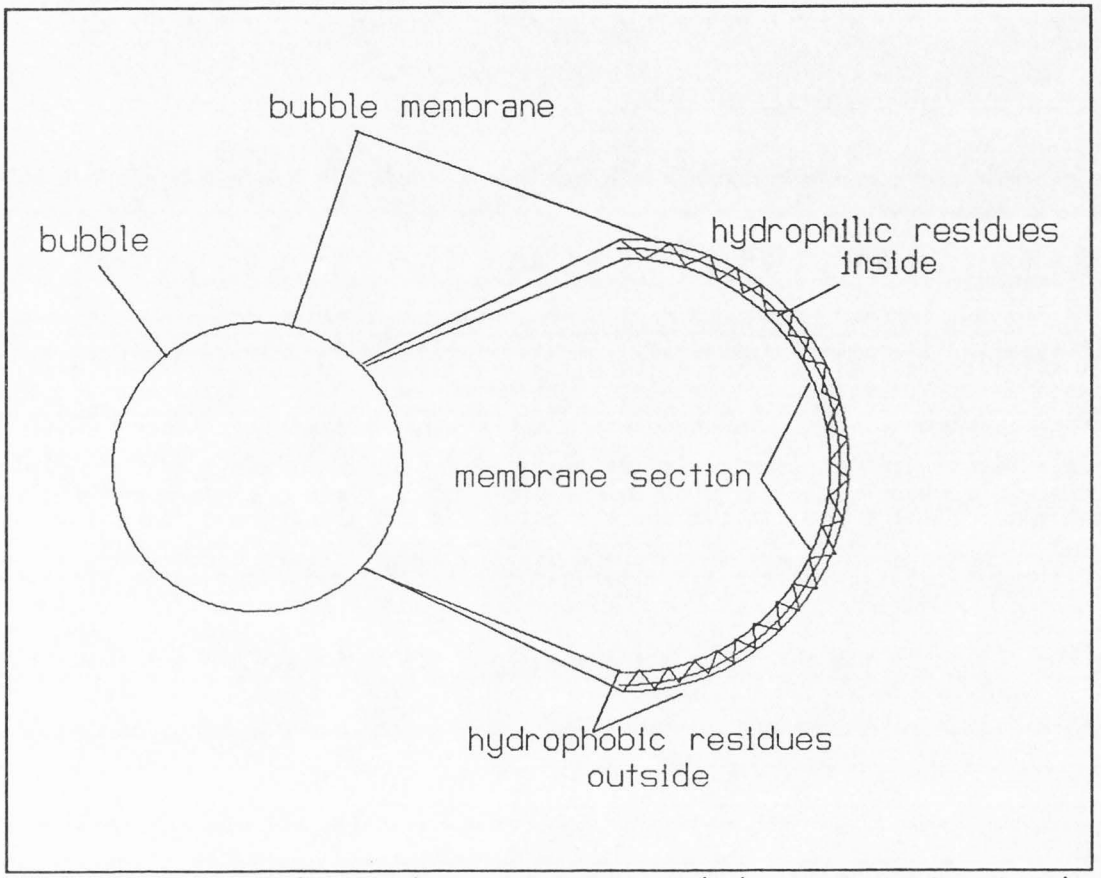

Figure 1. Orientation of hydrophilic and hydrophobic residues in a bubble membrane.

To form foams, proteins not only reduce surface and interfacial tension but also form membranous structures around air bubbles (29) as shown in Figure 2. The bubbles must be in the liquid phase long enough to obtain a stable interfacial film to prevent bubble coalescence (31). Surface-active proteins are able to encapsulate air droplets by surrounding the droplets with protein layers having enough mechanical strength to prevent coalescence (29).

Film on adjacent bubbles contact each other, trapping liquid and helping to prevent drainage (11). An elastic film aids in adapting to volume changes (29). The deformable

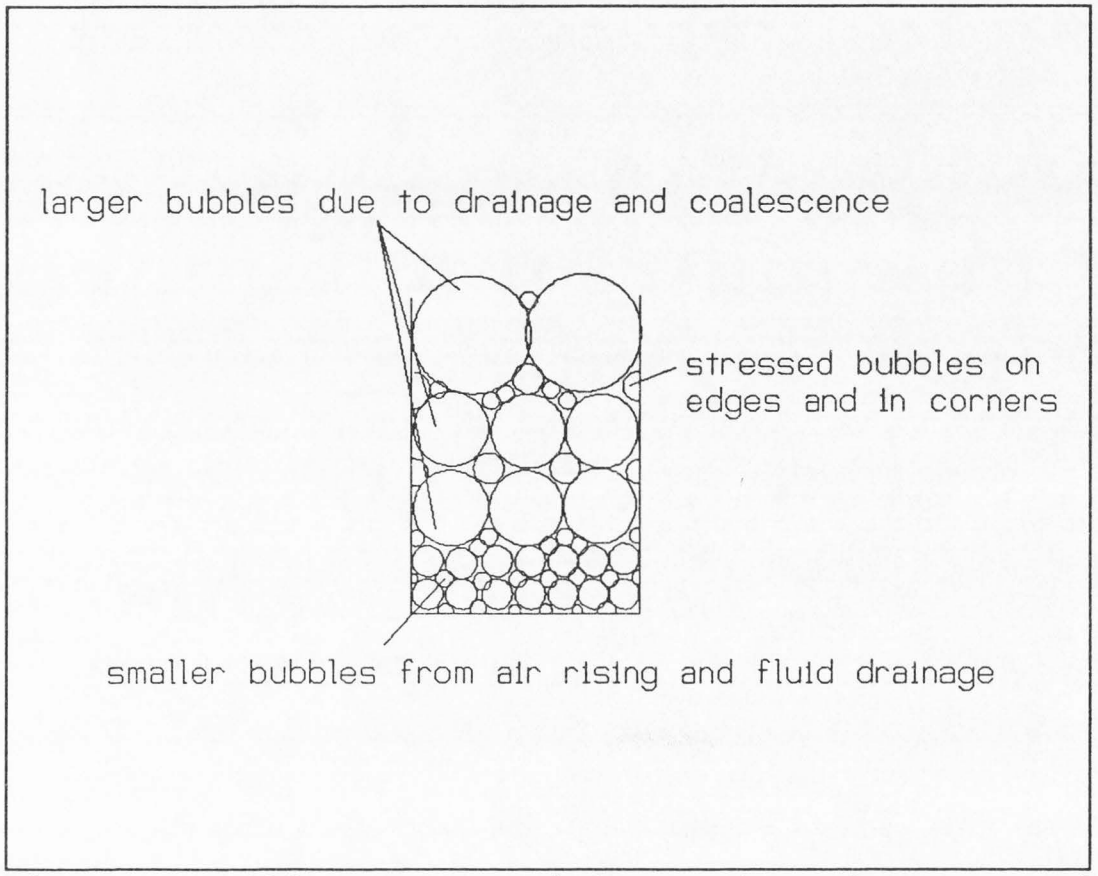

Figure 2. Bubbles; corner reinforcement, coalescence, and drainage.

interfacial film should resist thinning, which results from repulsion of surfactant between adjacent film layers (31).

Prolonged whipping gives smaller bubbles that are less stable. As the surface area increases from smaller bubbles, the need for interfacial material increases. So, the membrane can weaken, leak, and collapse if the surfactant protein is limited (29).

Without a surfactant, air bubbles will burst because of the high surface tension of water. The surfactant must be able to adapt to and reinforce stressed areas having thinner and weaker walls. A viscous fluid film allows for expansion

and compression due to stress and will flow into and reinforce stressed areas (31).

Foams drainage occurs due to surface tension and gravity flow and is regulated by the viscosity of the interfacial fluid. This contributes greatly to foam stability, as films that are viscous and elastic are more stable than rigid brittle films (11,31).

Emulsions. An emulsifier is able to lower the interfacial tension between the oil and water phases of a food. Emulsions consist of fat droplets surrounded by a protein membrane matrix (15,29). Emulsion capacity is the amount of oil emulsified by a protein before the system changes from an oil-in-water to a water-in-oil system (56). Emulsion stability is the ability of the emulsion to remain unseparated over time (29) and requires an unbalanced rather than an even distribution of hydrophobic and hydrophilic amino acids (48).

A higher number of molecules with smaller sizes may increase emulsion capacity but make a thinner layer around the oil droplets, decreasing stability (60). Stability is given when the continuous and dispersed phases are prevented from coalescing and is dependent on the balance between attractive and repulsive forces (45). Protein solubility is necessary so the protein will not be aggregated from denaturation and will be able to orient at interfaces (56). The hydrophilic amino acids turn to the aqueous phase, and the hydrophobic, to the

oil phase. Protein flexibility is important to unfold and expose hydrophobic areas to the oil phase. Molecules undergo surface denaturation during film formation at the interface (3). Free energy is reduced, contributing to stability. Breakdown comes from coalescence, flocculation, and separation of the two phases (45).

Gelation. Gels are three-dimensional networks of intertwining and partially associated proteins which trap water, carbohydrates, and flavors and have a high viscosity and elasticity. Pre-heating the protein causes partial denaturation and chain unfolding. Denatured gels occur when a balance between attractive and repulsive forces is reached (56,29).

In the first stage of heat gelation of whey proteins, they partially denature and unfold. Higher temperatures lead to firm gels (29,24). During the second stage, polypeptides associate through various types of bond formations into a matrix. Cooling during the second stage is required and also affects gel characteristics (29,56).

Water Binding. Bound water is considered that which binds and remains after filtration and centrifugation. Protein molecules are surrounded by several layers of water, making a hydration shell. The inner layers are more tightly bound than the outer layers (29). Proteins bind water through hydrogen bonds between water and polar groups on the polypeptide chain. The amount of water bound is affected by

the number and type of polar groups on the chain. Protein conformation affects the nature and availability of these polar sites (56). Undenatured and highly soluble proteins are not expected to bind water well. Absorption is dependent on protein source and concentration (29).

Viscosity. Swelling is the first step in water solvation and is the expansion due to the spontaneous uptake of a solvent by a protein (24). When there is insufficient water to dissolve, proteins swell and give body and thickening to the mixture. As proteins absorb water and swell, the system thickens and viscosity increases and can be measured with a viscometer (29,56). When swelling reaches its maximum and then decreases, the molecule is able to absorb water for solvation and lowered viscosity (24). Nonswelling soluble proteins such as albumins and globulins have low viscosity. When proteins have an initially high amount of swelling, their viscosity is concentration dependent. Proteins have high viscosity at lower concentrations if they have a limited swelling capacity (29).

Solubility. A protein's ability to rehydrate is dependent on the changes occurring during dehydration. As the protein macrostructure is altered during drying, the number of sites available for rehydration is limited due to protein interaction and alteration or reduction of the available surface area (45). Solubility helps determine the optimum conditions for protein extraction from its natural source

because solubility is an indication of the limitations and functionality of a protein in a given environment (29).

Modification of Milk Proteins

Modification methods are used to improve the functionality of a protein by intentionally changing protein structure and physical properties through physical, chemical, or enzymatic methods. These alterations may also change the protein's flavor or color but ideally not the nutritional value (30). Heat is used to denature whey proteins and complex them to casein micelles. Mineral composition is adjusted to control protein coagulation and other functional properties. Fractionation utilizes individual proteins, and enzymes are used to change the molecular structure and composition (53).

Fractionation. Milk proteins are fractionated based on their physiochemical properties. The fractions meet the general standards for food protein ingredients and are used in food formulations (53). Milk protein products are available in several forms, e.g., acid, lactic and rennet caseins, Na/Ca/K caseinates, casein/whey protein coprecipitates, and whey protein concentrates. Each product has its own characteristic functional properties (50).

Lactic casein is produced by fermentation to reach the isoelectric point and subsequent precipitation by *S. lactis* or *S. cremoris* (50). Alternatively, hydrochloric acid or sulfuric acid can be added for direct acidification. Acid

casein is insoluble but is still useful where dispersibility is more important than solubility (45,56).

Rennet casein has a different functionality than acid casein. The micelles are destabilized by rennet hydrolysis to induce coagulation, then washed and dried (53,56). Originally used in plastic manufacture for its elasticity, rennet casein is also used in food formulations, especially in cheese analogues (56).

Caseinates are acid-precipitated and neutralized by the addition of Na/K/NH_4/Ca hydroxide to the wet curd. This helps resolubilize the curd by altering the molecular charge enough to overcome hydrophobic bonding between molecules (49,50,53). Na/K/NH_4 caseinates are soluble above pH 5.5 while calcium caseinate forms a colloid, absorbing less water than Na caseinate because calcium is larger, divalent, and has stronger crosslinks with proteins (49,50,52).

Caseins and caseinates are heat stable and have good foaming and emulsifying properties because of their amphiphilic conformation and ordered aggregate structures, allowing them to rapidly migrate to interfaces. Na/K caseinates are good gelling agents and thickeners, are excellent water and fat binders, and have higher viscosity at higher temperatures (49,50,52).

Whey protein concentrates (WPC) are prepared from the whey proteins in skim milk, whole milk, cheese whey, and butter-milk. When whey proteins are denatured and partially

aggregated, emulsifying, gelling, and foaming properties decrease (59).

Processes to recover whey proteins have been developed in an effort to obtain them in their native and functional state (48,51,53). They must be concentrated and isolated by more expensive means than the caseins. Some processes used are ultrafiltration, reverse osmosis, and electrodialysis (50,53). Care must be taken at all steps to control heating, agitation, and pH to avoid protein denaturation which causes large molecular weight aggregates from protein-protein interactions, increased turbidity, and decreased solubility (48).

Functionality varies depending on the whey source and treatment history, differences in amount of lactose, salt, lipid and mineral removal, and denaturation. Whey protein concentrates have good solubility and functionality but do not function as well as caseins in some systems in stabilizing foams and emulsions because of their compact globular conformation that has fewer amphiphilic properties. Mild heat treatment, immediately before whipping, helps unfold the molecules for foam formation by exposing hydrophobic residues. Undenatured whey proteins are soluble in the acid pH range and thus can be used to fortify acidic beverages (49,50,51).

Heat-precipitated whey proteins have increased water binding which enables their use in meat formulations and for softer cheese curd. Whey protein solutions having the right concentration form heat-induced irreversible gels as

increasing the concentration affects protein-protein interactions and hydrophobic and ionic bonding (51).

When skim milk is heated, whey proteins complex with casein through disulfide links. Acidifying to the isoelectric point causes precipitation of the complex. The amount of calcium in the coprecipitate is determined by the manufacturing process used (45,50). High-calcium coprecipitates have a viscosity similar to rennet casein while medium- to low-calcium coprecipitates have viscosity similar to Na caseinate (65). The solubility increases as pH increases, but the coprecipitates are not totally soluble because of the insoluble, denatured whey proteins. Coprecipitates can partially solubilize by adding NaOH or Na-tripolyphosphate and resemble Na caseinate with insoluble lactalbumin added. Milk proteins and stabilizers can be added to the milk prior to precipitation for more product variation (45,50).

The coprecipitates are good surfactants for foaming and emulsifying and can be used as meat extenders and meat replacers for their texture qualities and have high water-binding capacity, viscosity, and gel strength (65).

Calcium and Ionic Complexes. Increased calcium, CaOH, and calcium sequestering agents (33) aid foaming and emulsion properties by the increased charge from the cations. Caseins bind calcium, reducing molecular charge and permitting association and aggregation (52). Calcium forms bridges between carboxyl groups on proteins at interfaces and reduces

electrostatic repulsion between adsorbed and adsorbing protein layers (59).

Calcium influences hydrophobic and ionic bonding, aiding the formation of stronger gels. The addition of calcium to WPC leads to heat-induced coagulation at all pH's, not just near the isoelectric point (51).

High-calcium coprecipitates and rennet casein have similar amounts of calcium and similar high viscosity, but acid coprecipitates and sodium caseinate have lower viscosity (65). The differences in viscosity are attributed to variations in calcium concentration (54).

Heat Treatments. Ultra-high temperature pasteurization, forewarming, evaporation, drying, and the pretreatment for coprecipitates all cause chemical and physiochemical reactions in milk and whey (54). Milk is relatively heat stable; however, its stability is affected by pH, with the optimum pH at 6.7. The heat coagulation time at that pH is affected by concentration of κ-casein, whey proteins, calcium, and phosphate. Heat stability is increased by calcium chelators (20).

During heating, other changes occur in the milk, including acid production, whey protein denaturation and interaction with casein micelles; dephosphorylation and hydrolysis of casein; and Maillard browning. Heat-induced acid formation is the biggest factor leading to heat coagulation of milk (20).

At temperatures near destabilization, the stabilizing forces are counterbalanced by the destabilizing forces, causing the molecule to unfold. At this point, depending on conditions present, the protein may return to its native state or polymerize and aggregate to an irreversibly denatured state (13).

β-casein is relatively hydrophobic, making it temperature sensitive. When fresh milk is cooled to 0-5°C, calcium and β-casein diffuse from the micelles (8). This is reversed when the milk is warmed to 28°C, causing changes in the micelles. Soft gelatinous micelles, easily dispersed in buffer, are found at 0-5°C, while micelles from 35-40°C milk are brittle and do not disperse in buffer as easily (47).

β-lactoglobulin is an important factor in the heat stability of milk. It complexes with κ-casein by disulfide linkages. Micelle properties change as a result of the β-lactoglobulin-κ-casein complex (54). Properties of casein micelles change when whey proteins bind to micelles during heat treatment (13,47). The micelles are less sensitive to changes in pH and calcium ion concentrations, affecting micelle stability at higher temperatures, and stabilizing the milk protein system (47).

Above 100°C, β-lactoglobulin complexes with other denatured whey protein molecules through calcium and disulfide linkages. These whey protein aggregates then react with casein micelles by calcium cross-linkages causing micelle

destabilization and polymerization. This destabilizes the milk protein system (47).

Whey proteins are globular, compact, and soluble at all pH's and do not associate with caseins in their native state. These two properties change upon denaturation, which occurs at temperatures above 60°C. The globular conformation unfolds to a random conformation. Whey proteins have sulfur containing amino acids and undergo disulfide crosslinkings with themselves and with caseins in the presence of heat (47).

α-lactalbumin denatures at 38°C and is least heat stable of the whey proteins but requires more heat per gram to unfold. β-lactoglobulin begins thermal denaturation and unfolding at 70°C. Within a short time period, up to that temperature, its unfolding is reversible. At 130°C, residual protein structure unfolds (13). Serum albumin denatures at 64°C and is the most easily denatured of the whey proteins (14).

During heat treatment β-lactoglobulin and bovine serum albumin influence aggregation through thiol/disulfide interchanges and oxidation/reduction reactions. α-lactalbumin is the most heat resistant, but its resistance is affected by sulfhydryl/disulfide interactions. It has been shown that the more numerous β-lactoglobulins govern the thermal behavior of the whey proteins. The immunoglobins are the most heat labile (13).

Whey protein denaturation from heat treatment of skim milk decreases as the total solids increases at temperatures

of 75-80°C (41). During heat treatment, the number of particles is important as the kinetics and specific surface area are correlated with particle number (41,47).

Denaturation can be determined by the Harland-Ashworth procedure as modified by Kuramoto et al. (34), which measures the solubility at pH 4.6-5.0. While whey proteins in skim milk at 90°C undergo aggregation, there is less aggregation at 146°C and 16 seconds than at 90°C for 10-30 minutes (47).

The Harland-Ashworth procedure has been standardized and is the basis of the method recommended by the American Dry Milk Institute. It is used routinely worldwide to classify milk powders based on the heat treatment received. This is an indirect method since it measures the amount of undenatured whey proteins. There is no method to directly measure denatured whey proteins (58).

The development of heat classifications for NDM powder resulted from specialized commercial uses of skim milk powders. These categories are based on the amount of undenatured whey protein per gram of powder: High heat has <1.5 mg/g, medium heat 1.5 to 5.9 mg/g and low heat ≥ 6.0 mg/g (45).

Low heat powder is used where high solubility and low whey protein denaturation are desired, such as in yogurt and renneted cheeses. Medium heat powder has good surfactant and water sorption properties. It is used in sour cream, ice cream, and frozen desserts. High heat powder has limited

dispersibility and solubility so is used in structured foods such as candy, comminuted meat products, and baked goods (45). The high heat treatment has eliminated milk's bread loaf-depressant factors, which lead to lower-volume bread loaves, making high heat powder desirable for use in breadmaking (45,66).

Although the caseins are heat stable, the whey proteins are heat labile and undergo heat induced denaturation. When the κ-casein-β-lactoglobulin complex forms, a new protein system is established (66). High heat treatment affects the degree of hydration and dispersion of reconstituted nonfat dry milk (NDM). The whey protein-casein micelle complexes in high heat NDM are poorly resolubilized when reconstituted, so the water of hydration in increased, but they are heat stable. Conversely, the whey proteins in low heat powder are undenatured so the powder is more readily solubilized (45). High heat NDM has high water absorption, which can be raised by increasing heat treatment and/or solids content in the pre-dried concentrate (66).

Although normal high\temperature\short\time pasteur-ization and spray drying conditions do not denature whey proteins, denaturation will occur upon prolonged holding at those temperatures. Denaturation at different stages of manufacture is cumulative and may vary with interacting effects. Protein-protein interactions due to preheating original milk before evaporation affects the degree of whey

protein denaturation (58). Yousif (70) and Dargan and Savello (12) found that whey protein denaturation in heated ultrafiltered milk increased as the solids increased.

In making rennet casein, the denatured whey protein-micelle complex inhibits rennet aggregation because κ-casein is not as susceptible to the enzyme due to the presence and interaction of β-lactoglobulin at disulfide groups (54).

Heat treatment causing complete denaturation impairs foaming in whey protein concentrates and leads to more protein aggregation, especially around the isoelectric point. Milder heat treatment that causes partial denaturation changes the protein/lipid/phospholipid complexes, hydration, and conformation to favor protein adsorption at interfaces (63).

Globular proteins can partially unfold to adsorb at interfaces, losing some tertiary and quaternary structure but keeping some secondary helix structure which is stable at interfaces. Some degree of thermal denaturation leads to more disorganization in the protein's macromolecular structure, so it is more surface active than in its untreated native state (15).

Denaturing whey proteins increases water of hydration and voluminosity, which can produce a whey protein gel with high viscosity (45,56).

Enzyme Treatments. Enzyme usage in foods is in three general categories: 1) as an integral part of a manufacturing process such as alcoholic beverage production or cheesemaking,

2) to improve product quality through meat tenderization or with lactose or protein hydrolysates, and 3) to improve the economics or technology of a process such as fruit juice extraction. There are several advantages to the use of enzymes. Enzyme specificity allows only the desired reaction to occur without changing other constituents. Enzymes are used in relatively small catalytic amounts and work under mild conditions. They are inactivated by relatively small changes in temperature or pH. Narrow pH activity ranges or substrate specificities can be disadvantages, but problems can be avoided with careful enzyme selection (19).

There are a variety of proteases used by industry, and each has its own specificity. Exoproteases cleave single amino acids off the end of a polypeptide chain while endoproteases attack in the middle of a chain. A protease's specificity is inversely proportional to the number of cleavages it makes in a chain. The amount of hydrolysis needed is dependent on the desired features of the end product. Hydrolysis may also be limited by the availability of the susceptible peptide bonds, as some may be buried inside a globular protein and not be accessible to the enzyme. Protein denaturation generally helps open up the tertiary structure of the protein to make the peptide bonds more accessible to the enzyme (7).

The most limiting feature of protein hydrolysis is bitterness, which has been attributed to hydrophobic amino

acids. These amino acids are normally on the inside of globular proteins so do not react with the tongue until being exposed through proteolysis (2).

The use of an exopeptidase from *Aspergillus oryzae* in combination with an endoproteinase on peptides has been shown to reduce bitterness. Other exopeptidases may be satisfactory if they have a specificity for hydrophobic amino acids (61). The use of aminopeptidase T to remove bitter flavors from hydrolyzed casein has been successful (43).

Extensive hydrolysis of casein leads to bitter peptides containing hydrophobic amino acids, but limited hydrolysis does not. Bitterness may be controlled through enzyme selection and by limiting hydrolysis (30,52) and is influenced by the substrate used (61).

Parameters for hydrolysis are the type and concentration of enzyme, concentration of substrate, temperature, pH, and time of the reaction (2,21). The degree of hydrolysis is defined as (the number of peptide bonds cleaved/the total number of peptide bonds) X 100%. Hydrolysis time decreases with increasing temperature until the enzyme is inactivated. The hydrolysis time is inversely proportional to the enzyme/substrate ratio (2).

In a hydrolysis reaction, water is also a reactant. Therefore, as the water percentage increases with decreasing solids, the reaction rate increases (Bob Beckerich, personal communication, Novo Industries, May 1991).

The most widespread application of enzymes in milk and dairy processing has been with the use of rennet and cheesemaking enzymes, which are widely used for curd formation and flavor development (9,19).

The theory behind the addition of proteases to accelerate cheese ripening is that the hydrolysis would provide more substrate for microbial enzymes that produce flavor precursors and compounds (17). Moskowitz and Noelck (55) discuss the technology of addition of proteolytic and lipolytic enzymes to curd during cheese manufacture.

Encapsulating enzymes, one of which was a neutral protease from *Bacillus subtilis*, into liposomes to be added to cheese milk is shown to be satisfactory for accelerated ripening (36), but Fernandez-Garcia, et al. (18) found the enzyme caused some bitterness. Law (35) and Law and Wigmore (37) also found the enzyme causes some bitterness but a stronger cheese flavor. These researchers also used the acid protease from the fungus *Aspergillus oryzae* and found more bitterness with it than the bacterial protease. Fedrick, et al. (17) used the neutral protease from *A. oryzae* to increase cheese ripening and also found bitterness but an increase in flavor development.

The hydrolysis of lactose with β-galactosidase is increasing for lactose-intolerant people (9). Enzymes have been used to resolubilize heat denatured cheese whey proteins.

The resulting high protein product can be a nutritional food ingredient (26,46).

Milk protein hydrolysates can be used as sources for a variety of meat flavors. A beef extract flavor is obtained from enzyme action during the fermentation of powdered milk by *Pseudomonas flourescens*. The flavor is also obtained from hydrolysis of yogurt by papain or trypsin (19).

The plastein reaction is often used by treating casein hydrolysates with α-chymotrypsin, resynthesizing new polypeptides from small peptides, making insoluble polymers with improved flavors (30,52).

Fatty acids have been esterified onto proteins to give surfactant properties (68). Caseins have been dephosphorylated by phosphatases to increase emulsion stability. Transglutaminase has been used to crosslink caseins (45) and whey proteins (1) into large polymers. Limited proteolysis of caseinates reduce viscosity to facilitate spray drying (20).

Enzymes have been used to hydrolyze fish and soy proteins to improve their whipping characteristics (2,21,42). Limited hydrolysis of WPC increases foaming capacity but decreases foam stability and emulsifying properties. The hydrolyzed proteins are able to incorporate more air but are too small to retain stability (32). Acid proteases are recommended for imparting excellent foaming and whipping characteristics (21,

and Mike Ernster, Excelpro, Inc., personal communication, Oct. 1990).

Acid fungal proteases attack nine bonds, mostly His-Leu, Gly-Phe, and Phe-Phe bonds in insulin. Alkaline fungal proteases vigorously break five bonds in insulin, primarily the Leu-Tyr, and Phe-Tyr bonds (9). While this is general information known about the action of fungal proteases on insulin, the action of these proteases on milk proteins is not known (Mike Ernster, Excelpro, Inc., personal communication, Nov. 1990).

Aspergillus oryzae and *A. niger*

Yamamoto (69) suggests that *A. oryzae* has three proteases, which have been designated as protease I for the alkaline, protease II for the neutral, and protease III for the acid protease and can be differentiated by activity at different pH values as shown in Figure 3.

The acid protease in the blend has a slight peak of 25% activity around pH 4-4.5, the neutral protease has a peak of 75% activity between pH 6 and pH 8, where the pH of milk fits, and the alkaline has a peak of 95% activity between pH 9 and pH 11.5. This is with an enzyme concentration of 15 units/ml, a substrate concentration of 0.5% Hammersten casein, a temperature of 30°C, and 10 minutes hydrolysis time (64).

As indicated in Figure 4, the activity of this enzyme mixture is 90% of its maximum at 55°C and pH 6 with the same

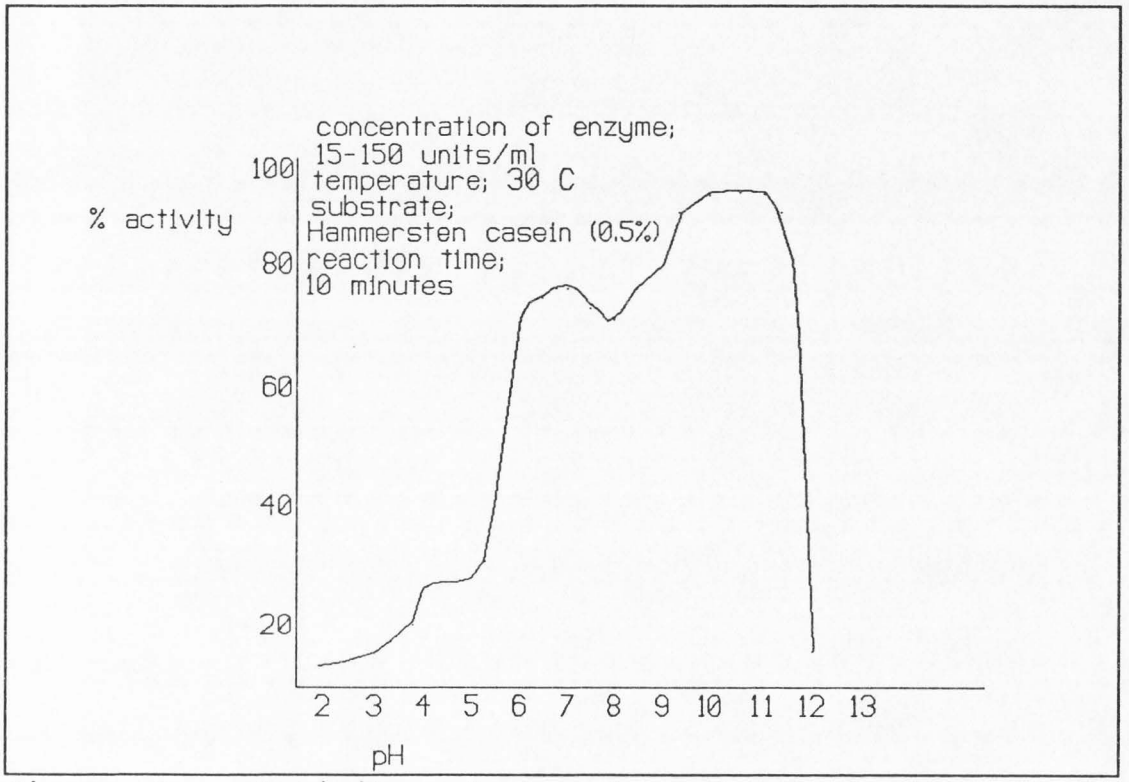

Figure 3. Activity of fungal protease concentrate at different pH values.

substrate, enzyme concentration, and reaction time as was used for the activity/pH curve in Figure 3 (64).

At 30°C and 25 units/ml, its stability is greatest between pH 5.5 and pH 8. With 25 units/ml, its stability is fairly constant at temperatures less than 45°C, with a sharp decrease in stability at temperatures above 45°C. It is more stable at pH 10 than pH 6 between temperatures of 25 to 55°C. Its effective pH range is fron pH 4.0 to 9.5 (64).

It is used on gluten to improve its elasticity for dough handling in breadmaking (64) and in attempts to modify the functional properties of soy proteins (62).

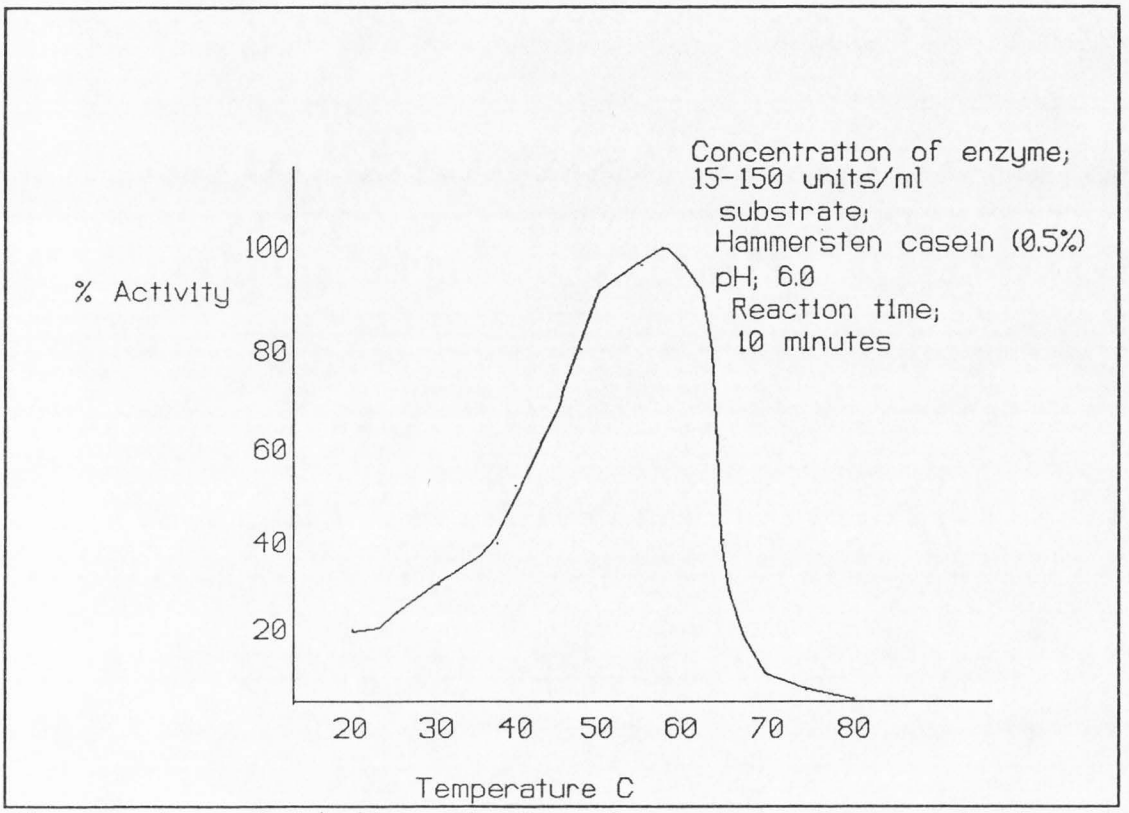

Figure 4. Activity of fungal protease concentrate at different temperatures.

Aspergillus niger has a broad substrate specificity and hydrolyzes most soluble proteins. The enzyme is an acid protease as it has an optimum activity at pH 3.0 and is stable between pH 3.0 and 5.0. Its optimum temperature range is between 50 and 60°C (64).

It also has been used in attempts to modify the functional properties of soy proteins (62).

Ultrafiltration

Ultrafiltration is designed to separate biological components in a liquid mixture. An ultrafiltration membrane is like a sieve that separates on a molecular level. When

milk is passed through such a membrane, water, salts, lactose, nonprotein nitrogen, free amino acids, and small polypeptides go through the membrane. This stream is called the permeate. The solids in the remaining stream, the retentate or concentrate, consist mostly of fat and protein. The retentate still has some of the permeate components. Milk that has had a third of its water removed by ultrafiltration also has had much of its salts, lactose, and other small molecules removed. A considerable amount of phosphorous and calcium is bound to the proteins, so is not all removed (71).

During ultrafiltration, a pressure difference is the driving force to push the permeate through the membrane and concentrate the retentate. Large molecules are retained and the smaller ones are transported with water. A diagram of solute movement is shown in Figure 5. Since most of the smaller molecules go in the permeate and the number of large molecules is relatively small, the osmotic pressure across the membrane is negligible (28).

A uniform pore size and high permeability are two of the features affecting the quality of separation. As ultrafiltration proceeds, the protein content in the retentate, volume of permeate, and viscosity of retentate increase (38).

Pressures between 0.1 and 1.0 MPa are used during ultrafiltration. An increase in pressure will also increase the permeate rate, up to a limiting pressure value. An

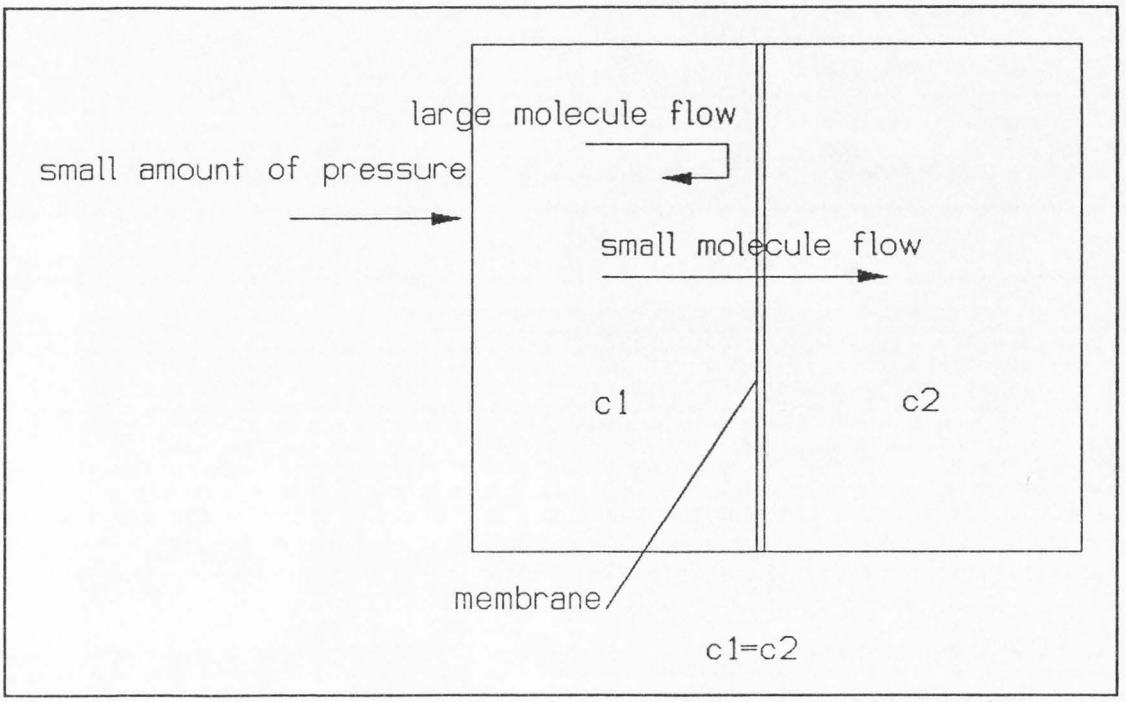

Figure 5. Solute concentration during ultrafiltration.

increase in operating temperature also increases permeate rate, while an increase in viscosity of the feedstock will decrease the permeate rate. An operating temperature between 5 and 45°C is not desirable from a microbiological standpoint (38).

Ultrafiltration minimizes whey protein denaturation during processing of whey protein concentrate, yielding a functional and nutritional protein source for various formulations. The use of ultrafiltered concentrate in final formulations is becoming more attractive as the cost of drying increases. Concentrates are used in soft and hard cheeses and yogurts (38).

Drying

Spray drying is used for a variety of products. During drying, the feed stock is atomized and water is evaporated from the droplets through contact with hot air. The powder is then separated from the drying medium. In a vertical dryer the spray nozzles are at the top or bottom of the drying chamber. The hot air inlets are at the top, bottom, or sides (5).

The atomized droplets and hot air flow in the same direction in a co-current flow system. The hottest air is in contact with the wettest droplets. The droplet temperature remains at the wet bulb temperature, minimizing heat denaturation. As the droplets are further dried and the hot air carries the powder further through the dryer, the air has cooled somewhat, lessening potential for heat damage (5).

In counter-current flow systems, the droplets and hot air flow in opposite directions. The driest particles are in contact with the hottest air so have more potential for heat damage. In a mixed-flow system, the hot air and spray droplets enter the drying chamber in paths at right angles to each other. Again, the particles are exposed to high temperatures and are more subject to heat damage (5).

Solids content and viscosity are among the most important characteristics of a feedstock that affect the drying process. Higher solids may generally increase particle density and bulk density. High viscosity feedstocks do not atomize as easily

or as finely as those with low viscosity. When the spray droplets are too large, they can cake on the walls of the drying chamber. A pre-heated feedstock usually has less viscosity and less nozzle clogging. A higher drying temperature decreases particle size because of faster evaporation. But, pre-heating that deaerates the feedstock may increase bulk density. A decreased outlet air temperature will increase bulk density and moisture of the powder (5).

Retentate Powder

As milk is ultrafiltered, many of the higher molecular weight particles kept in the retentate are proteins and fats while more of the lactose and minerals go through into the permeate. Retentate concentrated to 20-34% solids has a high number of protein and fat molecules compared to the concentration of minerals and lactose. The alteration in chemical composition is expected to affect the functionality of the retentate powder (27).

As total solids in whole milk retentate increase, the amount of fat and protein found in the retentate powder also increase (25,27).

Lactose and mineral percentages are lower in retentate powder (25,27). With less lactose, there is decreased lactose crystallization in the powder during storage (27). Although powder with less lactose has "flatter" flavor (25), low lactose retentate powder has been produced as an alternative

to enzymatic hydrolysis of lactose to make low lactose milk (16).

Foaming capacities of powders made with retentates of varying solids percentages increase as retentate solids increase (10,25). Solubility decreases as protein content in the powder increases (25). Water absorption and gelation increase with higher protein content in the powder (10).

Retentate that is heat treated before drying gives powder with increased water absorption but decreased whippability. Powders from heat treated retentate and used to make yogurt give it higher viscosity and less syneresis than powders from evaporated milk (10).

MATERIALS AND METHODS

Statistical Design

Randomized block designs were used for the retentate and powder samples. For the retentate, three solids levels and seven hydrolysis times were used. One hydrolysis time was for zero minutes hydrolysis and was the unhydrolyzed control. This gave a 3X7 randomized block, and was repeated in duplicate. A 3X4 randomized block was used for the powder, with three solids levels and four times of hydrolysis. Again, one time was for zero minutes hydrolysis and was the control. The powder samples were analyzed twice for duplication.

The results were analyzed using an analysis of variance by Minitab (44), a statistical package on Utah State

University's Vax mainframe computer. A Fisher's LSD test was used to test the difference between sample means.

Enzymes

A food-grade enzyme preparation extracted from *Aspergillus oryzae* was used and is a mixture of endo- and exo-peptidases, containing acid, neutral, and alkaline proteases. Its activity is 31,000 Hemoglobin Units (HU)/gram. One HU liberates 0.0447 mg. of non-protein nitrogen in 30 minutes under conditions of the assay (64).

The acid protease from *Aspergillus niger* was used in the initial stages of this project. The activity of the preparation is 2,000 Spectrophotometric Acid Protease Units (SAPU). One SAPU will liberate one micromole tyrosine per minute under conditions of the assay (64).

Both enzyme preparations have the lowest activity levels offered by the supplier, as only limited hydrolysis was wanted (Margie Mullin, personal communication, Genencor, Oct. 1990). In addition, the pH of milk is above the optimum pH for the second enzyme used, which would limit its activity somewhat, thereby limiting hydrolysis.

Ultrafiltration Membrane

The ultrafiltration membrane used is made by Osmonics, Inc., 5951 Clearwater Drive, Minnetonka, Minnesota 55343. Three Osmonics membranes were connected in a series and used on a module cart. They are made of SEPA-H polymer and have a

molecular weight cutoff at 20,000-30,000. Each membrane has a working surface area of 5 square meters, making a total of 15 square meters.

Dryer

The dryer used is a laboratory scale dryer supplied by Armfield, Ltd., Bridge House, West Street, Ringwood, Hampshire, England, BH24 1DY. Its schematics are shown in Figure 6.

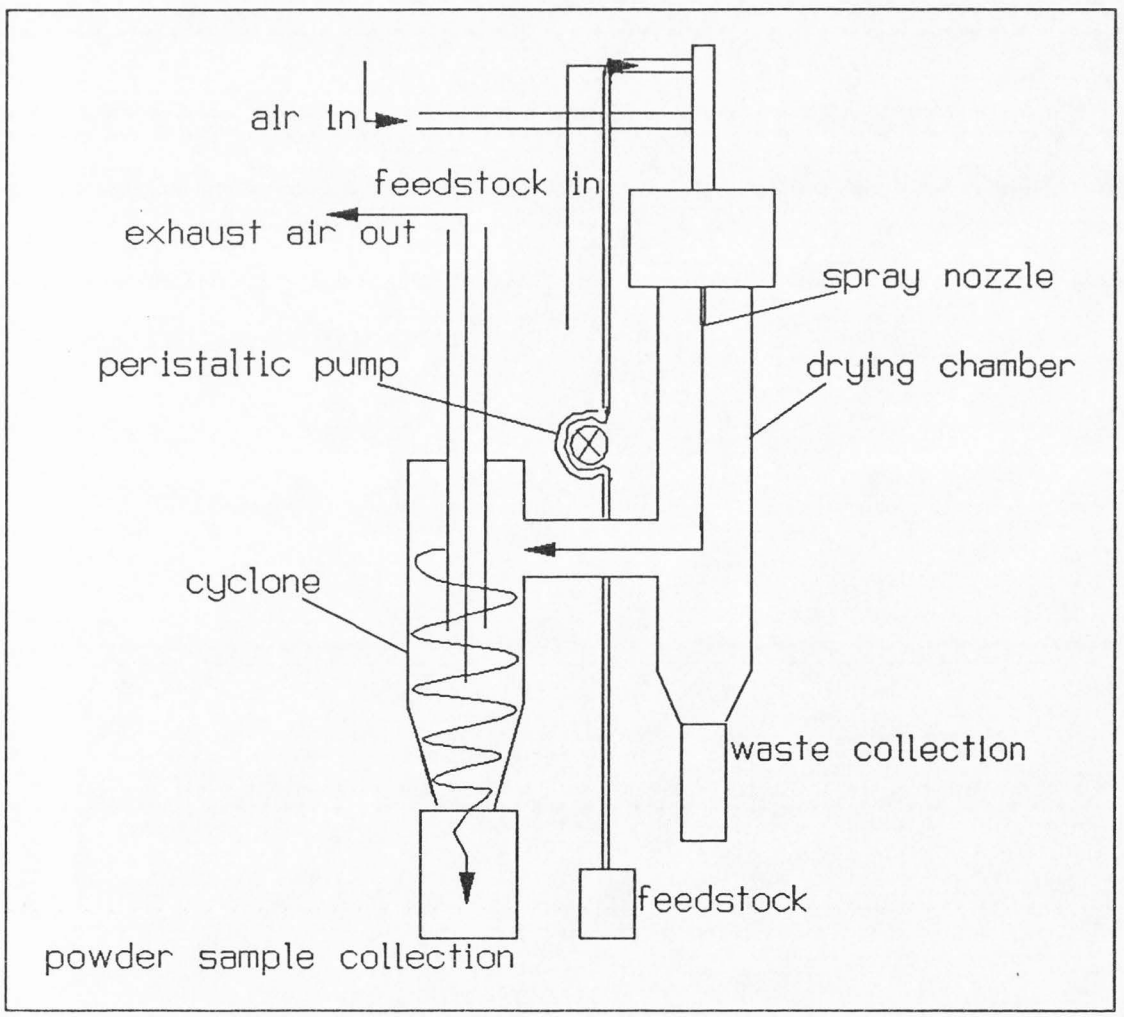

Figure 6. Dryer schematics.

The dryer has a flexible sample feed tube and uses a peristaltic pump. The feedstock and hot air enter the tower-type drying chamber at the top, in a co-current flow system. The powder is carried into a cyclone and then falls into a collection bottle as the hot air is exhausted to the outside. Inlet air temperature, air compression, and pump speed are controllable. Outlet air temperature is indirectly controlled by controlling the inlet temperature and pump speed.

Treatments

Ultrafiltration. Pasteurized skim milk was obtained from Utah State University's Caine Dairy Farm and later from MVM Wellsville, both in Wellsville, Utah. The temperature of the milk, held in ten gallon cans, was adjusted to 46°C in a vat. During ultrafiltration, the inlet pressure was 0.552 MPa and the outlet pressure was .345 MPa, giving a .207 MPa pressure differential across the membrane. The milk was concentrated to solids levels ranging from 18%-26%. The retentate was diluted to the desired solids levels with permeate. During ultrafiltration, the solids were approximated with a refractometer, made by Atago and supplied by VWR Scientific, Inc., while final determination was made by microwave analysis with a CEM AVC 80 microwave. The retentate was stored in a refrigerator until use.

Enzyme Hydrolysis. The retentate was divided into 250 ml portions, tempered to and held at 45°C, and treated with 1.0%,

.05%, and .01% w/v amounts of enzyme. The temperature was selected in accordance with the temperature stability curves provided by the supplier and with the suggestion of Mike Ernster (personal communication, Excelpro, Inc., Dec. 1990). To limit hydrolysis, the temperature used was not the enzyme's optimum, which would give 100% enzyme activity. The retentate samples were periodically stirred with a spoon. Visual observations were made on the appearance, viscosity and texture of the retentate. The time to reach syneresis was noted.

Based on initial results, .05% w/v was selected as the best enzyme concentration of the three. Samples of retentate were hydrolyzed at 45°C for 5, 10, 15, 20, 25, and 30 minutes before heat denaturing the enzyme. The samples were held in an 80°C water bath for 35 minutes to inactivate the enzyme. Retentate samples of 20%, 21%, and 22% solids were used. Untreated samples were used for controls. Samples to be whipped were put in the refrigerator to cool before whipping. Samples to be dried were dried immediately.

For the samples to be dried, solids concentrations in the retentate used were 20%, 22%, and 24%. Samples were treated for 10, 15, and 20 minutes before inactivating the enzyme. Unhydrolyzed samples were also dried for controls.

Enzyme Denaturation. A 250 ml sample of retentate at 45°C was inoculated with 0.05% enzyme. The retentate was then placed in an 80°C bath. After the temperature of the

retentate had reached 80°C, a sample was streaked with an inoculating loop every five minutes onto culture plates containing solidified Knox gelatin. The plates were held at room temperature for 24 hours and observed for gel softening due to enzyme hydrolysis of the gelatin. This method was used to determine the time at 80°C to denature the enzyme and was recommended by Mike Ernster (personal communication, Excelpro, Inc., Dec. 1990).

Drying. Approximately two to three liters of retentate were prepared for each sample of powder to be dried. The retentate was dried immediately after inactivating the enzyme to keep the viscosity at a minimum and to aid in the atomization of the feedstock. The nozzle diameter was 1.0 mm. The spray atomization air pressure was 0.412 MPa.

The inlet temperature of the dryer was 160°C and the outlet fluctuated between 85 and 95°C, depending on whether or not the nozzle was partially blocked or plugged by dried sample. The temperatures used were similar to those used at MVM Wellsville in the manufacture of spray-dried NDM and whey-soy protein hydrolysates (Lamar Jonsson, personal communication, NuSkin Products, April, 1991).

The powder was removed from the collection jar on the dryer and stored at room temperature in Whirl-pak bags.

Analyses

Air Capacity. The air capacity was determined by whipping 100 ml of retentate in a 600 ml beaker for five minutes and reported as density of g whip/ml (21). The refrigerated retentate samples were whipped at room temperature with an electric hand-held kitchen mixer by Black & Decker model M24S.

The powder samples were reconstituted to 20% w/w to make 100 ml and held in the refrigerator overnight. They were then whipped and tested for air capacity using the same method as that used with the retentate.

Whip Stability. Foam stability was determined using a variation of that used by Puski (62). Eighty mls of whipped sample were placed in a 100 ml beaker. The time for the top 20 ml of sample to coalesce and drain, leaving very large unstable bubbles was noted, reported, and compared among samples for stability measurements. This allowed, for those samples that collapsed within minutes of whipping, for comparison against those that held air for several hours.

Bitterness. The whipped samples were tasted by Dr. C. Hansen, S. Bond, S. Zhang, and J. Hansen. Because of the microbial load due to the processing and treatment temperatures, a formal taste panel was not done.

WPN Measurements. The modified Harland-Ashworth procedure was used as modified by Kuramoto et al. (34). The

WPN of the enzyme treated samples was compared with that of the unhydrolyzed samples.

Two grams of NDM were reconstituted in 20 ml distilled water in a 25 X 150 mm test tube. After adding 8 g NaCl, the tube was covered and placed in a 37°C water bath for 30 minutes. The tube was shaken eight to ten times during the first 15 minutes to completely saturate the sample with salt. Without cooling, the sample was then filtered through a Whatman No. 1 filter paper. A 1-ml aliquot was pipetted into 10 ml of saturated salt solution. The tube was inverted to mix, and two drops of 0.1 N HCl solution were added. After standing five to ten minutes, the absorbance at 420 nm was read. The spectrophotometer was adjusted to 100% transmittance using 1 ml of the original filtrate diluted with 10 ml saturated salt water.

A standard curve was prepared using 20 g of standard low-heat powder and 20 g of standard high-heat powder, supplied by the American Dairy Products Institute. Each 20 g sample was reconstituted with 200 mls distilled water and 80 g salt was added. The reference samples were incubated in a 37°C bath for 30 minutes, shaking eight to ten times during the first 15 minutes, and filtered. Proportions of the reference samples were pipetted into tubes as in Table 1.

The tubes containing combined filtrates were mixed. One ml aliquots were diluted and examined as previously described. The standard curve was plotted with the serum protein nitrogen

values per gram of powder on the vertical axis and the per cent transmittance on the horizontal axis (r^2 =1.00).

TABLE 1. Composition of tubes for WPN standard curve.

Tube No.	Low-Heat Filtrate	High-Heat Filtrate
	(ml)	
1	10	0
2	8	2
3	6	4
4	4	6
5	2	8
6	0	10

Chemical Composition. Three randomly selected samples were analyzed for fat, protein, and moisture. The fat and protein were analyzed according to methods of the AOAC (6). Moisture was tested following the procedure by Niro Atomizer (57). Protein analysis was done by analysing for total Kjeldahl nitrogen and using a conversion factor of 6.38 and was performed by Miller Laboratories, 3612 Lincoln Avenue, Ogden, Utah 84405.

RESULTS AND DISCUSSION

Retentate

Enzyme Hydrolysis. The action of the enzymes made the retentate thicken. The retentate initially looked like thick gravy. With more hydrolysis from the *A. oryzae* enzyme, the retentate began to resemble pudding, and eventually yogurt in thickness and body. The retentate looked like a soft yogurt gel with a slightly slimy appearance. Figure 7 shows the relationship between time and texture of the hydrolyzed retentate. For the relationship shown, the retentate was at 45°C and had 22% solids. An enzyme concentration of 0.05% w/v was used.

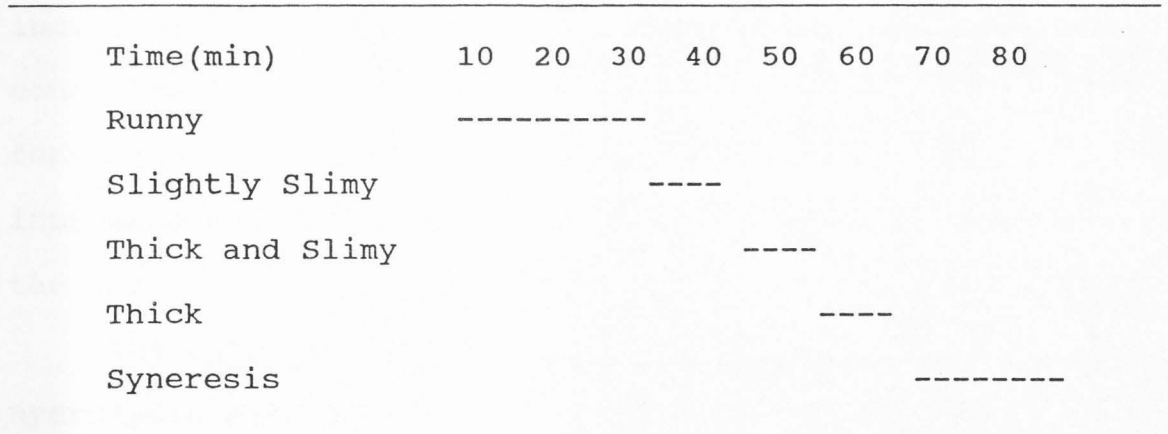

Figure 7. Hydrolysis time vs. texture appearance.

Slime, or ropiness, is caused in yogurt by the microbial production of mucopolysaccharides during fermentation. These polysaccharides get tangled up together and give the yogurt a slimy appearance. Another slimy food is egg white. Egg white does not have the mucopolysaccharides that yogurt has, but is

largely composed of protein. It also is the standard of comparison for whippability (29). The slightly slimy texture of the hydrolyzed retentate led to the belief that it might have good whipping properties.

The action of the *A. niger* enzyme only made the retentate thicken and did not produce a slimy appearance. Under the conditions of 45°C, 22% solids retentate, and 0.05% w/v enzyme, the retentate reached syneresis 30 minutes sooner.

Three enzyme concentrations were tried for both enzymes. The 1.0% w/v concentration gave too much hydrolysis too quickly. The retentate would have reached syneresis before enzyme inactivation in the hot water bath. The 0.1% w/v concentration gave no apparent change to the retentate after incubation at 45°C for over an hour. The 0.5% w/v concentration did not change the appearance of the retentate for almost an hour at 45°C. However, the rate of hydrolysis increased when the retentate was in the hot water bath until the enzyme was inactivated.

The objective at this point was to be able to stop the hydrolysis with *A. oryzae* enzyme at the "slime stage." This was accomplished. The retentate obtained and kept its slimy texture after the samples were put in the hot water bath to inactivate the enzyme after 10 to 15 minutes of hydrolysis at 45°C. The retentate samples reached syneresis after 35 minutes of hydrolysis before heat inactivation. Since there was no "slime stage" from hydrolysis from the *A. niger* enzyme,

there was no attempt to reach a desired degree of hydrolysis. The same time and temperature conditions were used with both enzymes.

Enzyme Denaturation. The temperature of 80°C was selected based on the information given by the supplier. The 250 ml samples of retentate took 20 minutes to reach the temperature of 80°C. Upon reaching 80°C, samples were streaked on gelatin plates every five minutes. After leaving the plates at room temperature for 24 hours, puddles of liquid gelatin were formed under samples streaked after five and ten minutes, but samples streaked after 15 minutes had no puddles. So, the total time to inactivate the enzyme was 35 minutes.

Only samples treated with the *A. oryzae* enzyme were used in the statistical design. The whipped samples developed soft but firm peaks. The results of analysis of variance tests on the whipped retentate are shown in Table 2.

TABLE 2. Retentate ANOVA levels of significance.

Source	Air Capacity	Whip Stability
	p Values	
Replication	.014	.532
Time of Hydrolysis	.015	.000
Retentate Solids	.000	.000
Time*Solids Interaction	.442	.006

Air Capacity. The whipped samples developed soft but firm peaks. The air capacity was reported as the whip density (grams whip/ml). The densities ranged from .25 to .575 grams/ml. Unwhipped samples had densities of 1.025 grams/ml. The samples were cooled in the refrigerator overnight before whipping because hot samples did not whip.

The variation in air capacity due to replication was significant at the .014 level of probability. The mean of one replication set was .37 grams while the mean of the other set was .333 grams. Forty ml samples were weighed, and there was a variation of ± 1 gram between duplications. The samples were whipped in a 600 ml beaker, and a portion was transferred to a 50 ml beaker for weighing. Thick samples were transferred with spoons. Sometimes, the samples would stick to the sides of the 50 ml beaker, and/or air pockets would form in the bottom of the beaker as the foam was being transferred. When trying to remove the air bubbles, extra sample would get on the sides of the beaker above the 40 ml mark. Any sample above the 40 ml mark affected its weight. These factors affected the weight of the sample. Whip densities at all hydrolysis times and averaged (n=6) for each solids level are shown in Figure 8.

In all graphs shown, the bars for zero time of hydrolysis are the controls. Those samples did not have any added enzyme. The variation in air capacity among all samples at all solids levels due to time of hydrolysis was significant

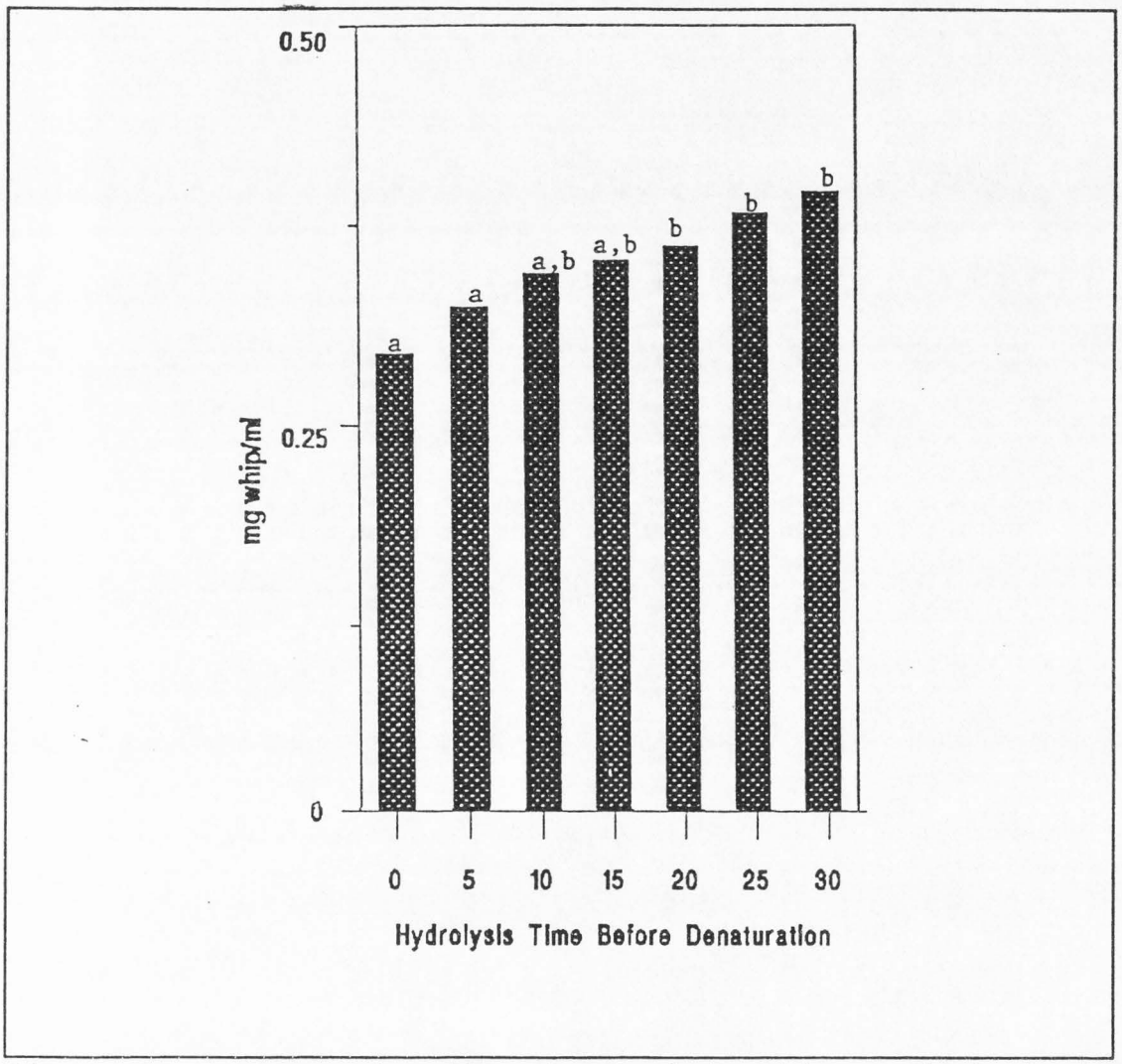

Figure 8. Average density of retentate whip vs. hydrolysis times at all solids levels. [a,b]Columns having the same letter are not different (P=.05).

(P=.015). There was a trend for increasing density with increasing hydrolysis. This indicated a decreased air capacity. There was not much difference between the average of the weights of 10, 15, and 20 minutes of hydrolysis, but there was an increase at 25 and 30 minutes.

This is in contrast to the findings of Gunther (21) and Kuehler and Stine (32), who used different substrates and

enzymes than those used in this project. Both researchers used pepsin, and Kuehler and Stine also used bacterial proteases.

They found that hydrolysis increased air capacity for hydrolyzed soy protein and whey protein concentrates, respectively. The smaller protein molecules resulting from hydrolysis were able to go quicker to the interface and form bubble membranes, increasing the air capacity of the solution (32). Possibly the increase in viscosity of the retentate due to hydrolysis decreased its air capacity.

The variation in whip density due to solids content and averaged (n=12) at all hydrolysis times is shown in Figure 9 and was significant ($P<.001$). There was no significant time vs. solids effect.

The samples at 22% solids and samples having incubation times longer than 20 minutes were thicker than the rest after hydrolysis and before whipping. They had not syneresed but looked like big semi-solid curd masses that resembled low quality yogurt having a firm body and texture from an excess of gelatin. The foams made from these samples were unable to hold as much air. The whip densities vs. hydrolysis times at each of the solids levels used are shown in Figures 10, 11, and 12.

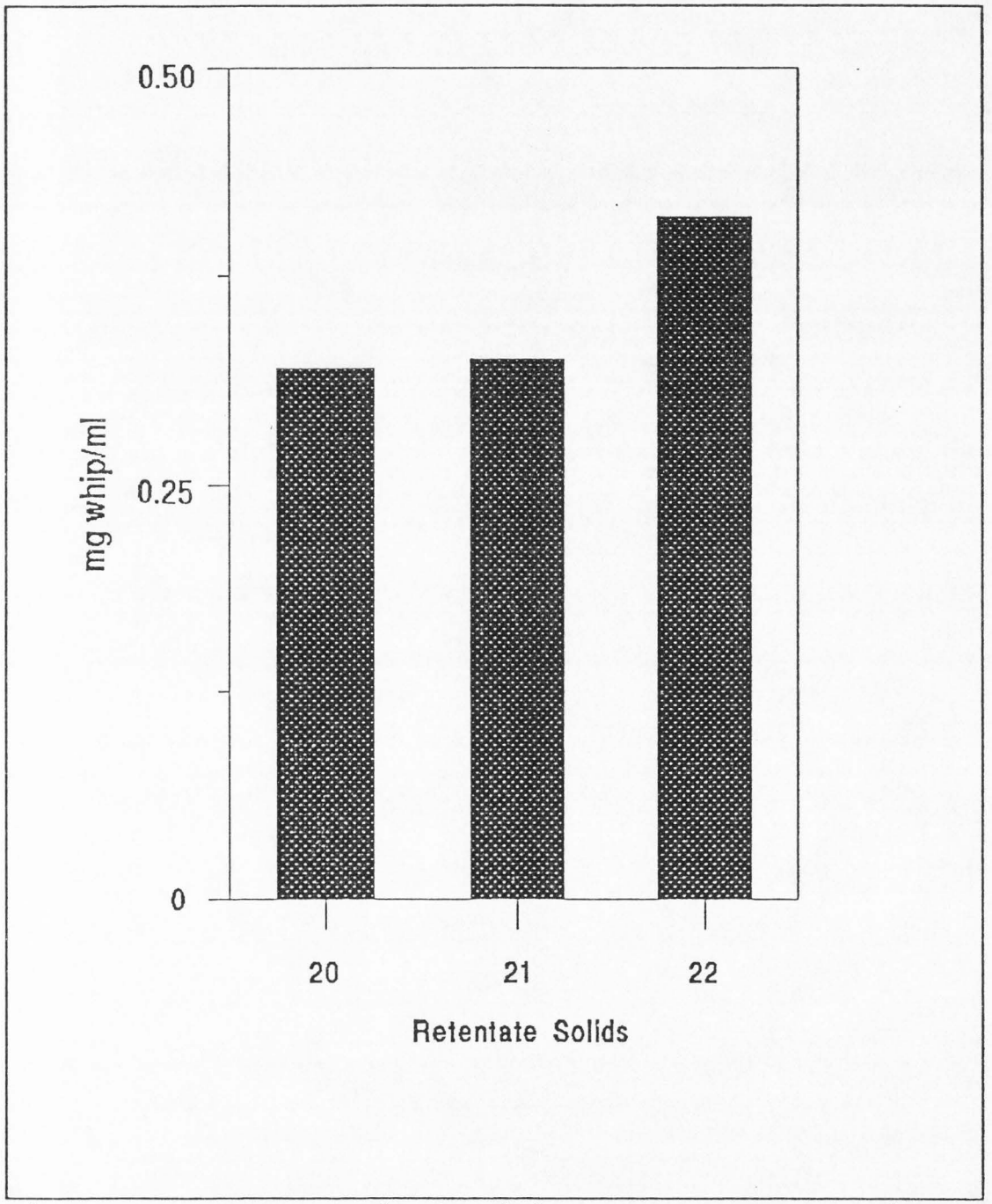

Figure 9. Average whip densities vs. retentate solids at all hydrolysis times. [a,b]Columns having the same letters are not different (P=.05).

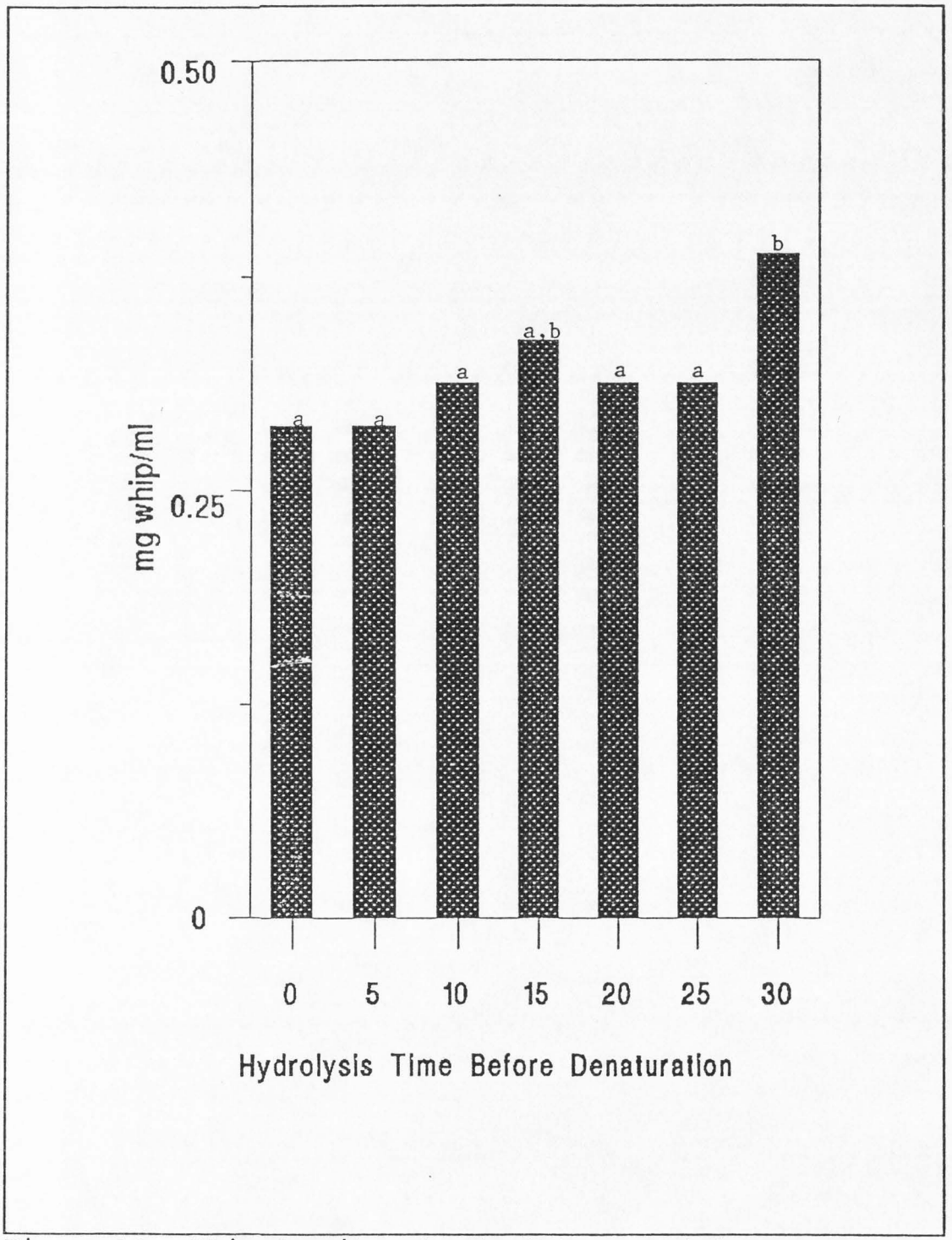

Figure 10. Whip density of retentate containing 20% solids.
[a,b]Columns having the same letters are not different (P=.05).

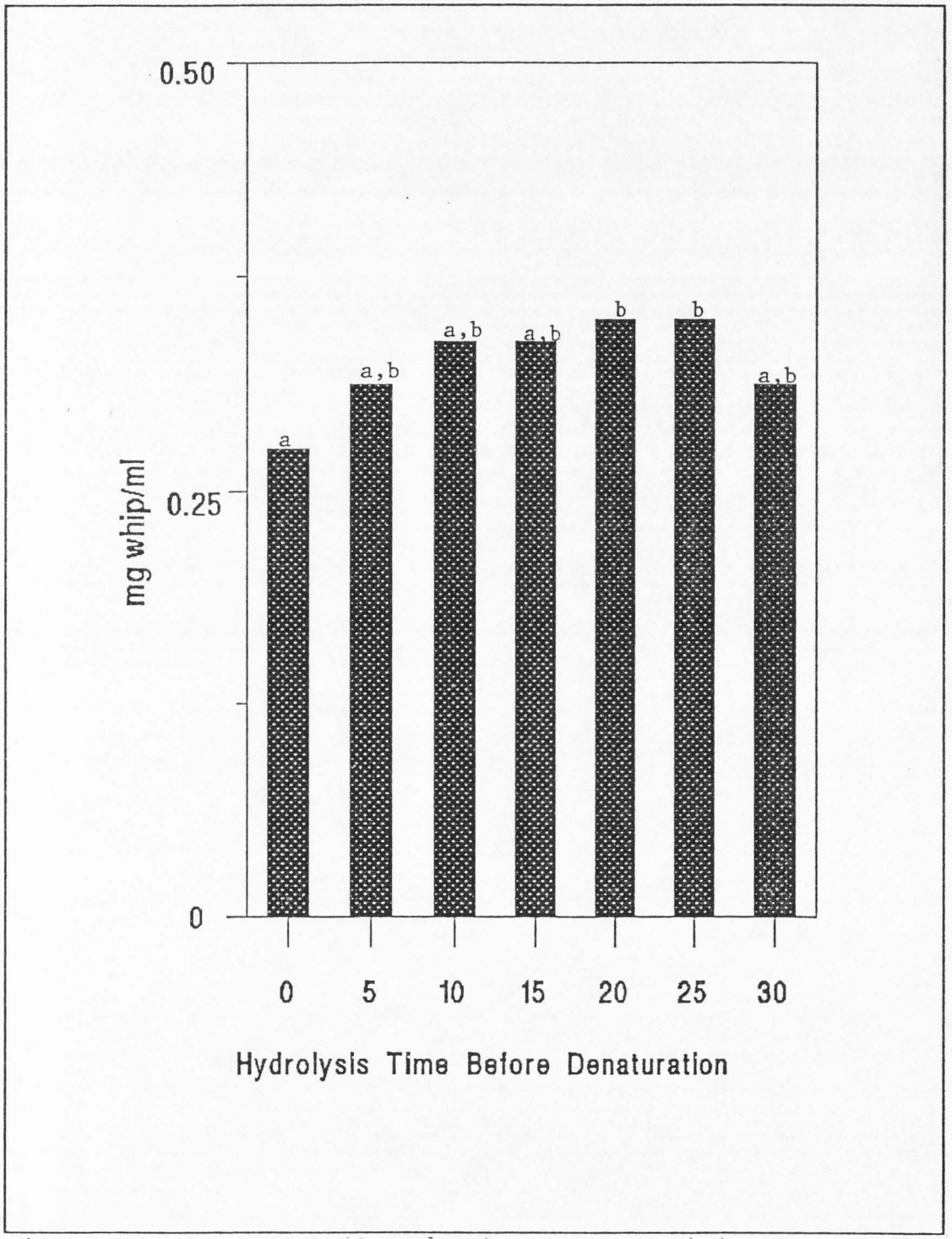

Figure 11. Whip density of retentate containing 21% solids.
[a,b]Columns having the same letter are not different (P=.05).

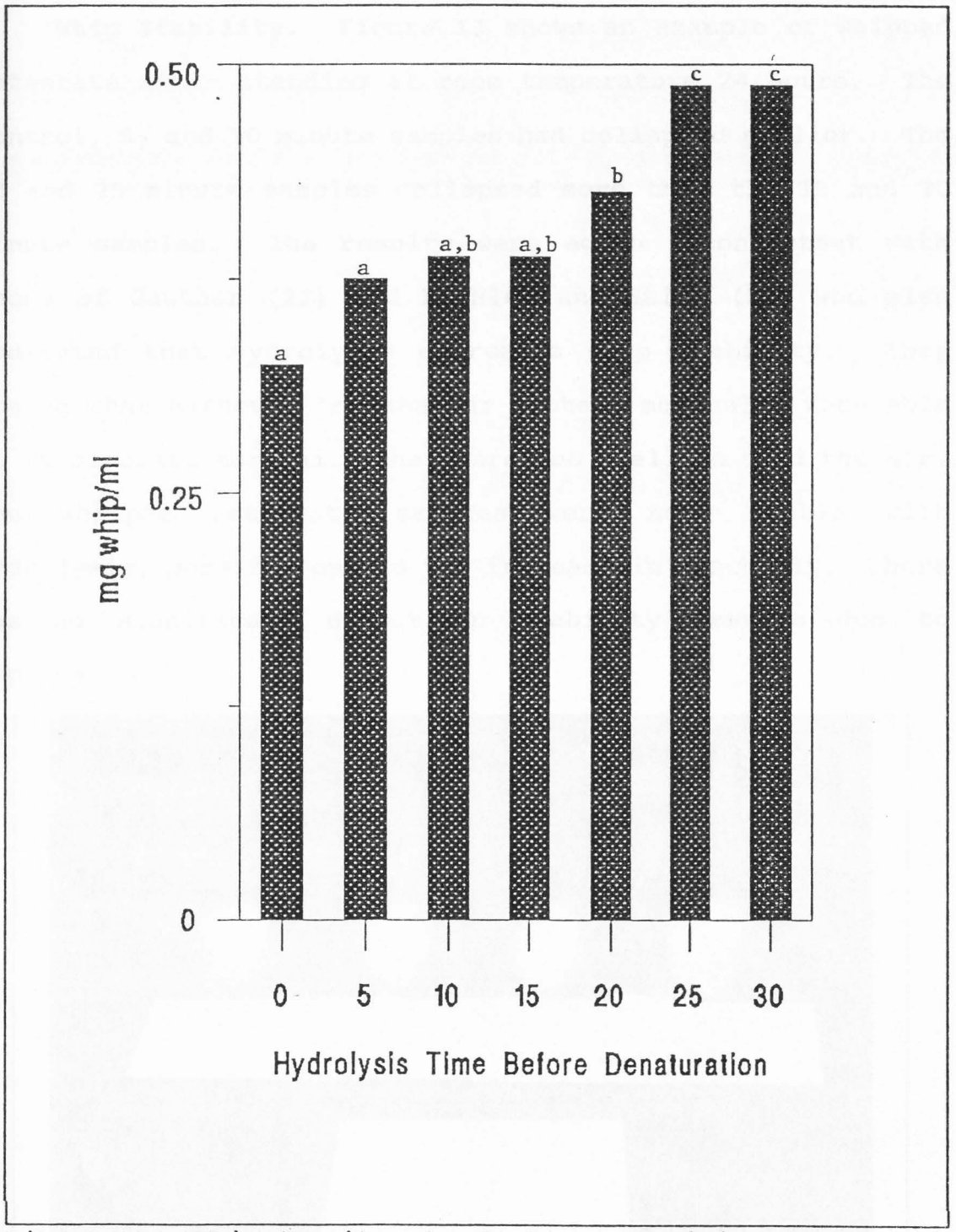

Figure 12. Whip density of retentate containing 22% solids.
[a,b,c]Columns having the same letter are not different (P=.05).

Whip Stability. Figure 13 shows an example of whipped retentate after standing at room temperature 24 hours. The control, 5, and 10 minute samples had collapsed earlier. The 20 and 25 minute samples collapsed more than the 15 and 30 minute samples. The results were again inconsistent with those of Gunther (21) and Kuehler and Stine (32) who also indicated that hydrolysis decreases whip stability. They stated that although the smaller protein molecules were able to incorporate more air, they were too small to hold the air. The whipped retentate samples were more stable with hydrolysis, possibly due to the increase in viscosity. There was no significant effect on stability results due to replication.

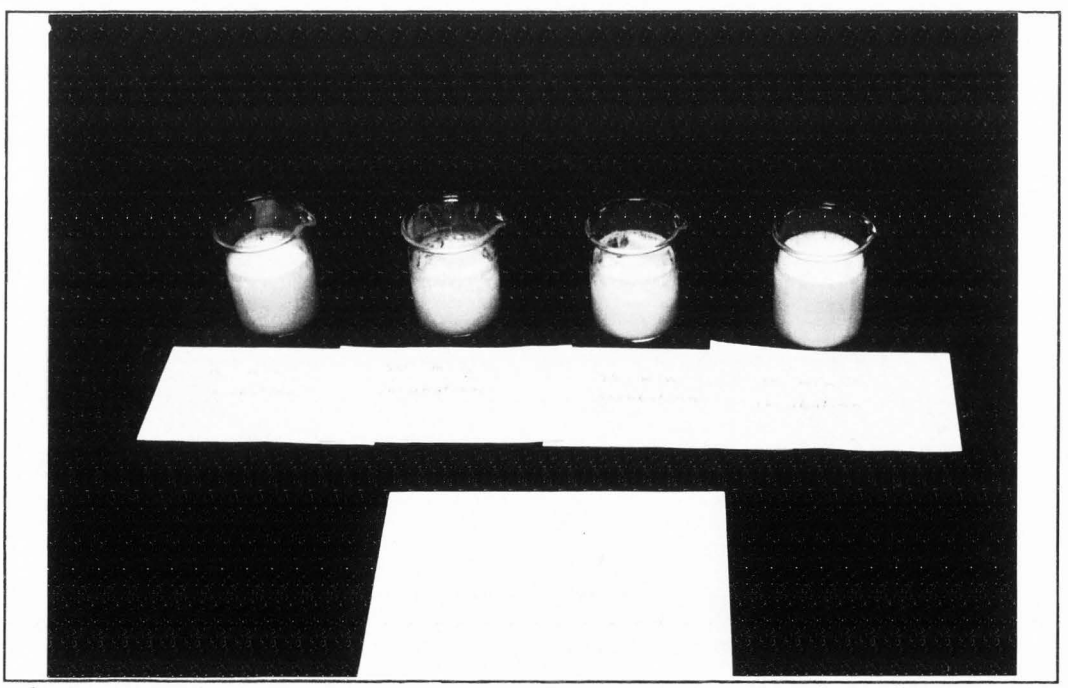

Figure 13. Photograph of samples indicating whip stability of retentate containing 19.5% solids and hydrolyzed at different times.

However, time and solids effects were both significant ($P<.001$) and the time*solids interaction was significant ($P=.006$). Figure 14 shows the means (n=6) of whip stability vs. the times of hydrolysis. There was a peak in stability at 15 minutes of hydrolysis--the consistent "slime point."

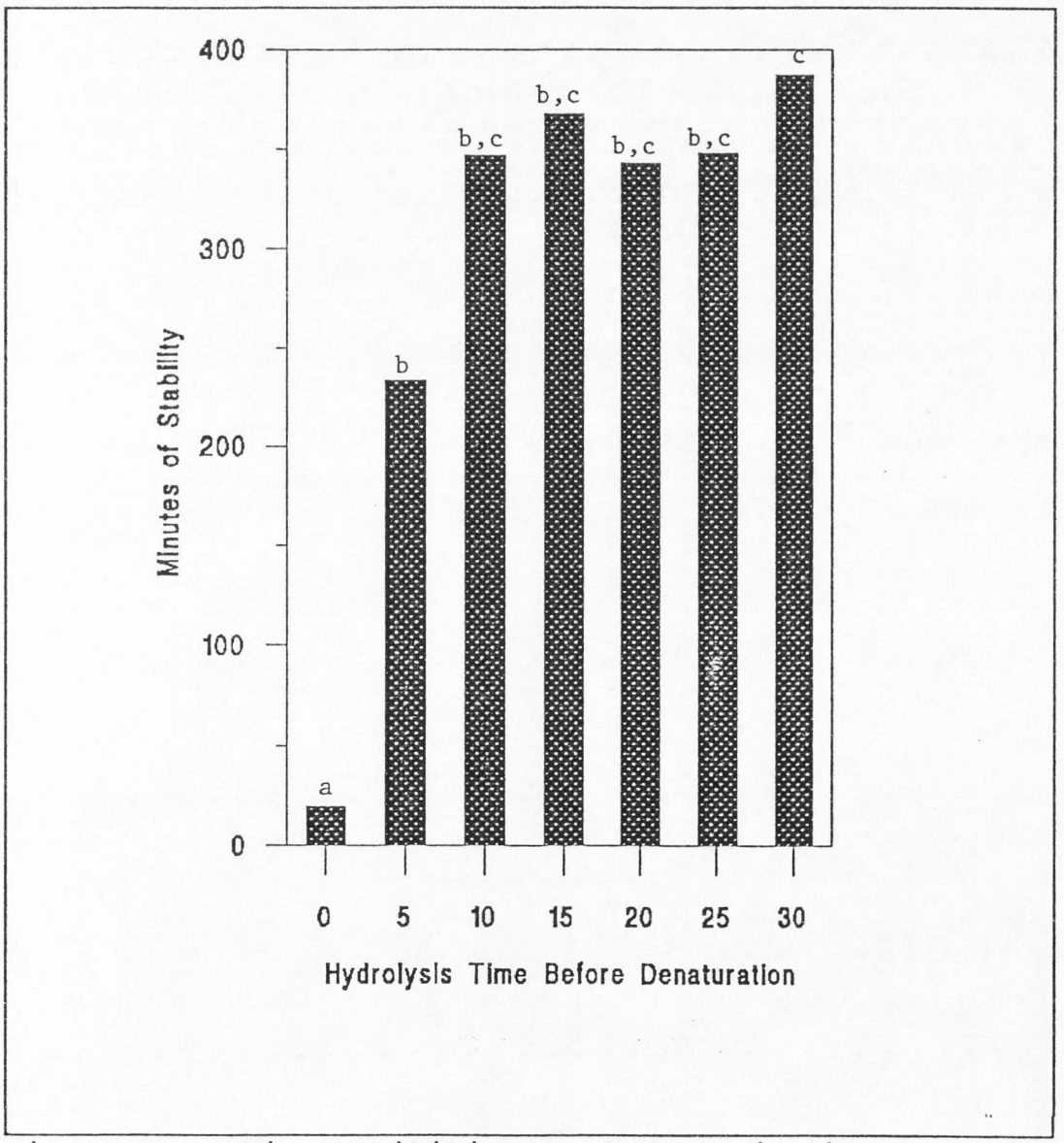

Figure 14. Whip stabilities vs. hydrolysis times averaged over all solids levels. [a,b,c]Columns having the same letter are not different ($P=.05$).

Stability decreased slightly for 20 and 25 minutes of hydrolysis, possibly due to the polypeptides being too small to keep the air as well (32). Stability went up again at 30 minutes of hydrolysis, but the air capacity at that level was the lowest.

Figure 15 illustrates the effect the different solids levels had on the stability of whipped retentate. As solids

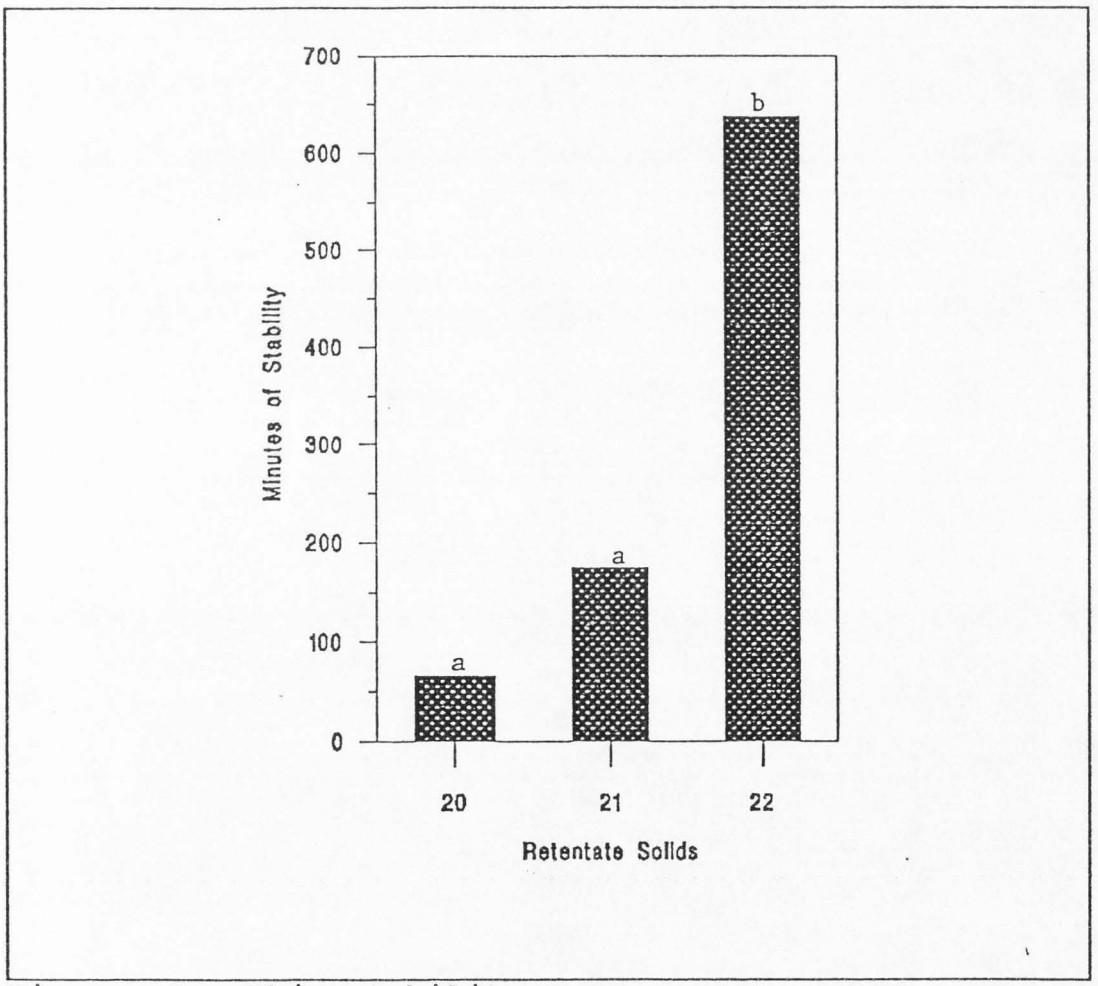

Figure 15. Whip stability vs. retentate solids averaged over all hydrolysis times. [a,b]Columns having the same letter are not different (P=.05).

increased, so did stability. With higher solids, the samples were more viscous. This made thicker films around the air bubbles, holding the air better and slowing coalescing and drainage. Figures 16, 17, and 18 show the whip stabilities at each of the individual solids concentrations.

In addition to time, the degree of hydrolysis is affected by the solids level as the substrate concentration in a hydrolysis reaction slows its rate (3). A faster reaction with lower solids would produce smaller, and already have fewer, polypeptides to hold the air bubbles. Therefore, the significant solids vs. time interaction was noted.

Powder

Drying. The retentate samples were dried immediately after inactivating the enzyme because the viscosity was lowest then. The viscosity increased with increasing hydrolysis time and increasing solids. The dryer is really not heavy-duty enough to dry materials having the viscosity of the hydrolyzed retentates. The unhydrolyzed samples dried without too much trouble at the 20 and 22% solids levels but had problems at the 24% level. Even the hot samples containing 24% solids did not go through the dryer well.

Thicker samples clog the nozzle and also do not atomize into droplets as well as those of less viscosity. The bigger droplets containing more sample build up on the sides of the drying chamber causing a significant decrease in yield due to product loss. The dryer dries at the rate of about a liter

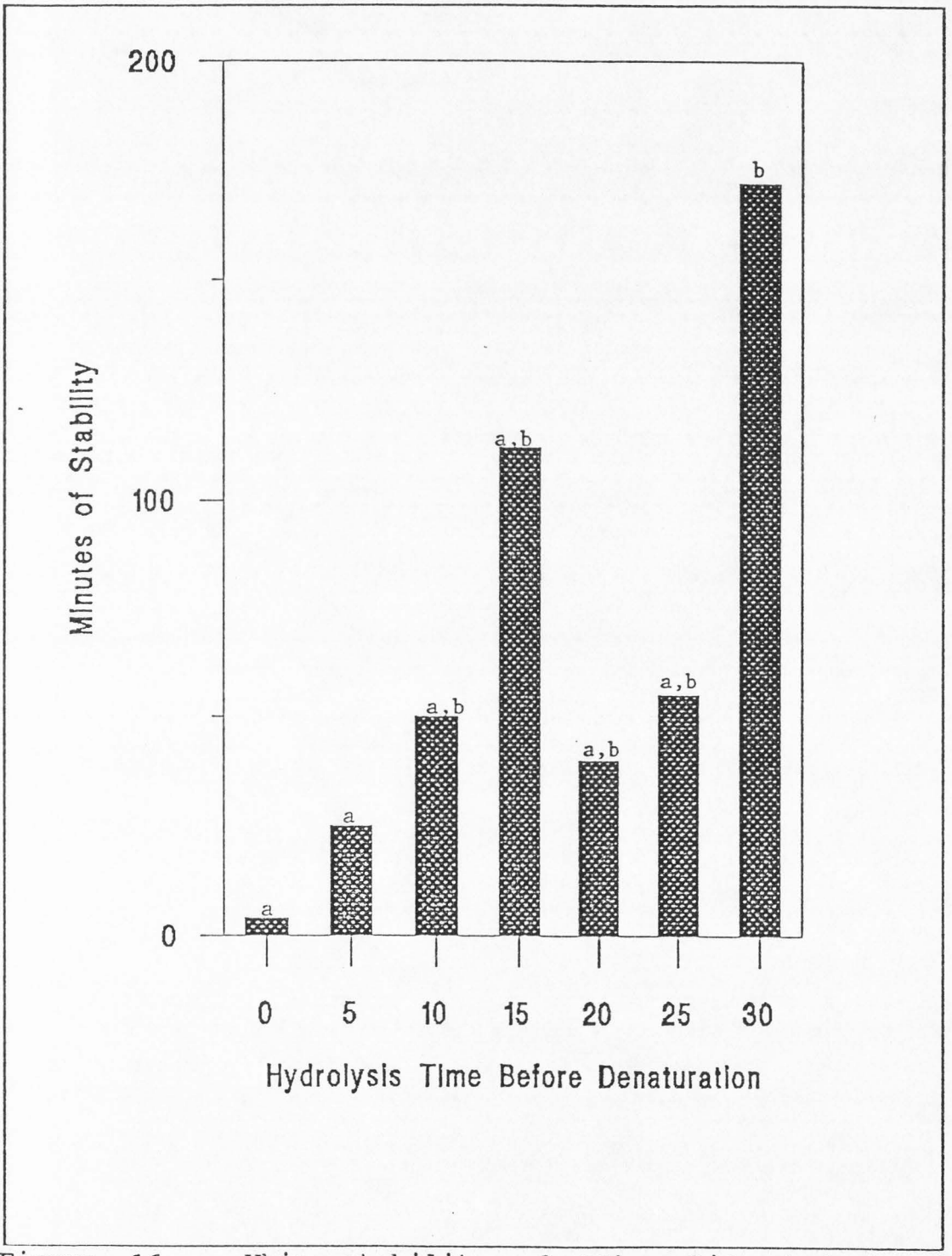

Figure 16. Whip stability of 20% solids hydrolyzed retentate. [a,b]Columns having the same letters are not different (P=.05).

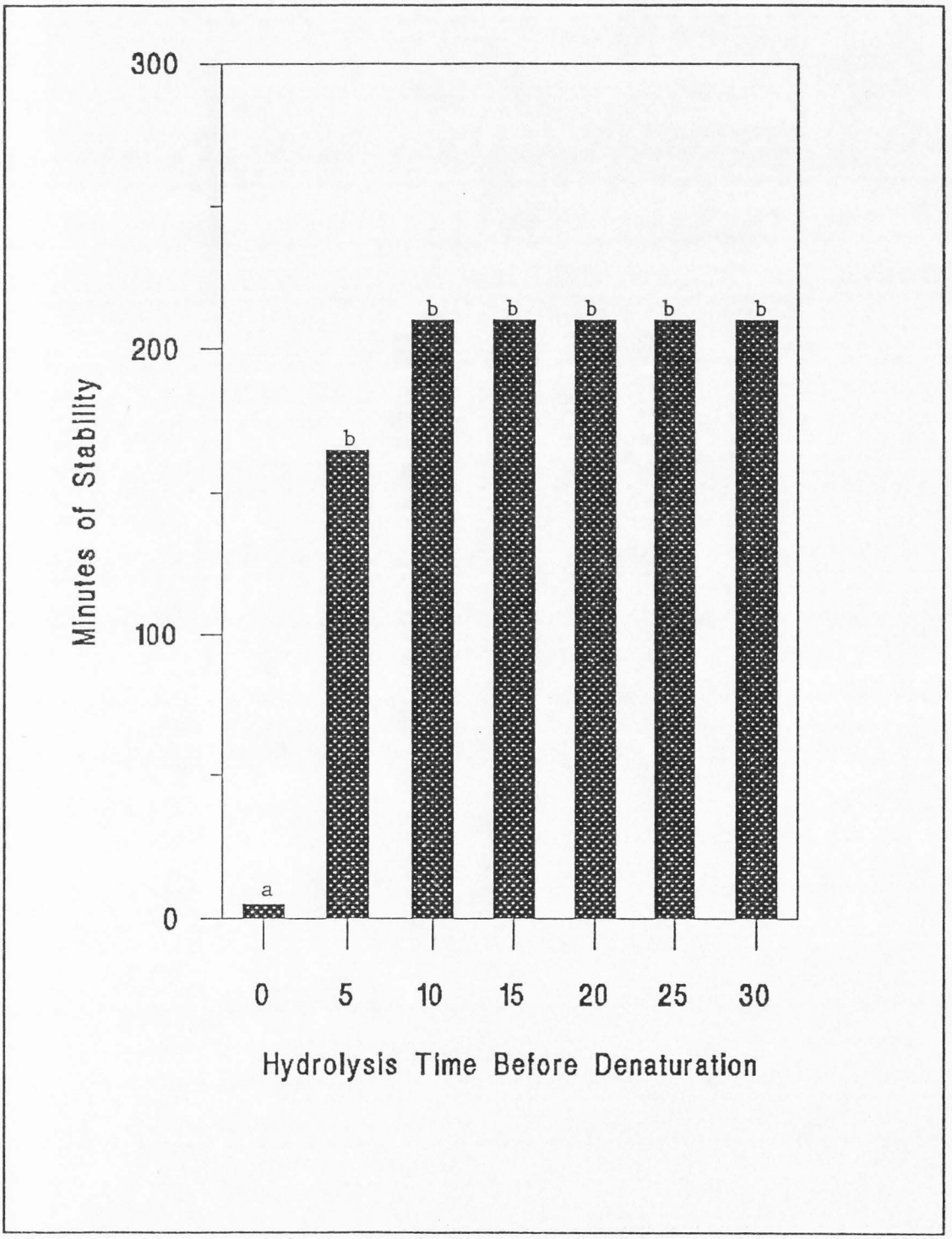

Figure 17. Whip stability of 21% solids hydrolyzed retentate. [a,b]Columns having the same letters are not different (P=.05).

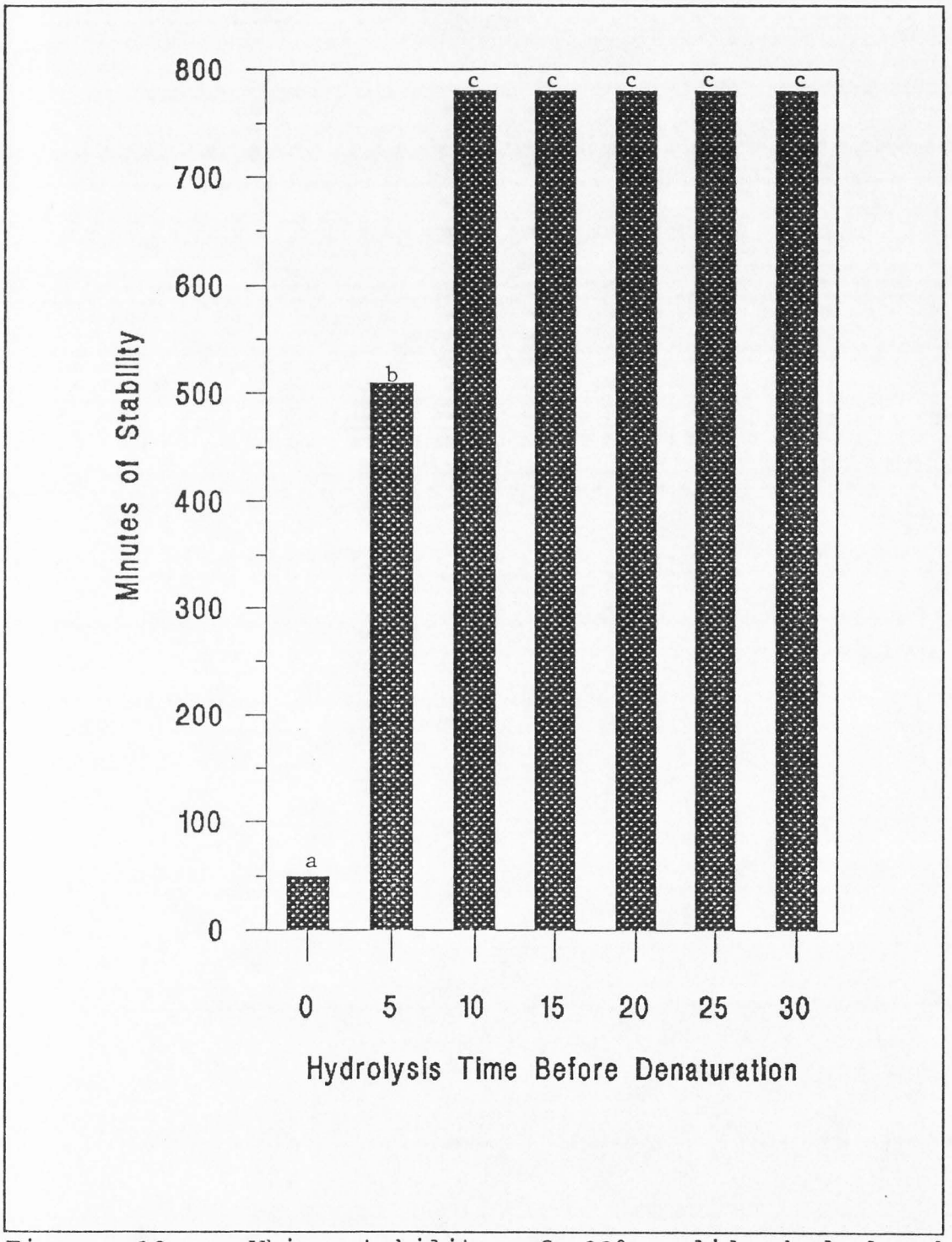

Figure 18. Whip stability of 22% solids hydrolyzed retentate. [a,b,c]Columns having the same letters are not different (P=.05).

per hour, which is not very fast. While some sample is being pumped into the dryer, the rest of it is cooling down. As it cools, it thickens, making drying more difficult. Preparing larger volumes of retentate to compensate for the decreased yield is futile because the dryer will only dry so much before the feedstock is too thick and lumpy to atomize. Added water would not mix well; so the lumps remained, preventing the sample from drying properly.

The results of analysis of variance on the air capacities, whip stabilities, and WPN measurements of the retentate powders are presented in Table 3.

TABLE 3. Retentate powder ANOVA levels of significance.

Source	Air Capacity	Whip Stability	WPN Values
	p Values		
Replication	0.131	0.887	0.040
Time of Hydrolysis	0.127	0.066	0.000
Retentate Solids	0.000	0.014	0.000
Time*Solids	0.013	0.019	0.000

Air Capacity. Retentate samples were dried after hydrolysis times of 10, 15, and 20 minutes before enzyme inactivation. Since the main objective of this part of the work was to obtain powder with acceptable properties, five-minute hydrolysis times were not used, as there was a marked improvement in the stability of whipped retentate between five

and ten minutes of hydrolysis. Samples hydrolyzed longer than 20 minutes were too thick for the dryer.

Figure 19 shows the effect of hydrolysis time averaged (n=6) over all solids levels. Time of hydrolysis was not significant (P=.127). With an insignificant F test, an LSD test would be misleading.

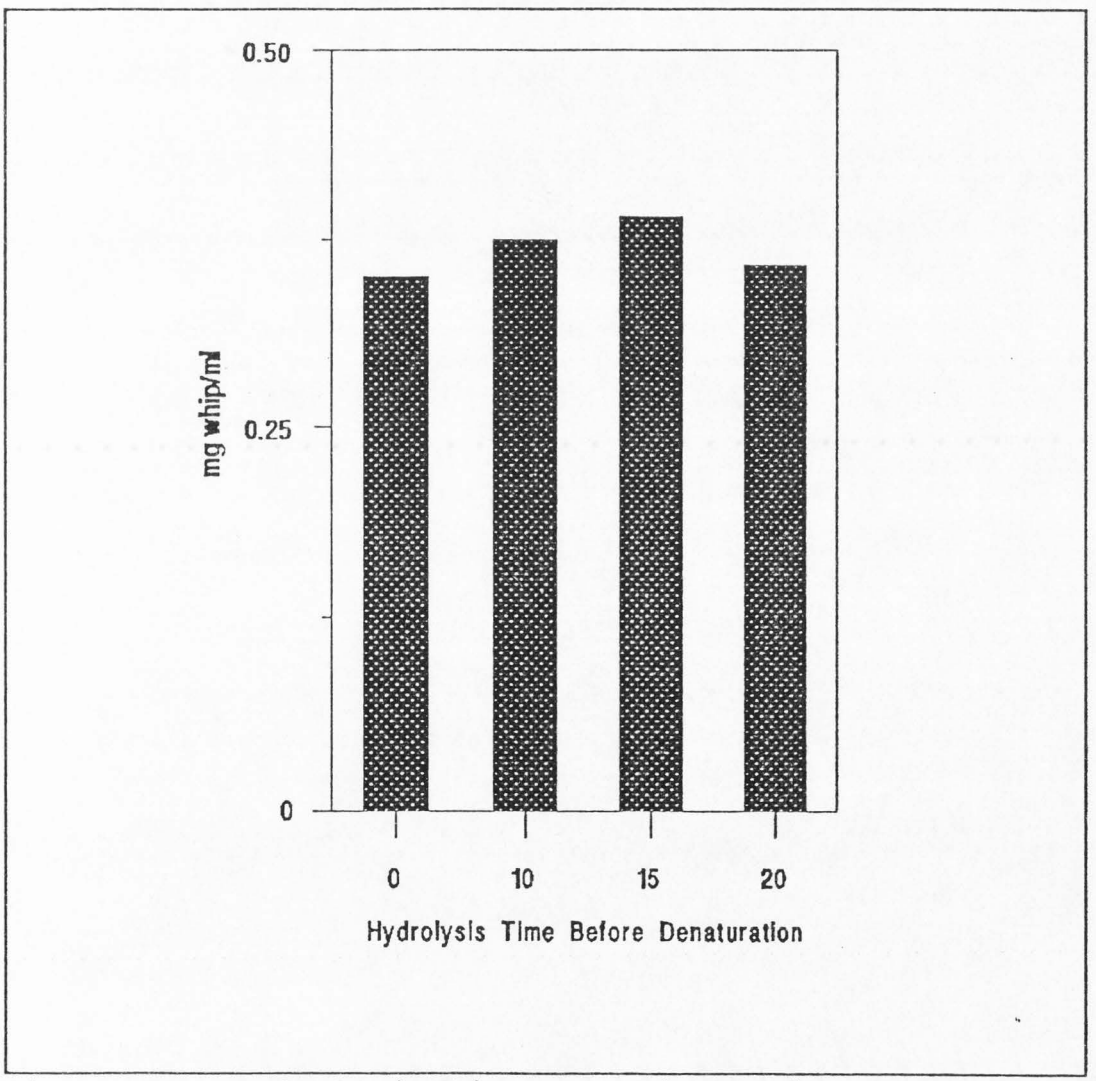

Figure 19. Hydrolysis time vs. whip density of retentate powder.

There was a significant effect (P<.001) of solids content in the retentate on the air capacity of the reconstituted powder. The averages of whip densities (n=8) for each of the solids levels are shown in Figure 20.

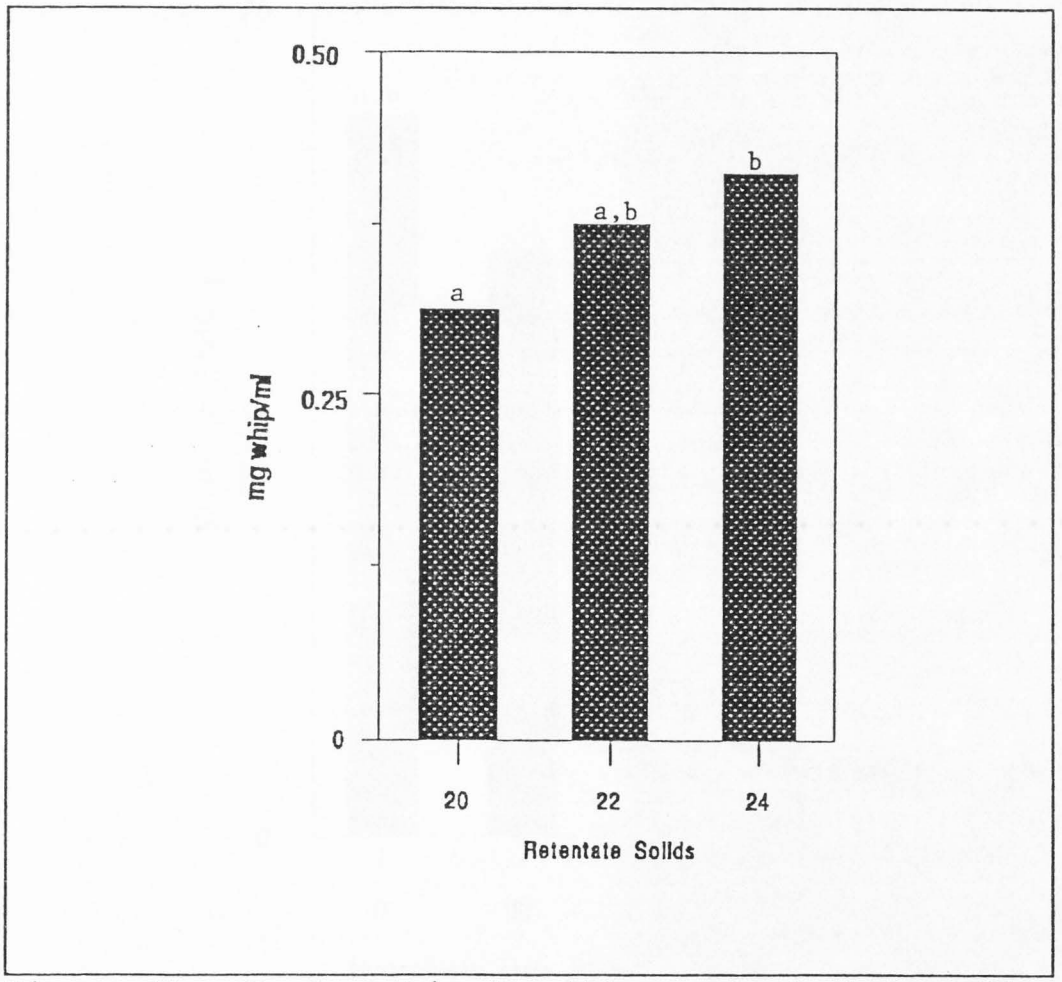

Figure 20. Powder whip densities vs. retentate solids averaged over all hydrolysis times. [a,b]Columns having the same letters are not different (P=.05).

Whip Stability. There was a significant effect (P=.066) of hydrolysis time on whip stability of the powder. Figure 21 compares the means (n=6), showing the whip stabilities at each hydrolysis time. The 15 minute samples

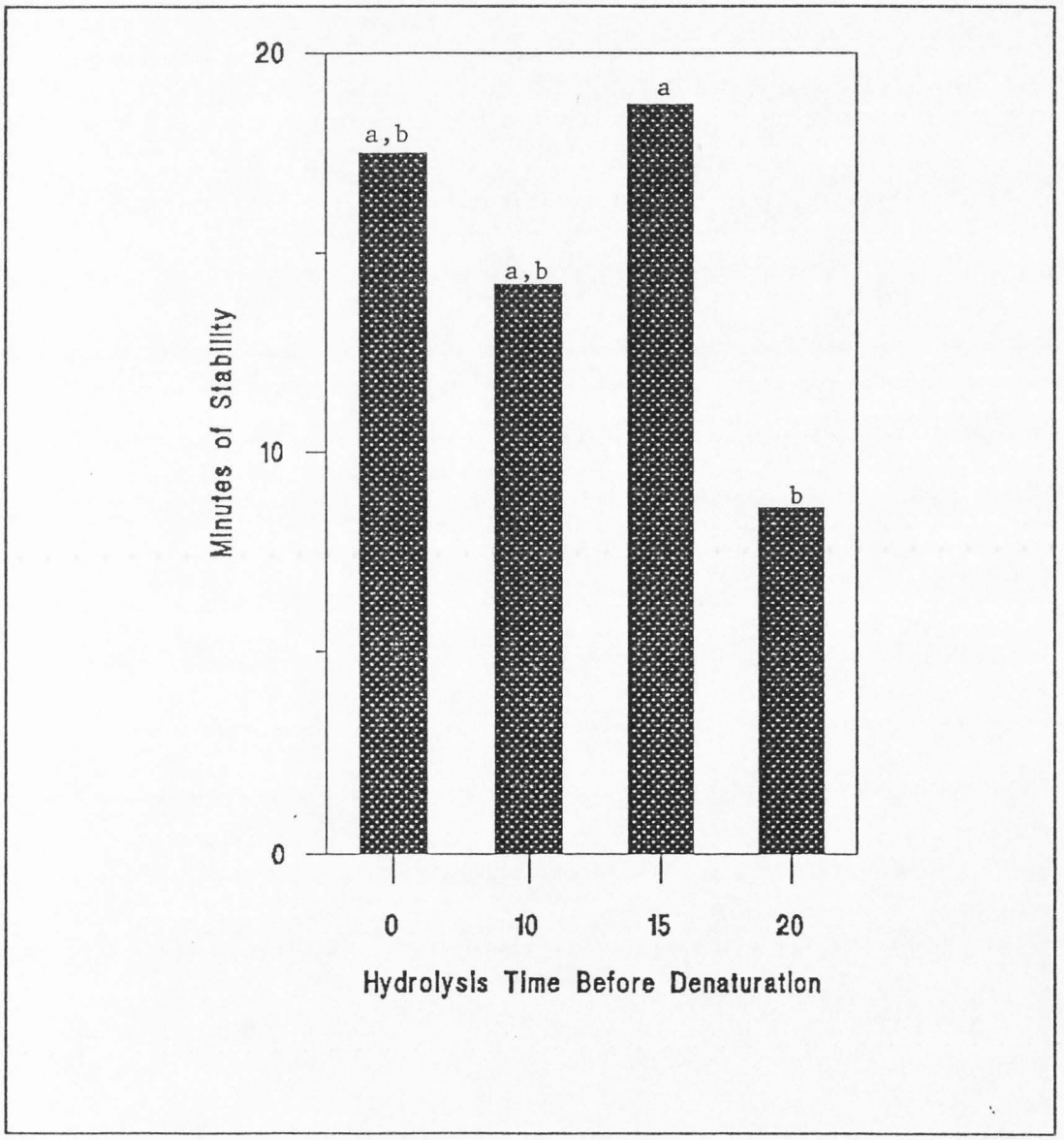

Figure 21. Average whip stabilities of retentate powder vs. hydrolysis times at all solids levels. [a,b]Columns having the same letters are not different (P=.1).

were more stable than the 10 and 20 minute samples, consistent with the peak in stability observed in the hydrolyzed retentate. This may indicate that during drying the proteins retained some of their whipping properties obtained from hydrolysis. Whip stabilities of the retentate powder samples vs. hydrolysis time are shown at 20, 22, and 24% retentate solids levels in Figures 22 and 23. Generally, the unhydrolyzed controls had more stability than the hydrolysates.

Retentate solids content had an effect on the whip stability of the powder (P=0.014) but Figure 24 indicates there was no difference between means (n=8) so the differences had to have been between individual samples. Powder from retentate containing 24% solids had less whip stability than powder from the 20 and 22% retentate solids.

This could be due to the problems in drying the thicker feedstock from the higher solids. The feedstock was watered down, but there were still lumps of hydrolyzed curd that would not completely break up so the water did not mix in completely. More soluble smaller polypeptides would have been mixed with the water, while larger polypeptides with better whipping properties may have stayed in the curd pieces, and did not get dried.

The stability decrease at 24% solids in the retentate could be from decreased hydrolysis due to the significant (P=.019) solids*time interaction.

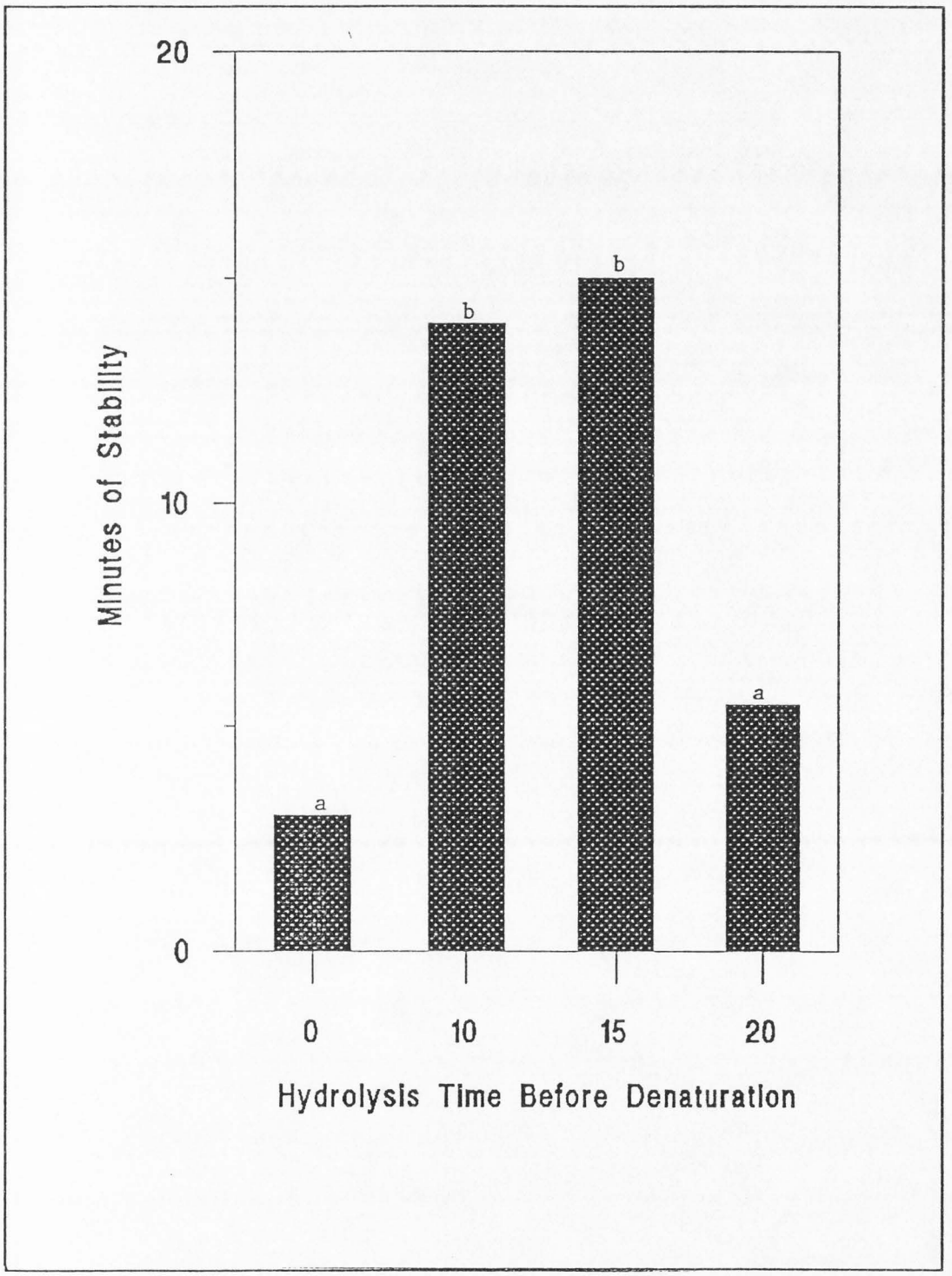

Figure 22. Whip stability of powder from hydrolyzed 20% solids retentate. [a,b]Columns having the same letters are not different (P=.1).

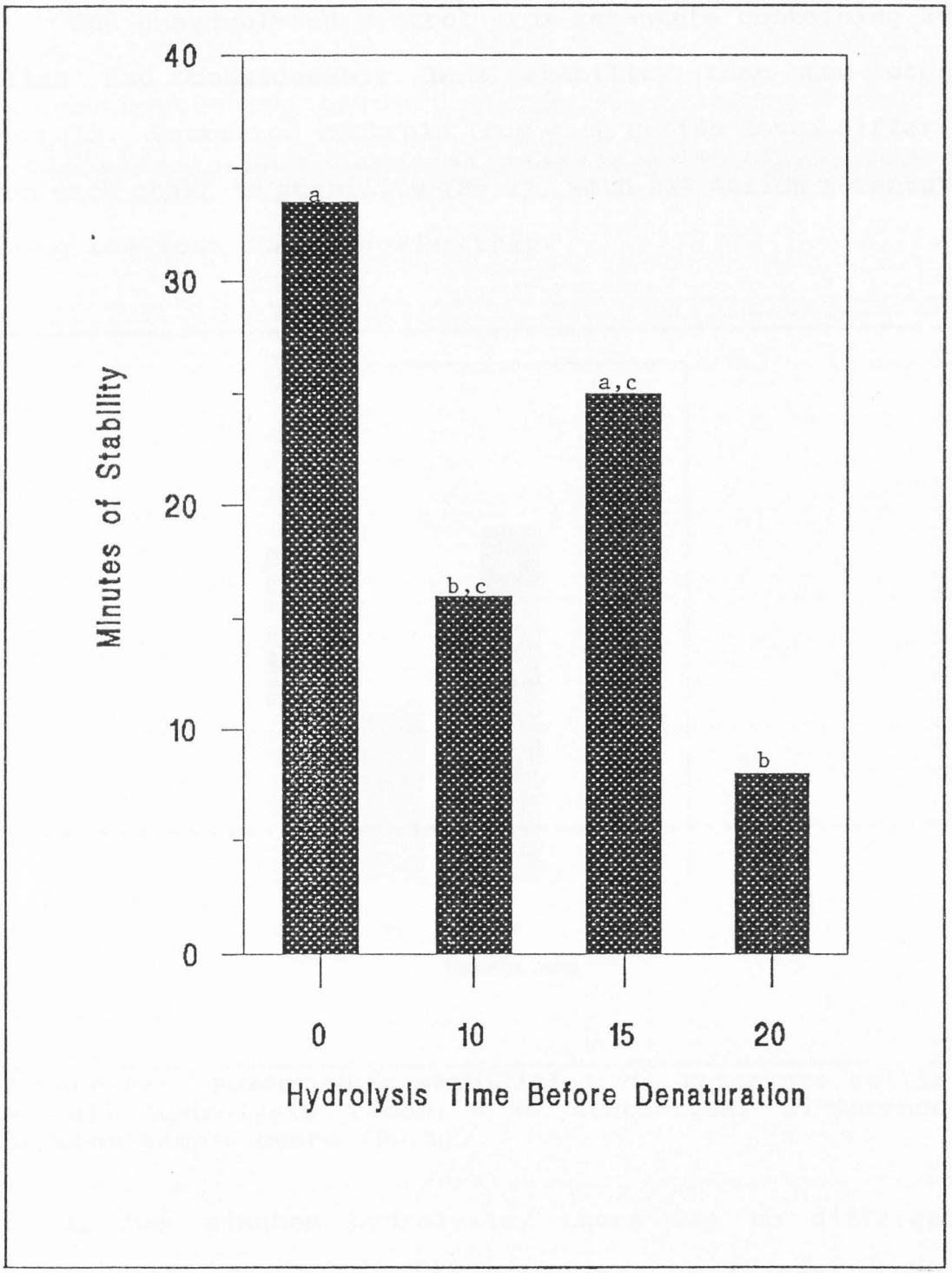

Figure 23. Whip stability of powder from 22% solids hydrolyzed retentate. [a,b,c]Columns having the same letters are not different (P=.1).

The unhydrolyzed control from retentate containing 20% solids had considerably less stability than the other controls. Untreated controls from each solids level differed from each other in stability (P=.1), with 22% solids retentate having the most stable powder whip.

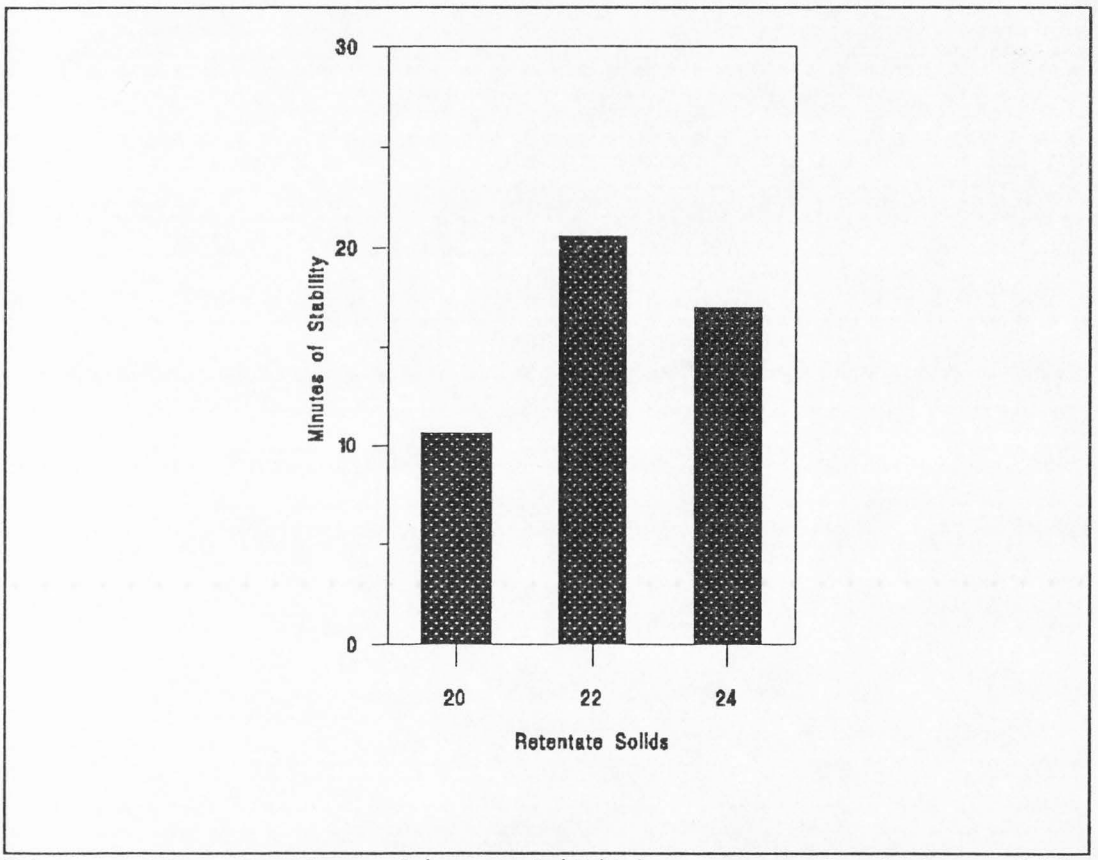

Figure 24. Powder whip stabilities vs. retentate solids at all hydrolysis times. No statistical difference between sample means (P=.1).

At ten minutes hydrolysis, there was no difference between solids levels in whip stability. Whip stability vs. retentate solids at 15 and 20 minutes of hydrolysis are shown in Figures 25 and 26.

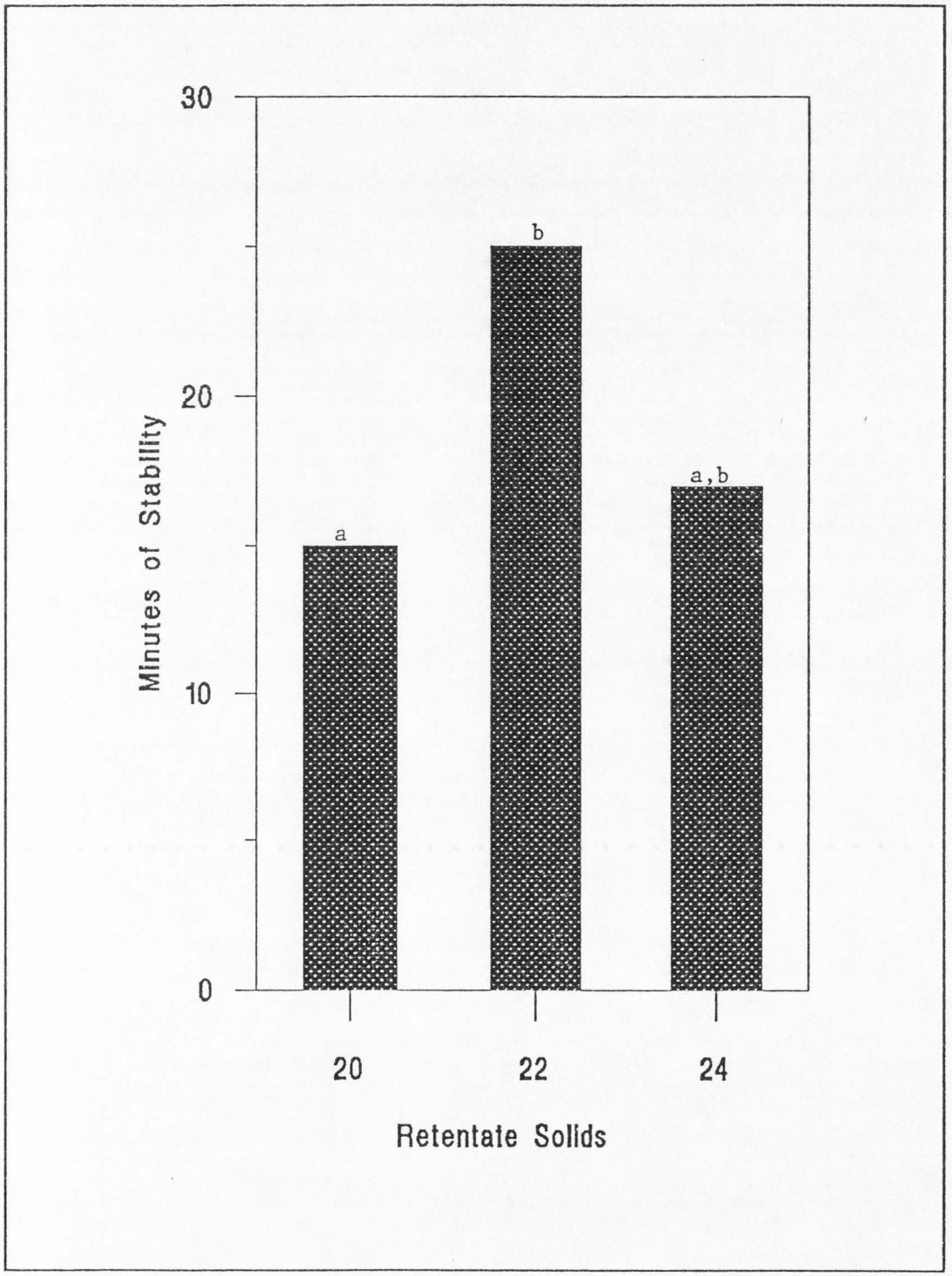

Figure 25. Powder whip stability vs. retentate solids at 15 minutes hydrolysis before denaturation. [a,b]Columns having the same letters are not different (P=.01).

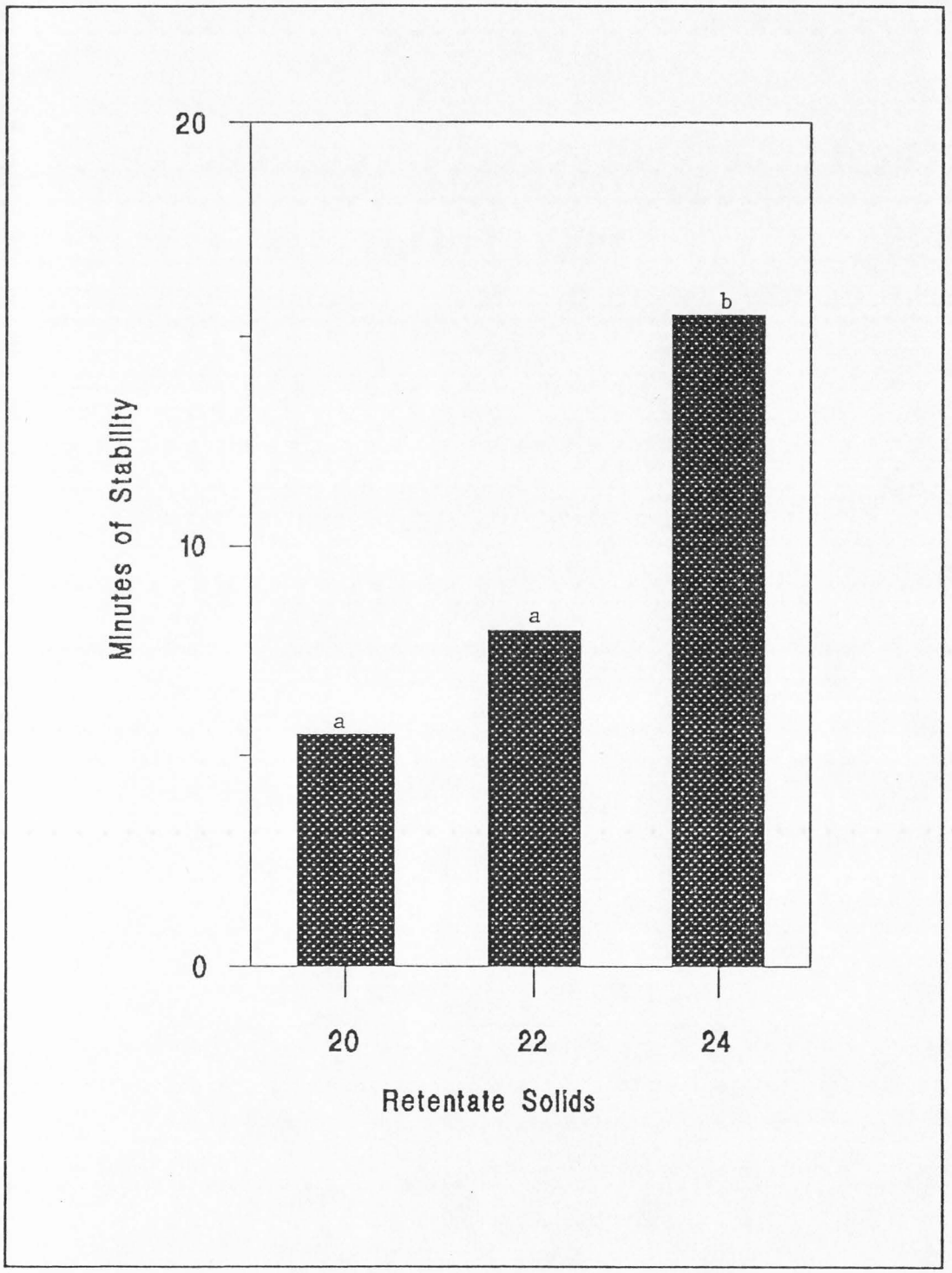

Figure 26. Powder whip stability vs. retentate solids hydrolyzed 20 minutes before denaturation. [a,b]Columns having the same letters are not different (P=.1).

The powder whip stabilities were disappointing when comparing them with those of the retentate. This may have been due to the problems with the dryer. Since the retentate was dried immediately after removal from the hot water bath when its viscosity was lowest, and since the dryer takes a long time to dry any considerable volume of feedstock, the hot retentate stayed hot for over an hour before getting dried, denaturing the milk proteins. Retentate that is heat treated before drying gives powder with increased water absorption but decreased whippability (10). As milk proteins are heat denatured, their water absorption increases (29).

WPN Measurements. The results are shown in Figure 27 and agreed with those of Mahmoud, et al., (40), who state that enzyme treatment has an effect on the results of the WPN test on the powder, and with Law (35) who found that the hydrolysis of cheese curd by bacterial and fungal proteases for accelerated ripening gave an increase in the amount of soluble nitrogen. The Harland-Ashworth procedure used, as modified by Kuramoto et al., (34), is designed to measure the amount of heat-undenatured, and therefore soluble, whey proteins in NDM at pH 4.6-5.0 (47). Since enzyme hydrolysis is also used to resolubilize proteins (26,46), the enzyme hydrolysis used on the retentate was expected to increase the WPN measurement of the powder even with no decrease in heat treatment. The results showed this as there was a significant effect of hydrolysis time on the WPN measurement at $P<.001$.

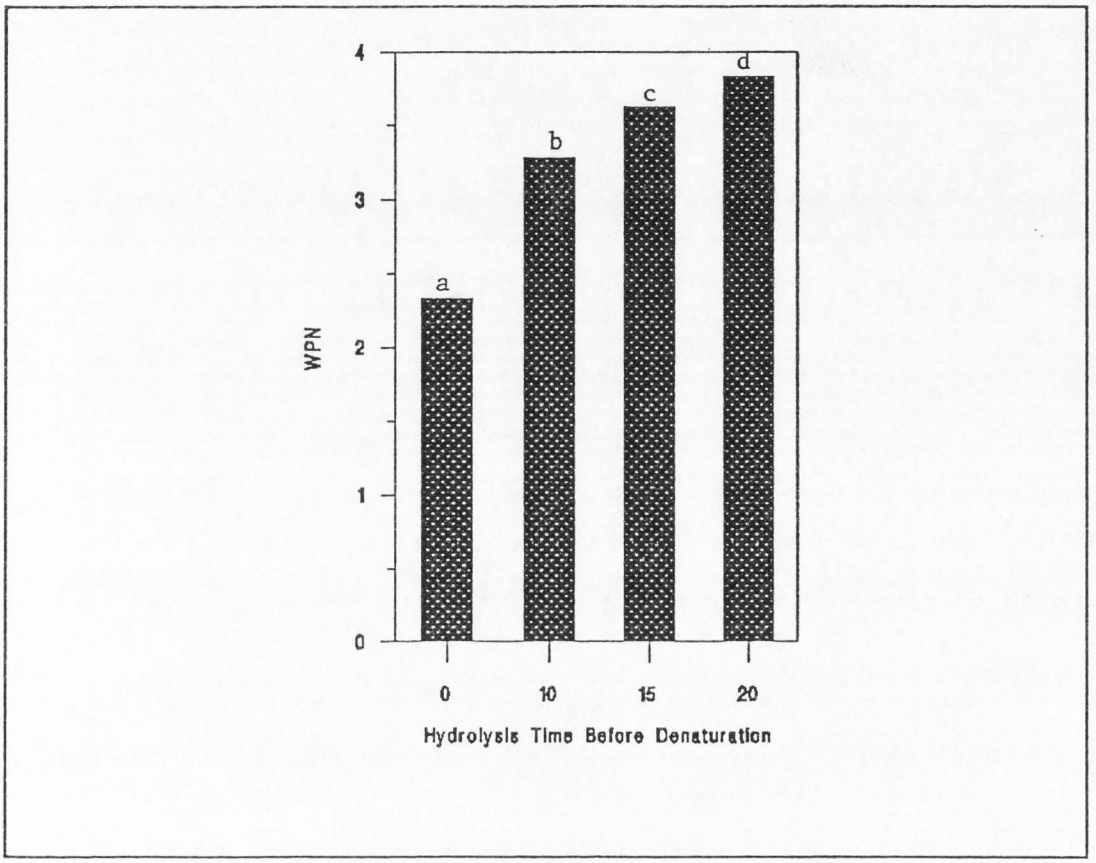

Figure 27. WPN measurement vs. hydrolysis time. [a,b,c,d]Columns are different (P=.01).

Figure 28 shows a significant effect of retentate solids content on the WPN of the powder, also at P<.001. As hydrolysis time and WPN values increased, the values still decreased with increasing solids. This was indicated by a significant solids*time effect at P<.001 on the WPN value of the retentate powder. There was a decrease in WPN values, indicating a decrease in hydrolysis, as the retentate solids increased.

There was also a variation due to replication. The statistics were done on the WPN values obtained from the standard curve in Appendix I. The percent transmittance

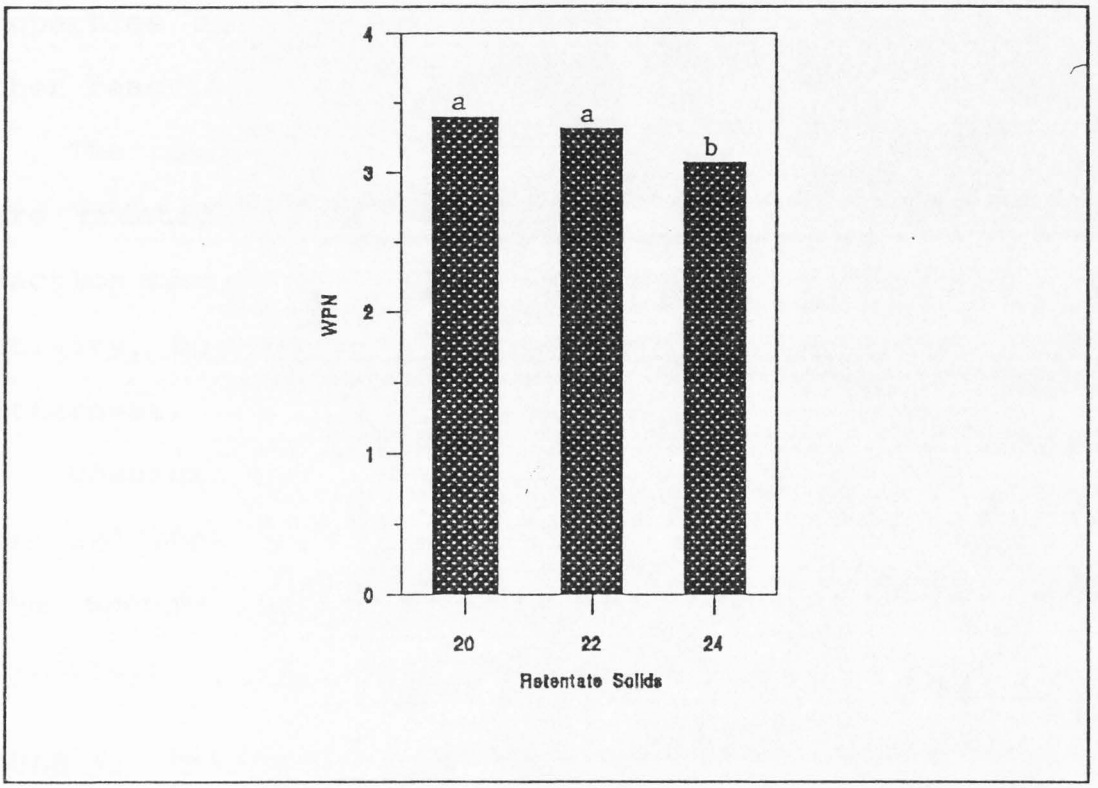

Figure 28. WPN measurement vs. retentate solids. [a,b]Columns having the same letters are not different (P=.05).

values for duplicates only varied ± 0.5% transmittance. Obtaining new readings is not necessary if the duplicates agree within 2% transmittance (4).

Bitterness. The whipped samples treated with *A. oryzae* enzyme were not bitter but were slightly sweet. The samples treated with *A. niger* enzyme had a bitter, unpleasant aftertaste. Enzyme selection and degree of hydrolysis are stated to be factors affecting bitterness (31,53).

The temperature used was not the optimum for the *A. oryzae* enzyme, decreasing its activity somewhat. The conditions used were favorable to obtaining good whipping

properties and still avoid bitterness. This enzyme, with other reaction conditions, can produce bitterness (22).

The conditions used produced bitter samples when they were treated with the *A. niger* enzyme. Milk pH and the reaction temperature were not in the range for maximum enzyme activity, but there was still enough hydrolysis to produce bitterness.

Chemical Composition. The three samples to be tested were selected based on the quantity of powder available, to have enough sample for all three tests. The chemical composition is shown in Table 4.

TABLE 4. Chemical composition of retentate powder.

Sample	Protein	Moisture	Fat
1	60.94	4.87	0.7
2	62.64	5.27	0.9
3	62.58	5.59	1.4
Average	62.05	5.24	1.0

The protein was higher in the retentate powder than that of evaporated skim milk powder which has a protein content of 33-35% (25,27).

However, these researchers also reported an increase in fat content of the retentate powder they sampled. There is not such a difference in the retentate powder made during these experiments. The fat percentage was comparable to that in the evaporated milk powder.

The moisture in the powder is a function of the outlet temperature of the dryer, not of the pre-drying concentration process. Either the inlet temperature should be raised or the pump speed should be slowed to raise the outlet temperature of the dryer if the standard moisture level for extra grade NDM of 4% moisture is desired (4,6).

CONCLUSIONS

Enzyme hydrolysis of ultrafiltered retentate with enzyme from *A. oryzae* increases the whipping properties of the proteins. Retentate concentrated to the 21% level would be acceptable as the basis for a formulation of a whipped product. It has better stability but similar air capacity to the whipped retentate of 20% solids. Although the whip stability of samples containing 22% solids was excellent, the samples were thick and viscous and held less air.

A hydrolysis time of 15 minutes at the enzyme activity level used in the project would be ideal. Even though there was a slight increase in whip density at this time, there was also a stability peak. With further hydrolysis and with higher retentate solids, the whipped samples were more stable but also had a much higher density and less air capacity.

The powder has potential if the equipment limitations can be overcome to enable drying without excessive denaturation. Since limited heat treatments increase the flexibility of

protein molecules and aid their surface properties, a small degree of heat denaturation would be advantageous.

The samples treated with the *A. niger* enzyme were unacceptable. In addition to their unpleasant aftertaste, preliminary trials indicated inferior whipping capacities when compared to those treated with *A. oryzae*. Perhaps reaction conditions which would further limit hydrolysis may increase whipping capacities and decrease bitterness.

The gelatin-streaking method used to determine the time necessary for enzyme denaturation worked. The puddles of hydrolyzed gelatin were obvious, making the test quick, easy, and repeatable.

In determining foam stability, some researchers (23) have measured the amount of liquid drainage from foam placed in a funnel in a graduated cylinder. This method did not work in this project as some of the bubbles would also drain from the funnel into the cylinder. Others (21) measured the foam volume after the sample had been standing for several minutes. This method also did not work since some samples collapsed after a few minutes, while others retained air for hours. The method developed for this project to determine whip stability was repeatable and was satisfactory for comparing the whip stabilities of the samples.

There is no standardized method for the evaluation of functional properties of food ingredients. Researchers must combine and adapt methods available and used by others to fit

their needs. This was also necessary for the determination of whip stability in this project.

RECOMMENDATIONS

It has been established that the action of the *A. oryzae* protease on retentate proteins imparts desirable whipping characteristics to the proteins. The reaction needs to be studied further to be able to use chemical analytical methods to determine the degree of hydrolysis that creates the desired endpoint. The pH-stat method measures any drop in pH as more acidic terminal ends are exposed through hydrolysis and is recommended by Adler-Nissen (2).

For study of the action of the enzyme on milk protein and its effect on molecular weight, gel filtration chromatography (67) and SDS-Page electrophoresis (39) can be done. These tests would allow the enzymatic reaction to be monitored in an industrial setting for commercial production.

A study should be done to determine the degree of heat denaturation in combination with enzyme hydrolysis that would maximize the whipping properties of the product.

The drying process using the dryer available in the Nutrition and Food Sciences laboratory needs to be analyzed for any possible modifications that can be done to dry the retentate without excessive protein denaturation. A stronger pump and more rigid tubing for the thicker feedstock would enable the dryer to handle more viscous feedstocks at a faster

drying rate. The use of a larger nozzle is desirable for more viscous feedstocks (J.M. Robinson, personal communication, MVM Wellsville, August, 1991). This would aid in atomization and prevent so much product loss due to caking on the drying-chamber walls.

The retentate can be used as a basis in formulations for fruit salads, whipped toppings, and ice cream. These formulations should be developed and tested through consumer taste panels.

Foam stabilizers may be necessary to retain air during stirring and mixing of other ingredients. Hansen and Black (23) used carboxymethylcellulose to increase stability of a whey protein whip. Cellulose-based stabilizing gums are available from Dow Chemical and FMC Corporation that are designed to aid foam stability.

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APPENDIX 1

TABLE 5. Raw data for whipped retentate.

Solids	Time	Air(mg whip/40 ml)	Stability(min)
20%	0	.275	3
20%	5	.3	45
20%	10	.325	60
20%	15	.375	120
20%	20	.325	5
20%	25	.325	45
20%	30	.325	45
21%	0	.3	5
21%	5	.325	240
21%	10	.375	240
21%	15	.375	240
21%	20	.4	240
21%	25	.35	240
21%	30	.35	240
22%	0	.325	25
22%	5	.35	240
22%	10	.4	780
22%	15	.4	780
22%	20	.45	780
22%	25	.575	780
22%	30	.55	780

TABLE 5. (Cont.)

Solids	Time	Air (mg Whip/40 ml)	Stability(min)
20%	0	.3	5
20%	5	.275	5
20%	10	.3	40
20%	15	.3	105
20%	20	.3	75
20%	25	.3	65
20%	30	.45	300
21%	0	.25	5
21%	5	.3	90
21%	10	.3	180
21%	15	.3	180
21%	20	.3	180
21%	25	.35	180
21%	30	.275	180
22%	0	.325	75
22%	5	.4	780
22%	10	.375	780
22%	15	.375	780
22%	20	.4	780
22%	25	.4	780
22%	30	.425	780

APPENDIX 2

TABLE 6. Raw data for whipped retentate powder.

Retentate Solids	Time	Air(mg Whip/40 ml)	Stability(min)	WPN
20%	0	.325	4	2.39
20%	10	.325	12	3.59
20%	15	.325	12	3.75
20%	20	.275	6	3.88
22%	0	.325	37	2.62
22%	10	.35	12	3.29
22%	15	.425	24	3.43
22%	20	.375	8	3.78
24%	0	.425	22	1.97
24%	10	.4	10	2.87
24%	15	.4	23	3.57
24%	20	.375	18	3.82
20%	0	.3	2	2.39
20%	10	.325	26	3.55
20%	15	.325	18	3.86
20%	20	.325	5	3.83
22%	0	.325	30	2.68
22%	10	.375	20	3.41
22%	15	.45	26	3.46
22%	20	.375	8	3.87
24%	0	.425	10	1.95
24%	10	.475	15	2.99
24%	15	.375	11	3.61
24%	20	.425	13	3.85

APPENDIX 3

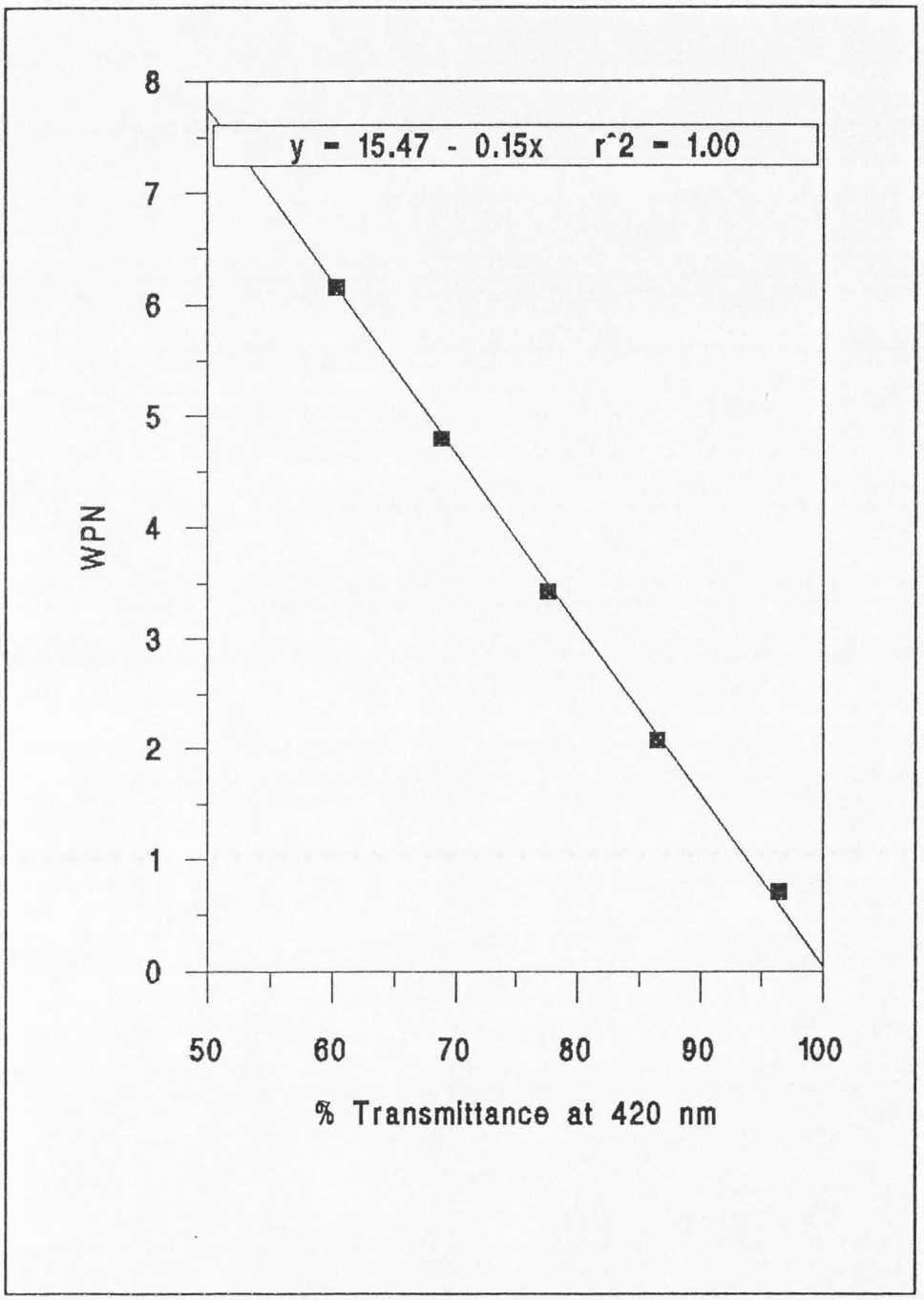

Figure 29. Standard curve for WPN measurements.