STUDIES ON THE HYDROPHOBICITY
OF PROTEINS AND ENZYMES

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in
THE FACULTY OF GRADUATE STUDIES
(Department of Food Science)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April 1982
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ABSTRACT

This thesis deals with studies on the hydrophobicity of proteins and enzymes and is divided in four chapters summarized separately below.

(1) Traditional methods of coagulating milk for the manufacture of cheese suffer from two major drawbacks, namely, the high cost of the enzyme and the fact that they are batch systems. An obvious solution to both problems would be the use of immobilized enzymes to coagulate milk. Hydrophobic adsorption offers certain potential advantages over other techniques of enzyme immobilization. The objective of this part of the thesis was to immobilize the milk clotting enzymes chymosin and pepsin on various hydrophobic carriers and to assess their suitability for continuous coagulation of skimmilk. All enzyme-carrier preparations exhibited high initial activity on exposure to milk. However, the deactivation rates were very high. The main reason for this rapid deactivation appeared to be the loss of enzyme from the carriers, since soluble activity was detected in all enzyme preparations. The enzyme loss was due to the physical desorption of enzyme from the carriers as well as to the relatively rapid leakage of the ligand from the carrier (phenoxyacetyl cellulose). The best enzyme preparation was obtained with phenoxyacetyl cellulose. However, a study indicated that the continuous coagulation of skimmilk with proteases immobilized on the hydrophobic supports used in this study was not economically feasible.

(2) The fat binding capacity (FBC) of food proteins is an essential functional property. However, fat binding as determined by existing methods has been mainly attributed to physical entrapment of the oil rather than to the binding with proteins. A simple turbidimetric
method, thus, was developed for determining the FBC of various proteins. The turbidity was dependent on wavelength, blending time and volume of oil. The regression equation for predicting FBC was:

\[ \text{FBC} (%) = 30.271 + 1.381 S_o - 0.014 S_o \times s \]

where \( S_o \) and \( s \) are surface hydrophobicity and solubility index, respectively.

A highly significant correlation \( (R^2 = 0.802, P < 0.01) \) was found between \( S_o, S_o \times s, \) and FBC of 11 food proteins tested. Advantages of the method developed include the small amount of sample required and the fact that the measured values would reflect the true fat binding capacity of proteins by minimizing the fat-entrapping effects.

(3) The objectives of this part of the thesis were to determine the effects of heating on the emulsifying properties of selected food proteins, and, to assess the value of \( S_o \) as a predictor of these properties. The results obtained indicated that the emulsifying properties of the proteins studied were differently affected by heating, and that heat-denaturation was not always accompanied by loss of functionality, but, on the contrary, resulted in great improvement. The emulsifying properties could well be predicted solely on the basis of \( S_o \) level but not on the basis of solubility level, which indicated that \( S_o \) is a very important property determining protein functionality. However, the emulsion activity, emulsion stability and fat binding of the proteins studied could be well explained and more accurately predicted by \( S_o \) and solubility together.

(4) The objectives of this part were to evaluate the thermal properties (thickening, coagulation and gelation) of selected food proteins and to assess the value of hydrophobicity as their predictor. The results obtained indicated that the average \( (S_e) \) and not the surface
hydrophobicity was important for these properties. The thermal properties studied could not be explained by either the average hydrophobicity or sulfhydryls alone. Instead, they could well be predicted using average hydrophobicity and sulfhydryls together.
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ACKNOWLEDGEMENT

I wish to express my sincere gratitude to my research supervisor, Dr. S. Nakai, for his constant advice, assistance, encouragement and constructive criticism throughout the course of this study, and in the preparation of the thesis.

I am also thankful to the members of my supervisory committee, Drs. W. D. Powrie and P. M. Townsley of the Department of Food Science and to Dr. B. Roufogalis of the Faculty of Pharmaceutical Sciences, for their interest in and review of this thesis.

Finally, I would like to thank my wife, Maria, for her encouragement and patience during my years of graduate studies.
Chapter I

Coagulation of Skimmilk with Proteases

Immobilized on Hydrophobic Carriers
INTRODUCTION

Traditional methods of curd formation in the manufacture of cheese suffer from two major drawbacks; that is, the high cost of rennet and the labour intensiveness of batch systems (Cheryan et al., 1975b). A solution to both problems would be the use of immobilized proteolytic enzymes to clot milk, since the enzyme can be reused and a continuous throughput of milk can be employed.

The major problem encountered by Cheryan et al. (1975a, 1975b, 1976) and Taylor et al. (1977) was a rapid loss in activity of the immobilized enzymes. The longest active lifetime of proteases was about two days of continuous treatment of milk. Other disadvantages encountered in these studies were the high cost of carriers, the lengthy and complicated immobilization methods and the excessive desorption of pepsin from alumina into the effluent milk.

Hydrophobic adsorption offers certain potential advantages over other techniques for enzyme immobilization (Brash and Lyman, 1969; Butler, 1975). These are: (1) essentially irreversible enzyme binding; (2) the enzymes typically retain a very high percentage of their native activity; (3) since hydrophobic interactions tend to stabilize protein conformation, immobilized enzymes show increased resistance to denaturation; (4) reusability of the carrier; (5) simplicity and convenience; and (6) reproducibility in preparing enzyme catalysts. The objectives of this part of the thesis were to
immobilize the milk-clotting enzymes, chymosin and pepsin, on several hydrophobic carriers and to assess their suitability for continuous coagulation of skimmilk.
A. Introduction to immobilized enzymes

(a) Methods for enzyme immobilization

There have been a large number of methods reported in the literature for immobilizing enzymes. Most of these methods can be classified under one of the following four general categories:

1. Adsorption onto a water-insoluble carrier.
2. Entrapment, by occlusion within cross-linked gels or by encapsulation within microcapsules, hollow fibers, and fibers.
3. Cross-linking of the enzyme by bi- or multi-functional reagents.
4. Covalent binding to a reactive insoluble carrier, through functional groups which are not involved in the activity of the enzyme.

1. Adsorption

Historically, the earliest method of enzyme immobilization, namely adsorption, is also the easiest way of preparing enzyme-carrier conjugates. Adsorption of an enzyme can be achieved by simply mixing an enzyme solution with the finely-powdered adsorbent for some period of time after which the excess enzyme is washed off the insoluble adsorbent. A wide variety of solids have been used to adsorb enzymes. Some of the most popular adsorbents are activated carbon, organic polymers, glass, mineral salts, and silica gel. Adsorption for immobilizing enzymes has the following advantages: (1) it is cheap and easily carried out, since no reagents are required and the process involves only a minimum of activation steps (Barker and Kay, 1975); (2) the enzyme is less likely to be denatured during
the process of immobilization when compared with chemical methods of immobilizing enzymes (Kilara and Shahani, 1979); (3) the nature of the interactions are such that a high level of activity is retained by the adsorbed enzyme; (4) a wide variety of adsorbents can be used; (5) reusability of the adsorbent (Carr and Bowers, 1980); and (6) adsorption can yield reproducible preparations of the immobilized enzyme.

The binding forces between an enzyme and a carrier may be ionic, hydrophobic, hydrogen bonds, and/or Van der Waal's interactions, depending on the nature of the surface of the carrier. Since the bond energies are weak desorption of the bound enzyme can be brought about by changes in temperature, pH, ionic strength or the presence of substrate (Barker and Kay, 1975). Another disadvantage of adsorption immobilization is that the adsorption surface is non-specific, i.e., the surface does not selectively bind the enzyme, but will adsorb proteins or other substances to which it is exposed in the course of its use (Barker and Kay, 1975). A suitable adsorbent should possess high affinity for the enzyme and cause minimal denaturation (Brodelius, 1978; Goldstein and Manecke, 1976).

The reversibility of the enzyme attachment by adsorption requires a control of process conditions such as ionic strength, pH of the reactant solution, and temperature, in order to avoid desorption of the enzyme. If desorption of the enzyme is detrimental to the overall process, such as contaminating the product or allowing the catalytic process to proceed in solution, this reversible binding may be a serious
drawback (Stanley and Olson, 1974). Irreversible behaviour of enzymes has been reported, however, for a number of adsorption systems (Sundaram and Crook, 1971). Moreover, it is possible to stabilize the enzyme which has been temporarily adsorbed onto the matrix by cross-linking the enzyme molecules in a chemical reaction subsequent to its adsorption (Barker and Kay, 1975). Thus, some of the objectionable desorption characteristics can be removed, but carrier regenerability is sacrificed (Carr and Bowers, 1980).

By using the principles of affinity chromatography, more specific techniques for adsorption of enzymes have been developed. For example, "hydrophobic chromatography" has been used to achieve the virtually irreversible adsorption of several enzymes onto N-alkyl derivatives of Sepharose (Hofstee, 1973c; Visser and Strating, 1975). The bound enzymes (alkaline phosphatase, lactate dehydrogenase, urease, and xanthine oxidase) were not easily desorbed by aqueous solvents such as 1 M NaCl. The strong binding of these acidic enzymes to N-alkyl-Sepharose, known to carry residual positive charges could be attributed to a combination of hydrophobic and electrostatic interactions (Hofstee, 1973a, 1973b, 1973c). Therefore, insoluble polymers for use as biospecific adsorbents in affinity chromatography should not contain groups which would cause a decrease in specificity. Porath et al. (1973) have described the adsorption of proteins on a completely nonionic substituent of agarose as a new approach to hydrophobic adsorption. In aqueous solutions, the adsorbent showed an affinity for solutes with hydrophobic groups. Nonionic agarose exhibited hydrophobic interaction with hydrophobic regions on the molecular surface of proteins and other solutes. Porath
and his coworkers (Caldwell et al., 1976a, 1976b, 1976c, 1976d), subsequently, studied the immobilization of enzymes through hydrophobic interactions. They immobilized β-amylase and amyloglucosidase through adsorption onto hexyl-substituted epichlorohydrin-cross-linked agarose gels (Sepharose 6B), and subsequently used these adsorbates for continuous hydrolysis of starch over extended periods of time. The adsorption was rapid and the retention of the catalytic activity upon immobilization was high for both enzymes. The operational stability of the adsorbates was good during month-long continuous substrate percolation. Activity leakage (due to release of enzyme) was demonstrated but was low.

A second type of hydrophobic carrier has been produced by the reaction of phenoxyacetyl chloride with cellulose (Butler, 1975). Butler (1975) reported that phenoxyacetyl cellulose very strongly bound all 10 of the enzymes tested. The bound enzymes, which were not desorbed by 1 M (NH₄)₂SO₄ or moderate concentrations (25 - 50%) of nonaqueous solvents (e.g. ethylene glycol), but which effectively desorbed in solutions containing nonionic detergents (0.1% Triton X-100 in buffer), exhibited nearly complete retention of catalytic activity. Butler (1975), moreover, suggested that the strong noncovalent adsorption of the enzymes was due solely to hydrophobic interactions. The fact that all of the 10 enzymes tested were strongly bound to phenoxyacetyl cellulose suggested that affinity for certain hydrophobic groups may be a general characteristic of enzymes and other proteins. Phenoxyacetyl cellulose is commercially available from Regis Chemical Co., under the trade name ENZORB-A.
2. **Entrapment**

In principle, all entrapment methods are based on confining an enzyme in the lattice of a polymer matrix or enclosing an enzyme in a semipermeable membrane. Immobilization by entrapment, therefore, differs from other methods of immobilization (e.g. covalent binding or cross-linking) in the respect that the enzyme molecules are free in solution, but restricted in the room by a gel lattice or a membrane. The structure of the entrapping polymer should thus be tight enough to prevent the enzyme from diffusing out and at the same time be loose enough to allow diffusion of substrate and product. The most popular matrices for entrapment include polyacrylamide, silicone rubber, starch and silica gel. Occlusion within cross-linked polyacrylamide gels has been the most widely used entrapment technique.

In general, entrapment techniques have the following advantages: (1) as there is no bond formation between the enzyme and the polymer matrix, entrapment provides a method which is generally applicable to any enzyme (Barker and Kay, 1975; Goldstein and Manecke, 1976); (2) enzymes can be contained without any chemical modification of bonding that may lead to activity loss (Pitcher, 1980a); (3) they allow high local concentration of enzymes; and (4) multiple enzyme systems can be handled readily since entrapment is essentially the method living cells use to retain their enzyme systems (Pitcher, 1980a). Entrapment techniques, however, have several limitations: (1) the generality of this method (due to advantage No. 1) is limited by the fact that entrapment methods are suitable mainly for enzymes that utilize substrates of molecular weights low enough to diffuse through the matrix (Goldstein and Manecke, 1976). This disadvantage may serve as a limitation for their use in food
technology, since food systems often contain macromolecules (Kilara and Shahani, 1979); (2) entrapment results in enzyme loss by leakage of the enzyme from the matrix. This is due to the broad distribution in the pore size of the polymer. It should be noted, however, that ultrafiltration methods may be used to overcome the problem of leakage, because of the uniform pore size of the semipermeable membranes (Barker and Kay, 1975); (3) none of the stabilizing effects of bonding to a rigid support are available to the entrapped enzyme (Pitcher, 1980a); (4) reproducible preparations are difficult to make (Stanley and Olson, 1974); and (5) mass transfer limitations in conjunction with the physical properties of the entrapped enzyme devices places some constraints on the reactor design (Pitcher, 1980a).

3. **Cross-linking**

This immobilization method is based on the formation of intermolecular cross-linkages between the enzyme molecules or between protein and enzyme molecules by means of bi- or multi-functional reagents. The most commonly used bifunctional reagent is glutaraldehyde, a dialdehyde that reacts with primary amines to form stable linkages. Although the matrix may contain just enzyme molecules, it is usually in the interest of economy to copolymerize the enzyme with an inert protein such as albumin in order to increase the bulk of the final product (Trevan, 1980). The advantages of the cross-linking are its relative simplicity and the fact that a single reagent can be used to prepare numerous enzyme derivatives. This method has the following disadvantages: (1) cross-linking an enzyme to itself is both expensive and inefficient, as part of the enzyme will be inevitably functioning as a support, resulting in relatively low enzymic activity
(Barker and Kay, 1975; Kilara and Shahani, 1979); (2) the polymerization of the enzyme directly with bifunctional reagents suffers from a lack of selectivity (Carr and Bowers, 1980), that is, it is extremely difficult to control intramolecular cross-linking while obtaining a high degree of intermolecular cross-linking; (3) it is difficult to control the size and mechanical properties of the polymer; and (4) many enzymes are sensitive to the coupling reagents and lose activity in the process (Guilbault, 1975). Cross-linking of the enzyme, however, has proven valuable in combination with other immobilization techniques. For example, enzymes adsorbed on insoluble supports have been cross-linked to minimize leakage of the enzyme from the support and thus to increase stability of the immobilized enzyme preparation (Goldman et al., 1968; Liu et al., 1975; Taylor et al., 1977).

4. Covalent binding

The covalent binding method involves the linking of enzymes and water-insoluble carriers by covalent bonds, and is the most intensely studied of the enzyme immobilization techniques. The functional groups of enzymes available for covalent binding include N-terminal amino groups, ε-amino groups of lysine and arginine, C-terminal carboxyl groups, β- and γ-carboxyl groups of aspartic and glutamic acids, respectively, phenol group of tyrosine, the sulfhydryl group of cysteine, the hydroxyl groups of serine and threonine, the imidazole group of histidine, and the indole group of tryptophan (Barker and Kay, 1975; Kilara and Shahani, 1979). Of these, the most widely used are the first three. In coupling reactions, these functional groups react with carriers containing reactive groups such as diazonium, acid azide, isocyanate and halides. Undoubtedly, the greatest
advantage of this method is the durability of the derivatives. Thus, variations in pH, ionic strength, substrate, solvents, and temperature will not normally cause the enzyme desorption problem which is usually exhibited by certain adsorbed enzyme systems (Guilbault, 1975; Pitcher, 1980a). Binding is not necessarily permanent, however, as diazo and sulfur-sulfur linkages have been reported to be unstable under certain conditions (Stanley and Olson, 1974). Another advantage is that enzymes attached by covalent binding may be stabilized by virtue of being tied down in several places (Falb, 1972). The major disadvantage of this method is that the reaction conditions required for covalent binding are relatively complicated and not particularly mild. Covalent binding, therefore, frequently alters the conformational structure of the enzymes and may change their reactivity. Moreover, in the extreme case, the active site of the enzyme may be blocked through the chemical reaction used in the attachment reaction and the enzyme is rendered inactive (Falb, 1972).

Covalent binding has not been found to be used as extensively in the food processing industry as one might expect from the numerous articles describing different chemical coupling schemes. One obvious reason, is that many of the highly toxic reagents used in the coupling processes would not be suitable for applications where they would come in contact with foods (Pitcher, 1980a).

From the above description of the methods for enzyme immobilization, it is evident that there is no single method that is universally applicable, and a number of factors (such as enzyme stability, mechanical properties and cost of the carrier, as well as carrier regenerability and capacity for enzyme binding) have to be considered prior to choosing a method. In practice, therefore, it is necessary to find a suitable method and conditions
for the immobilization of a particular enzyme according to the intended application.

(b) Properties of immobilized enzymes

The properties of an immobilized enzyme system can be quite different from those of the corresponding soluble enzyme system. These changes can be attributed to alterations of the enzyme conformation or to the physico-chemical nature of the carrier. The activity of enzymes almost always decreases as a result of the immobilization. This decrease may be ascribed to the following factors (Chibata, 1978): (1) functional groups of essential amino acids in the active center of the enzyme participate in binding the enzyme to water-insoluble carriers; (2) conformational change of the enzyme occurs when the enzyme is bound to an insoluble carrier; and (3) the enzyme may be bound without loss of activity, but the interaction of substrate with the enzyme is affected by steric hindrance. The extent of the decrease in activity depends on the immobilization technique, conditions required for enzymic treatment, characteristics of supporting materials, diffusion rates of substrate to and product from the enzyme, and molecular weight of the substrate (Olson and Richardson, 1974).

One of the most important features of an immobilized enzyme used in a large scale operation is its stability. Most enzymes when immobilized show a greater stability than their soluble counterparts. It has been suggested that this increased stability could be the result of reduced conformational inactivation, reduced autolysis (autodigestion), and reduced attack by reactive solutes due to steric shielding (Brodelius, 1978; Trevan, 1980). Stability of immobilized enzymes refers to several factors including operational stability, storage stability, and temperature
and pH stability. The operational stability of the activity of immobilized enzymes is one of the most important factors affecting the success of industrialization of an immobilized system. Operational stability is usually expressed as half-life. The half-life of various immobilized enzymes varies widely. A few immobilized enzymes (e.g. glucose isomerase, amino acylase) have long half-lives permitting industrial application. Most of them, however, exhibit short half-lives. The storage stability of an immobilized enzyme becomes important when the enzyme preparation is used only intermittently. Immobilized enzymes may exhibit better storage stability than soluble enzymes. The storage stability of 50 immobilized enzyme systems have been reviewed by Melrose (1971). Of these, 12 systems showed no difference in stability relative to their soluble counterparts, 8 showed decreased stability and 30 exhibited greater storage stability. This indicates that one can generally expect enhanced storage stability.

The catalytic activity of enzymes increases with elevation of the temperature. However, as enzymes are proteins and thus are susceptible to heat denaturation (inactivation), the enzyme reaction cannot be practically carried out at high temperature. If the heat stability of an enzyme is, therefore, increased by immobilization, the potential utilization of such enzymes will be extensive. The heat stability of many immobilized enzymes has been studied, and there are examples showing increases, no change, and decreases of heat stability of enzymes upon immobilization.

Stability of immobilized enzymes toward variation in pH values depends upon physical and chemical characteristics of the enzyme carrier and chemical modification of the enzyme brought about by binding of enzyme to carrier. It has been reported, that polyanionic derivatives of proteases (trypsin, chymotrypsin and papain) exhibited improved stabilities toward
alkaline pH values (Goldstein, 1970; Levin et al., 1964). On the other hand, polycationic derivatives possessed greater stability in the acidic pH range (Goldman et al., 1971). Shifts in pH optima upon immobilization have been found for many enzymes. The changes in optimum pH (and pH-activity curve) depend on the charge of the water-insoluble carrier and/or of the enzyme protein. The shifting of the pH optimum for an enzyme may be rationalized as a microenvironmental effect. The environment in the immediate vicinity of an immobilized enzyme often differs from that in the bulk phase. Thus, if the enzyme is bound to a polyelectrolytic support, electrostatic interactions will lead to an unequal distribution of the ions between the carrier phase and the bulk phase. Within a polyanionic carrier (e.g. carboxymethyl cellulose) the concentration of the positively charged ions will be higher than in the external solution. Consequently, the pH inside such a carrier will be lower than in the surrounding media and the pH optimum of the immobilized enzyme will be shifted toward a higher value (i.e. more alkaline pH) to compensate for the increased local hydrogen-ion concentration. On the other hand, in a polycationic carrier (e.g. DEAE-cellulose), the opposite effect will be expected, i.e. the pH optimum for the immobilized enzyme will be lower than for the native enzyme (Brodelius, 1978; Carr and Bowers, 1980). By proper selection of support material, therefore, such shifts in pH optima could permit the use of enzymes in environments which would normally inactivate them. Thus, it may be advantageous to raise the pH optimum of acid proteases in treating foods at pH values closer to neutrality. On the other hand, shifting the pH toward acidic values could aid in controlling microbial growth during continuous processing or it could increase the activity of enzymes in acidic foods (Olson and Richardson, 1974).
(c) Advantages of immobilized enzymes

The rationale for the replacement of existing applications of soluble enzymes by immobilized enzymes is based on the following advantages which immobilized enzymes can offer over soluble enzymes in many areas such as study of enzymes, analytical biochemistry, pharmacology, and industrial processing including food processing (Barker and Kay, 1975; Olson and Korus, 1977; Taylor et al., 1976): (1) an immobilized enzyme acts as a stable biocatalyst to bring about chemical reactions on a continuous basis. In this respect, the enzyme cost would be lower; (2) the reaction is easily terminated by separating the substrate from the enzyme; (3) the enzyme is not left in the product. Thus, enzymes can be used which are presently unusable for various reasons (e.g. microbial rennets). Moreover, not only the cost of heat inactivation of the enzyme is saved but also the detrimental effect of heating to some foods is avoided; (4) increased pH and temperature stability; (5) choice of continuous or batch operation; (6) better quality control of the produce (because of the continuous process); (7) less product inhibition due to the easier separation of the product and substrate; (8) advantageous use of multiple enzyme systems; (9) greater reactor design flexibility due to the large number of different physical forms of solid matrices; and (10) potential operation over greater pH range by modifying the charge characteristics of the carrier.

Immobilized enzymes have potential disadvantages which include: (1) the cost of support and immobilization procedure; (2) the lower activity or loss of enzyme activity upon immobilization; (3) the inactivation with continued operation; (4) greater initial plant investment; (5) more technically complex process requiring more skilled supervision; and (6) applicable mainly to soluble substrates.
Although the above generalizations can provide some guidelines, the final considerations which determine whether it is advantageous to immobilize an enzyme depend on the particular process one is considering (Olson and Korus, 1977).

(d) Potential applications of immobilized enzymes in the food industry

Although every field that utilizes enzymes has found potential uses of immobilized enzymes, their greatest potential is in the food industry. Table 1 summarizes some of the enzymes used in the food industry and their applications.

Despite the large volume of research work carried out on the potential applications of immobilized enzymes, relatively few processes using immobilized enzymes have been commercialized. Currently there are only three immobilized systems in commercial use due to their economy and practicality. These are the glucose isomerase system for producing high fructose corn syrup (HFCS), the amino acylase system used for the resolution of D- and L-amino acids, and the penicillin acylase system used in the pharmaceutical industry to obtain 6-aminopenicillanic acid (Kilara and Shahani, 1979). The large-scale use of immobilized enzymes in food processing has been prevented by two major problems (Cheryan, 1974). One major problem has been the frequent plugging of the reactors possibly due to microbial growth in the columns, but more probably due to the presence of suspended matter in the substrate which clogs the interstices of the catalyst bed. This problem, however, can be overcome by the proper selection and design of the reactor and proper choice of operating parameters. Another major problem has been the relatively poor stability characteristics shown by some immobilized enzymes in contact with
Table 1 - Potential applications of immobilized enzymes in the food industry

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acylase</td>
<td>Resolution of D- and L-amino acids</td>
</tr>
<tr>
<td>a-Amylase</td>
<td>Starch degradation (liquefaction)</td>
</tr>
<tr>
<td>3-Amylase</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>Cold Sterilization of milk</td>
</tr>
<tr>
<td>Cellulase</td>
<td>Convert cellulose to glucose</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>Hydrolyze lactose in milk and whey</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>Deoxygenate foods; desugar eggs</td>
</tr>
<tr>
<td>Glycoamylase</td>
<td>Production of glucose from starch</td>
</tr>
<tr>
<td>Glucose isomerase</td>
<td>Production of fructose from glucose</td>
</tr>
<tr>
<td>Invertase</td>
<td>Hydrolyze sucrose from production of invert sugar and confections</td>
</tr>
<tr>
<td>Lipase</td>
<td>Enzymic modification of flavor of foods</td>
</tr>
<tr>
<td>Naringinase</td>
<td>Debitter clarified citrus juices</td>
</tr>
<tr>
<td>Papain, Ficin, Bromelain</td>
<td>Chillproofing of beer</td>
</tr>
<tr>
<td>Pectinase</td>
<td>Fruit juice clarification and yield improvement</td>
</tr>
<tr>
<td>Pepsin, Rennin, Chymotrypsin</td>
<td>Continuous coagulation of skim milk for cheese-making; production of protein hydrolysates</td>
</tr>
<tr>
<td>or other proteases</td>
<td></td>
</tr>
<tr>
<td>Peroxidases</td>
<td>Cold sterilization of foods</td>
</tr>
<tr>
<td>Sulphydryl oxidase</td>
<td>Elimination of the cooked flavor in UHT sterilized milk</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Inhibition of oxidative rancidity in milk</td>
</tr>
</tbody>
</table>
their natural substrates (especially protein-containing substrates). Thus, the immobilized enzyme preparations tend to lose activity too rapidly for commercial acceptability.

B. Continuous coagulation of skimmilk with immobilized proteases

(a) Mechanism of milk coagulation and theoretical basis for the system

Milk is a complex fluid consisting of numerous proteins, fat, lactose and minerals. The major proteins are the caseins, comprising 80% of the total protein. The remaining proteins, whey proteins, are soluble in the serum. Casein is a quite complicated mixture of proteins, which can be generally fractionated into \( \alpha_s, \beta, \kappa, \) and \( \gamma \)-caseins although many other subfractions have been described. The casein proteins are present in milk as stable micelles (agglomerated macromolecular spherical particles) with the various components together as "calcium caseinate". Additional complexity occurs with calcium phosphate as well as with magnesium and citrate ions (Gordon and Kalan, 1974). When milk is coagulated, it is the stability of this casein micelle that is disrupted.

Coagulation of milk by milk-clotting enzymes is a remarkably complex process. In this process, two rather distinct phases can be distinguished: a primary or enzymatic phase and a secondary or non-enzymatic phase (Ernstorm and Wong, 1974). In the primary phase the proteolytic enzyme cleaves a specific phenylalanyl-methionine bond of \( \kappa \)-casein, releasing a negatively charged hydrophilic glycomacropeptide and para-\( \kappa \)-casein, thereby destabilizing the micelles. In the secondary phase, which requires calcium, there is a physical aggregation of the destabilized micelles which results in the formation of a coagulum. The mechanism of micelle aggregation is not completely elucidated, but the negative charge on micelles is reduced by the loss of the glycopeptides allowing further
interaction of micelles (Green and Crutchfield, 1971; Green and Marshall, 1977; Payens, 1966; Pearce, 1976).

It is feasible to develop a system for continuous coagulation of milk employing immobilized proteolytic enzymes because of a peculiarity of the coagulation process. The large temperature coefficient ($Q_{10}$) of the secondary phase ($Q_{10}=11-12$) but the low $Q_{10}$ of the primary phase ($Q_{10}=2$) permits separation of the two phases by lowering the temperature in the immobilized enzyme reactor (Cheryal et al., 1975a). Thus, the immobilized enzyme retains sufficient activity at lower temperatures (e.g. 5 - 15°C) to complete the primary enzymatic phase but clotting does not occur until after the milk is removed from the immobilized enzyme reactor and warmed. The two phases can also be separated by maintaining the milk at high pH (6.7), since both phases of enzymatic milk coagulation are pH dependent - and the secondary phase is highly pH dependent. A 30-fold decrease in the clotting time, when the pH of the secondary phase was lowered from 6.7 to 5.6, has been reported by Cheryan et al. (1975a).

(b) Advantages of using immobilized proteases in cheese manufacture

Clotting milk with immobilized proteases for the manufacture of cheese offers the following specific (and potential) advantages over the traditional method: (1) there has been an ever increasing worldwide shortage (and thus high cost) of calf rennet for the past 20 years, created by the increasing demand for both beef and cheese, prompting use of other suitable proteases in traditional cheesemaking systems (Taylor et al., 1976). However, these substitutes have some limitations (due to excessive proteolysis, defective flavor, body and texture, color development and the production of toxins and pathogens). Shortage of milk-clotting enzymes may
be eased by employing immobilized enzymes which can be reused; (2) since
the immobilized enzyme does not contaminate the curd, other proteases,
which are not suitable for use in the soluble form because of excessive
uncontrolled proteolysis, could be used in an immobilized state. Thus, it
may be possible to substitute a less expensive, less desirable, but more
readily available enzyme which normally cannot be used, such as crude
microbial proteases, instead of commercially available milk-clotting
enzymes (Richardson and Olson, 1974); (3) the lack of contamination of
cheese with milk-clotting enzymes would allow, moreover, separation of
the milk-clotting and cheese-ripening functions of immobilized proteases.
Consequently, the immobilized proteases could be chosen for their optimal
milk-clotting activity and for the effects on cheese yield and characteristics of cheese curd, while soluble proteases added to the curd could be
selected for their optimum cheese-ripening action (Taylor et al., 1979).
The enzyme used in the traditional process is chosen for its milk-clotting
capacity as well as for its influence on cheese-ripening process, that is,
the choice of enzyme is a compromise between its milk-clotting and cheese-
ripening activities (Brodelius, 1978; Richardson and Olson, 1974). As
a result, cheese-ripening is much less controllable in the traditional
method; (4) greater flexibility and control over cheese ripening and
merchandising. Thus, if less enzyme is added, the cheese could be stored
longer (because of the longer ripening time), allowing better coordination
between seasonal fluctuations in milk supply and cheese demand (Taylor
et al., 1979); and (5) immobilized proteases could fit into a continuous,
semi-continuous or batch cheese-making process, depending upon requirements
(Taylor et al., 1979).

The above specific advantages of a continuous cheese making process
based on an immobilized protease system help in explaining the continual efforts made to develop such a system.

(c) **Enzymes, carriers, methods of immobilization and reactor designs used in preparing immobilized milk-clotting proteases**

Several proteolytic enzymes such as chymotrypsin, *Mucor miehei* proteases, papain, pepsin, chymosin, rennet and trypsin have been immobilized for use in milk coagulation. Although a direct comparison of the performance of these catalysts is difficult (since the carrier and immobilization technique have also a great effect on the catalyst activity), Taylor *et al.* (1976) suggested that, based on the literature, pepsin appears to be the best immobilized protease for coagulating milk. Pepsin, an enzyme with a low pH optimum and not particularly active nor stable at the normal pH of milk, is quite active and stable upon immobilization (Cheryan *et al.*, 1975b; Cheryan *et al.*, 1976). Presumably immobilization of pepsin places it in a microenvironment of lower pH than the bulk solution which stabilizes its tertiary structure (Taylor *et al.*, 1979). Rennet was found to be more active but less stable than pepsin (Cheryan *et al.*, 1975b). It is interesting to note that, contrary to the behaviour of commercial calf rennet, chymosin exhibited very low (Arima *et al.*, 1974; Green and Crutchfield, 1969; Thonart *et al.*, 1978) or no activity at all (Cheryan *et al.*, 1975b) when immobilized. Since most commercial rennet preparations contain bovine pepsin, it has been suggested by Cheryan *et al.* (1975b) that the activity observed with immobilized rennet was probably due to the immobilized bovine pepsin.

In order for the coagulation of milk with immobilized proteases to be commercially feasible, the carrier material should be reasonably
inexpensive, physically suitable and stable for the reactor design, non-toxic, and allow good enzyme-substrate contact and proper flow of substrate. A great variety of carrier materials have been used to immobilize milk-clotting proteases. Agarose (Angelo and Shahani, 1979; Arima et al., 1974; Green and Crutchfield, 1969), aminoethyl cellulose (Arima et al., 1974; Green and Crutchfield, 1969), and paraffin wax (Savangikar and Joshi, 1978; Shindo et al., 1980a, 1980b) are some of the organic carriers used for immobilizing milk-clotting enzymes. However, the most successful immobilized enzyme-carrier preparations have been obtained with inorganic carriers such as alumina, porous glass, and titania (Cheryan et al., 1975b; Cheryan et al., 1976; Taylor et al., 1977).

Covalent attachment and adsorption are the two major methods reported in the literature for immobilizing milk-clotting proteases. Entrapment of milk-clotting enzyme within a gel or membrane has not been attempted because such a method will not be workable since the substrate (casein) is too large to diffuse in and out of the enzyme-trapping matrix. Covalent binding has been the most widely used method for immobilizing milk-clotting enzymes. This is due to the fact that covalent binding allows little or no desorption of the milk-clotting enzymes into the curd (Cheryan et al., 1975a, 1975b; Ferrier et al., 1972; Hicks et al., 1975). Although this method is preferable for laboratory studies, for practical application to commercial cheese-making, it may be too complicated and costly. On the other hand, adsorption is a simple, mild and inexpensive method, but desorption of protease may be a problem, since the ionic strength of milk may promote enzyme desorption. Prevention of the desorption of the immobilized milk-clotting enzyme is very important in
order to minimize catalyst inactivation and to control proteolysis in cheese curd during aging.

One of the great advantages of using immobilized enzymes to coagulate milk is the flexibility in reactor design. Three common reactor designs - fluidized bed, packed bed and stirred tank - have been reported in the literature. The packed bed reactor is usually limited by milk plugging of the column. The plugging problem can be avoided by using a continuously stirred tank reactor, but the shear forces developed limit the types of carriers to those having sufficient structural integrity (Taylor et al., 1976). Plugging can also be eliminated by using a fluidized bed reactor. Cheryan et al. (1975b) reported that fluidized bed reactor performed equal or superior to the fixed bed reactor under almost all conditions. Another advantage of fluidized bed is that it can be used with carriers (e.g. porous glass) that would disintegrate in a stirred tank reactor.

(d) Activity and stability of immobilized milk-clotting proteases

Performance of an immobilized enzyme-carrier preparation depends upon both activity and stability. A catalyst preparation with high initial activity and a slow inactivation rate is preferred.

A relatively great number of studies have been conducted on the immobilization of milk-clotting enzymes and their use in cheese production. Green and Crutchfield (1969) prepared enzymically active insoluble derivatives of chymotrypsin and chymosin, using cyanogen bromide activated agarose as the carrier (matrix). They also immobilized chymosin on aminoethyl cellulose. Each of the insoluble enzyme derivatives apparently catalysed the clotting of milk, but in all cases this activity was shown to be almost entirely due to enzyme released into solution (milk) from
the carrier. Thonart et al. (1978) immobilized chymosin on glass bead by diazotization. The initial activity of this preparation was very low (8 - 10% of the activity of free enzyme), and after one hour of continuous coagulation of milk, immobilized chymosin retained only 40% of its initial activity. It was, furthermore, suggested by these authors that this loss of activity was due to adsorption of milk protein onto the carrier and desorption or solubilization of the active enzyme molecules from the carrier. Ohmiya et al. (1978, 1979) immobilized an alkaline protease from Bacillus subtilis on an anion exchange resin (Dowex MWA-1) by glutaraldehyde and studied the feasibility of using this preparation for cheese-making. They concluded that the alkaline protease could be used for cheese making as a milk-clotting enzyme instead of calf rennet, if the proteolytic activity to release sialic acid was properly controlled. These workers, however, failed to report any economic study substantiating their claim. Shindo and coworkers (Shindo and Arima, 1979a, 1979b; Shindo et al., 1980a, 1980b) have extensively studied the preparation, properties and utilization of immobilized chymosin in cheese-making. Immobilized chymosin prepared by paraffin wax showed relatively high stability to repeated enzymatic reaction (Shindo et al., 1980a).

Furthermore, Gouda type cheese was produced by Shindo et al. (1980b) with this immobilized chymosin preparation. These authors concluded that immobilized chymosin using paraffin wax could be useful for cheese-making if a suitable manufacturing method was applied. It should be noted, however, that no economic comparison between free and immobilizing chymosin preparation was reported, and therefore, these authors failed to reveal any economic advantage of their batch method over the traditional cheese-making process. Angelo and Shahani (1979) immobilized rennet on
Sepharose 4B through covalent bonding with an objective to assess its suitability for milk coagulation in continuous cheese-making. Immobilized rennet coagulated skim milk within an average of 18 min (range 7 to 30 min). A decline in the milk clotting activity of a packed bed reactor of immobilized rennet occurred in 3 hr run. No "soluble activity" of immobilized rennet was detected. The most successful system among the immobilized milk-clotting proteases reported in the literature appear to be that described by Taylor et al. (1977). The three best pepsin-carrier preparations were obtained with titania (controlled pore ceramic) and glass (zirconium oxide-coated controlled pore), using a covalent attachment procedure (glutaraldehyde) and alumina (controlled pore ceramic), using adsorption at pH 1.2. The reactor was operated as a fluidized bed at low temperature, and coagulation of the milk was achieved by acidifying (pH 6.1) and then warming (30°C) after the milk emerged from the reactor. Although the initial clotting activity was high, the active lifetime of the catalyst was not more than 2 days. Loss of enzymatic activity was very rapid during the initial stages of continuous coagulation of skim milk. The rate of loss of activity was slower but exponential during subsequent operation of the reactor. Inactivation during use of the reactor appeared to be caused in part by deposition of proteins and peptides on the immobilized enzyme (Cheryan et al., 1975; Ferrier et al., 1972). Accumulation of nitrogenous material and sialic acid paralleled the initial rapid loss in enzymatic activity. Both whey proteins and caseins appeared to be responsible for loss in activity. In addition, desorption of pepsin from the alumina carrier during the continuous reactor operation was observed (Taylor et al., 1977). Inactivation during continuous usage was minimized by precoating the carrier with proteins (BSA) and then coupling pepsin to
the coated supports (Cheryan et al., 1976). Coating the supports with proteins probably increased catalyst stability by decreasing the positive charge of the carrier and thus decreasing the adsorption of negatively charged milk proteins. Attempts to restore the activity of inactivated catalysts were only partially successful. Thus, washing immobilized pepsin with water restored some activity but maximum regeneration was obtained by washing with dilute hydrochloric acid (0.1 N) or urea at pH 3.5 (Cheryan et al., 1975b; Taylor et al., 1977). On subsequent reuse of the regenerated immobilized pepsin, a rapid loss in activity was observed and the catalyst exhibited a memory in returning to the activity level prior to regeneration. Reasons for this memory effect, which has been observed with porous glass as well as alumina have not been defined. Furthermore, two other attempts to regenerate immobilized pepsin activity using polyethylene glycol and Triton X-100 resulted in complete loss of activity (Taylor et al., 1977).

Taylor et al. (1977) suggested that pepsin adsorbed on alumina may have commercial potential for cheese making and promised an economic study which has never been been reported. It should be pointed out that the encouraging results presented by Taylor et al. (1977) were obtained by using controlled-pore alumina as enzyme carrier which is an expensive chromatographic material. However, adsorption of pepsin to a number of inexpensive industrial-grade aluminas did not reproduce the catalytic activity obtained with the expensive controlled-pore alumina (Skogberg, 1976; Taylor et al., 1977). Differences in surface area and/or surface charge may be responsible for the poor results obtained with the industrial-grade aluminas (Taylor et al., 1979). Olson and Richardson (1979) of the same group in their last communication suggested that
immobilized enzymes may have application in cheese manufacturing if the economics of the process are improved, and that this would require cheaper enzyme supports, more complete immobilization of enzyme, better enzyme stability on exposure to milk or means of regenerating lost enzymatic activity. According to Sardinas (1976) the undeniable promise of the continuous coagulation of milk with immobilized proteases is sufficient challenge to assure its continued development.

MATERIALS AND METHODS

A. Materials

Distilled, deionized water was used throughout except for washing of catalysts, which was done with distilled water. All chemicals used in this study were reagent grade or better. Pasteurized skimmilk, pH 6.7, was purchased from the local market. Enzymes used included pepsin (from porcine stomach mucosa, crystallized and lyophilized), from Sigma Chemical Company, Saint Louis, Mo., and rennin (N.F.), from Nutritional Biochemical Corporation, Cleveland, Ohio. Other reagents included epichlorohydrin, from Fisher Scientific Company, Fair Lawn, N.J.; Sodium borohydride ($\text{NaBH}_4$), from Sigma Chemical Company, Saint Louis, Mo.; 1-bromohexane, from Eastman Kodak Co., Rochester, N.Y. Support materials (carriers) used include Sepharose 6B, from Pharmacia Fine Chemicals, Uppsala, Sweden; Phenoxyacetyl cellulose, containing 0.3 to 0.5 phenoxyacetyl groups per glucose moiety, from Regis Chemical Co., Morton Grove, Ill.; and activated carbon 12 x 20 mesh, from Darco ICI America Inc., Wilmington, Del.
B. Preparation of hexyl-substituted epichlorohydrin-crosslinked desulfated Sepharose 6B

Agarose gel of type Sepharose 6B was stabilized through crosslinking with epichlorohydrin prior to the introduction of the aliphatic ether substituents. Epichlorohydrin crosslinked desulfated Sepharose 6B (ECD-Sepharose 6B) was prepared according to the method of Porath et al. (1971) as follows: 500 ml of swollen agarose beads were mixed at room temperature with 500 ml of 1N NaOH containing 50 ml epichlorohydrin and 2.5 g sodium borohydride. The mixture was heated to 60°C for 2 hr with adequate stirring. The crosslinked gel so obtained was washed with hot distilled water to neutral pH. 500 ml of 2N NaOH and 2.5 g NaBH₄ were added to 500 ml of the suspension and the mixture was heated in an autoclave at 120°C for 1 hr. The gel was washed with 1.5 L of hot solution of 1N NaOH containing 0.5% NaBH₄ and then with 1.5 L of cold solution of the same composition. The gel was quickly transferred to a beaker containing finely crushed ice and acetic acid was added to bring the pH down to 4.0. The gel was again transferred to a Büchner funnel and washed with hot distilled water (to remove the remaining traces of boric acid) and finally with ice water. The gel was stored as a suspension in 0.02% sodium azide solution.

Hexyl-substituted ECD-Sepharose 6B (hexyl-ECD-Sepharose 6B) was prepared according to the method of Caldwell et al. (1975) as follows: 100 mg of sedimented ECD-Sepharose 6B, suspended in 100 ml of 5 N NaOH containing 0.5% NaBH₄, were mixed with 50 ml of 1-bromohexane. The reaction mixture was kept at 100°C for 35 hr under gentle stirring. After careful washing with water, and subsequently with ethanol, until a neutral pH was reached, the product was again washed with distilled water and stored as
an aqueous suspension.

C. Immobilization of pepsin on hexyl-ECD-Sepharose 6B

Slurry of the gel was packed in a column of 1.0 cm internal diameter and the amount adjusted so as to give a bed volume of 0.6 ml. The bed was washed with distilled water for settling and then with 10 ml of 0.1 M acetate buffer pH 4.6 containing 3 M NaCl for equilibration. A solution of pepsin (10 mg porcine pepsin in 3 ml of 0.1 M acetate buffer containing 3 M NaCl) was passed through the gel which was subsequently washed with the equilibration buffer until the eluate showed negligible UV absorption at 280 nm. The amount of enzyme adsorbed at this point was estimated from the difference in absorbance at 280 nm of the pepsin solution before and after adsorption to the gel. The column was then washed with distilled water pH 6.7 until the pH of the eluate was 6.7. Subsequently, cold pasteurized milk pH 6.7 was applied to the column which was operated as a fixed bed reactor at a flow rate of 8 ml/hr in a cold room (4°C).

D. Immobilization of pepsin and chymosin on phenoxyacetyl cellulose

To 2.5 g of phenoxyacetyl cellulose (PAC) sufficient mixture of ethanol-water (1:1) was added to cover the phenoxyacetyl cellulose powder in a beaker. The resulting slurry was stirred and the trapped air bubbles were allowed to rise. The slurry was then poured into a column (1 x 30 cm). The bed was washed with 30 ml of ethanol-water to settle packing, then with 30 ml of deionized water to expose the phenoxyacetyl cellulose to aqueous environment, and finally with 30 ml of 0.1 M acetate buffer pH 4.6. A solution of pepsin (15 mg pepsin in 4 ml 0.1 M acetate buffer pH 4.6 containing 3 M NaCl) was pumped through the PAC column. The unbound
enzyme was washed from the bed by passing fresh buffer through the column. The column was, subsequently, washed with distilled water pH 6.7, until the pH of the effluent was 6.7. Then, cold pasteurized skimmilk was applied to the column. A constant flow rate of 36 ml/hr was maintained with a peristaltic pump. For chymosin immobilization, a dispersion of 300 mg rennin in 5 ml of 0.1M acetate buffer, pH 6.2 containing 3M NaCl was centrifuged at 6,950 x g (7,500 rpm) for 10 min and only the supernatant was applied to the column.

E. Immobilization of pepsin on activated carbon

The activated carbon granules (12 x 20 mesh) were ground in a mortar with a pestle and they were sieved with standard sieves (30/40 mesh) to obtain 425-589 μm diameter particles for enzyme immobilization, washed thoroughly with 1 L of warm deionized water and dried at 105°C overnight. The carbon, thus obtained, was designated as untreated activated carbon (UAC). Hydrochloric acid-treated activated carbon (TAC) was prepared according to the method of Cho and Bailey (1979) as follows: 20 g of UAC were treated in a Soxhlet extraction apparatus for 48 hr with 1.7N HCl. The carbon particles were then rinsed with warm distilled water and then warm deionized water until no chloride ion was detected and dried at 110°C overnight. Chloride ion detection was done according to Hogness et al. (1966).

Two methods of enzyme immobilization were investigated. Adsorption and covalent binding. The adsorption immobilization was carried out as follows: 100 mg of pepsin were dissolved in 12.5 ml of distilled water. To this solution 12.5 ml of 0.4N HCl were added (dropwise with gentle stirring) to bring the pH down to 1.2. This enzyme solution was poured
into a 125 ml flask and a 5 g of UAC or TAC were added. The mixture was
gently shaken at room temperature for 30 min on a shaking bath. The
immobilized preparation was thoroughly washed with 4 L of water at pH
3.5 and 0.5 L water at pH 6.7, and then transferred into a column
(1.6 x 20 cm).

Covalent immobilization of pepsin was carried out according to the
method of Cho and Bailey (1977). Thus, 5 g of UAC or TAC were mixed with
10 ml of 0.1M acetate buffer pH 4.6. To this slurry 100 mg of water
soluble diimide [l-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide
metho-p-toluene sulfonate] was added with gentle shaking. After 10 min,
100 mg of pepsin in 5 ml buffer was added, and the immobilization was
carried out at room temperature for 2 hr with mild shaking. Finally, the
immobilized enzyme-carbon preparation was washed with 100 ml of 0.1M
acetate buffer and then with 3 L of distilled water pH 6.7 prior to
transfer into the reactor. The column was operated as a fluidized bed
(upward flow), since preliminary studies with a fixed bed column proved
this design to be unsuitable due to plugging of the column.

F. Immobilized enzyme assays

Activity of milk-clotting enzymes is normally expressed as the time
(min) for milk to clot under standardized conditions after adding the
enzyme to the milk (Ernstrom and Wong, 1974). Clotting time is related
inversely to enzymic activity (Hicks et al., 1975). Immobilized enzyme
preparations in this study were assayed for activity and stability as
described by Cheryan et al. (1975b). Activity was expressed as clotting
time, which was the-time in minutes for effluent skimmilk from the reactor
to clot under standardized conditions after treatment with immobilized
enzyme. Effluent skimmilk was collected in a 5 ml graduated cylinder cooled in an ice-bath and 2 ml sample was quickly added to a prewarmed (30°C) 125 ml flask with a pipette chilled in ice. The required amount of 2N phosphoric acid was added (generally about 28 μl/2 ml skimmilk) to bring the pH of the milk to 6.1. The flask was then quickly attached to the arm of a rotor (forming a constant angle of about 30° with the water surface) and placed in a water bath of 30°C and rotated continuously at a speed of about 10 rpm. The timer was started just after the flask was immersed into the water. Clotting time was taken when the first visible signs of coagulum formed on the sides of the flask.

"Soluble activity" of an immobilized enzyme preparation is the activity due to desorption or solubilization of the enzyme from the carrier. This activity was determined by adding the whey from a coagulated milk sample to a fresh untreated milk sample (2 ml) at the same pH and measuring the clotting time as described above. The clotting time of the mixture was used as an index of soluble activity.

Catalyst stability was described by the rate of catalyst deactivation at any point of operation (time on stream) of the reactor. Since the rates of deactivation of milk-clotting enzymes were shown to be logarithmic with the time (Cheryan et al., 1975b), stabilities on exposure to milk were most conveniently expressed in terms of "D values", i.e.: the time in hours for the clotting time to increase by one logarithmic cycle. The "D value" was determined from the plot of log clotting time versus time on stream by drawing a tangent to the clotting time curve. The time for the tangent to transcend one log cycle was a "D value". Higher D values would indicate greater catalyst stability.
RESULTS AND DISCUSSION

A. Immobilization of pepsin on hexyl-ECD-Sepharose 6B

Sepharose has been widely used as a support material for enzyme immobilization (Angelo and Shahani, 1979; Green and Crutchfield, 1969; Caldwell et al. 1975, 1976a, 1976b, 1976c, 1976d). Its usefulness is mainly due to features such as a large porosity in combination with mechanical stability. If the immobilized enzyme is to serve as a catalyst for a reaction involving a high molecular weight substrate, it is evident that the porosity of the carrier will be of importance for substrate and product transports. Agarose can be prepared in a beaded form well suited for column chromatographic procedures in which resolution of large molecules is desired. Caldwell et al. (1975) have reported on the immobilization of β-amylase based on hydrophobic interactions between the enzyme and hydrocarbon chains attached to a carrier gel. A hexyl-substituted agarose (hexyl-ECD-Sepharose 6B) produces an absorbate of high stability suitable for month-long continuous starch hydrolysis at room temperature. Furthermore, Caldwell et al. (1976c) immobilized amyloglucosidase through adsorption onto the same carrier (hexyl-ECD-Sepharose 6B). This preparation exhibited high retention of its activity and was used for continuous production of glucose from starch for three months with only 40% reduction in its activity. Hexyl-Sepharose is an adsorbent quite permeable and highly resistant to chemical and biological attack (Caldwell et al., 1976b). Crosslinking with epichlorohydrin renders the agarose polymer stable without changing its molecular sieving properties or its ability to swell in water (Porath et al., 1971). One advantage of hexyl-ECD-Sepharose 6B as a carrier is the fact that it is a non-charged carrier...
(as opposed to cyanogen bromide activated agarose derivatives). Therefore, it should not exhibit mixed ionic-hydrophobic adsorption but, instead, "pure" hydrophobic interactions with the hydrophobic regions on the molecular surface of proteins (Porath et al., 1973). Therefore, neither desorption of the enzyme from the carrier (due to increased ionic strength of the substrate) nor adsorption of protein from the substrate on the carrier (due to electrostatic interactions) should be expected. Another advantage of hexyl-ECD Sepharose 6B is the great stability of the ether bond (linkage) between ligand and the matrix (carrier) as opposed to the unstable bond obtained with cyanogen bromide-activated Sepharose resulting in the leakage of the ligand from the matrix in alkaline medium (Matsumoto et al., 1979).

As it can be seen from Table 2, pepsin immobilized on hexyl-ECD Sepharose 6B exhibited small initial clotting activity and extremely rapid inactivation. Thus, a limit of 10 min coagulation time at 30°C (considered as the maximum desirable clotting time for evaluation purposes) was reached after 5 hr of continuous operation at a flow rate of 8 ml/hr (Table 3). Binding capacity of this carrier was 3.7 mg pepsin per 0.6 ml gel, corresponding to a binding efficiency of 40%. No "soluble activity" of immobilized pepsin preparation was detected in the treated milk. It should also be reported that rennin exhibited no activity at all when immobilized on hexyl-ECD-Sepharose 6B. Although the reasons for the small initial activity and extremely rapid inactivation of pepsin-hexyl-ECD-Sepharose 6B preparation were not investigated, the following (reasons) are very plausible. One reason for the low initial activity exhibited by this preparation may be the "steric hindrance" by the carrier when the enzyme approaches the substrate. This effect has been also reported by
<table>
<thead>
<tr>
<th>Support</th>
<th>Enzyme</th>
<th>Immobil. Method</th>
<th>Clotting time (min)</th>
<th>D value (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 - 14 14 - 20 20 - 30</td>
<td>4 14 20</td>
</tr>
<tr>
<td>PAC</td>
<td>Pepsin</td>
<td>Adsorption</td>
<td>0.75(7.4)</td>
<td>8.5 23.0 55.0</td>
</tr>
<tr>
<td>PAC</td>
<td>Chymosin</td>
<td>Adsorption</td>
<td>0.69(5.7)</td>
<td>7.5 24.0 41.7</td>
</tr>
<tr>
<td>UAC</td>
<td>Pepsin</td>
<td>Adsorption</td>
<td>0.50(3.0)</td>
<td>6.0 24.5 45.5</td>
</tr>
<tr>
<td>TAC</td>
<td>Pepsin</td>
<td>Adsorption</td>
<td>0.80(4.5)</td>
<td>8.5 23.5 36.0</td>
</tr>
<tr>
<td>UAC</td>
<td>Pepsin</td>
<td>Covalent B.</td>
<td>0.85(7.2)</td>
<td>8.4 35.0 61.0</td>
</tr>
<tr>
<td>TAC</td>
<td>Pepsin</td>
<td>Covalent B.</td>
<td>0.35(3.2)</td>
<td>10.0 22.0 38.0</td>
</tr>
<tr>
<td>Hexyl-ECD-Sepharose 6B</td>
<td>Pepsin</td>
<td>Adsorption</td>
<td>8.05(0.0)</td>
<td></td>
</tr>
</tbody>
</table>

PAC, Phenoxyacetyl cellulose
UAC, Untreated activated carbon
TAC, HCl-treated activated carbon

b Number in parenthesis shows corresponding soluble activity

c Average of two trials

d D value is a measure of stability of the immobilized enzyme, as defined in the text
Table 3 - Calculation of the economics of using immobilized proteases for the continuous coagulation of skimmilk (Reactor: pH 6.7, Temper. 4°C; Coagulation: pH 6.1, Temper. 30°C)

<table>
<thead>
<tr>
<th>Enzyme-Carrier Preparation</th>
<th>Flow Rate (ml/hr)</th>
<th>Time on Stream (hr)</th>
<th>Amount of Enzyme Applied (mg)</th>
<th>Amount of Milk Treated (L)</th>
<th>Enzyme Required Per L Milk (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin-PAC(Ad.)b</td>
<td>36</td>
<td>33</td>
<td>15</td>
<td>6.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Rennin-PAC(Ad.)</td>
<td>36</td>
<td>30</td>
<td>300</td>
<td>5.5</td>
<td>54.5</td>
</tr>
<tr>
<td>Pepsin-UAC(Ad.)</td>
<td>100</td>
<td>30</td>
<td>100</td>
<td>22.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Pepsin-TAC(Ad.)</td>
<td>100</td>
<td>30</td>
<td>100</td>
<td>14.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Pepsin-UAC(C.B.)</td>
<td>100</td>
<td>32</td>
<td>100</td>
<td>13.2</td>
<td>7.6</td>
</tr>
<tr>
<td>Pepsin-TAC(C.B.)</td>
<td>100</td>
<td>45</td>
<td>100</td>
<td>32.7</td>
<td>3.1</td>
</tr>
<tr>
<td>Pepsin-Hexyl-ECD-Sepharose 6B(Ad.)</td>
<td>8</td>
<td>5</td>
<td>10</td>
<td>0.08</td>
<td>125.00</td>
</tr>
<tr>
<td>Pepsin (soluble)</td>
<td>---</td>
<td>--</td>
<td>---</td>
<td>---</td>
<td>----</td>
</tr>
<tr>
<td>Rennin NF (soluble)</td>
<td>---</td>
<td>--</td>
<td>---</td>
<td>---</td>
<td>17.60</td>
</tr>
</tbody>
</table>

a PAC, Phenoxyacetyl cellulose
UAC, Untreated activated carbon
TAC, HCl-treated activated carbon

b Letters in parenthesis indicate immobilization method: Ad., adsorption; C.B., covalent binding

C Clotting time limit of 10 min reached
Green and Crutchfield (1969). Their chymosin-agarose (Sepharose 2B) derivative displayed very low or zero activity on casein micelles in milk, although it did hydrolyse \( \kappa \)-casein. However, \( \kappa \)-casein is only about one-fifth of the size of a casein micelle, so steric hindrance was less. A second manifestation of steric hindrance was deduced from the data obtained for \( \kappa \)-casein as substrate indicating that the agarose-chymosin derivative exhibited constant enzymic activity regardless of the amount of enzyme bound. Green (1980) has recently drawn an idealized version of chymosin-agarose derivative of Green and Crutchfield (1969). This model suggested that the steric hindrance may have prevented even the surface of the casein micelle from making contact with most of the enzyme molecules. The author concluded that steric hindrance is likely to be a serious problem with immobilized enzymes, particularly if the substrate is large. The steric hindrance effect is expected to be more pronounced in the hexyl-ECD-Sepharose used in this study than in the simple (noncross-linked) Sepharose of Green and Crutchfield (1969). This is due to the fact that, the epichlorohydrin cross-linking increases the stability and the rigidity of the gel but, at the same time, decreases its permeability, and this will be a serious problem in enzymatic treatment of high molecular weight substances (e.g. casein micelles) due to diffusional limitation in the substrate supply. Another reason for the low initial activity exhibited by the pepsin-hexyl-ECD-Sepharose 6B preparation may be masking of the active center of a certain number of enzyme molecules upon immobilization. These molecules become immediately inactive and, therefore, do not contribute to the activity of the immobilized preparation.

The extremely fast inactivation of the pepsin-hexyl-ECD-Sepharose 6B derivative could not be due to microbial growth, since pasteurized fresh
milk and low temperature ($4^\circ$C) could not facilitate such a growth. Moreover, adsorption of milk proteins on the gel could also be eliminated as possible cause of the observed inactivation, since hexyl-ECD-Sepharose 6B is a non-charge carrier and, thus, does not promote electrostatic interactions. It is suggested that one possible reason for the rapid inactivation observed may be denaturation of the enzyme derivative. This denaturation may be due to certain conformational changes of the enzyme. Such changes could be due to a steady increase in the number of attachment points, with a resulting increase in binding stability, as the molecule experiences free motion. A multipoint attachment will, however, impose a strain on the conformation, with subsequent loss of activity (Caldwell et al., 1976b). Caldwell et al. (1976b) reported that the enzymatic decay suffered by the $\beta$-amylase adsorbate in continuous operation could not only be ascribed to enzyme leakage, but also to a denaturation of the enzyme attached to hexyl-ECD-Sepharose 6B. Subsequently, Caldwell et al. (1976d) investigated the effect of substituent density (on the gel) on the activity and stability of the adsorbed enzyme. They found that hydrophobic immobilization of $\beta$-amylase on hexyl-ECD-Sepharose gels was optimally performed, as far as enzyme activity and stability were concerned, with gels of a hexyl to galactose ratio around 0.5. A lower degree of substitution (0.3) resulted in lower activity and faster decay of enzymatic activity due to substantial enzyme leakage from the gel. On the other hand, an adsorbate with higher degree of substitution (0.7) also exhibited lower activity and less favorable operational stability. It is possible that the degree of substitution (0.5) of the gel used in this study may have not been satisfactory for optimum activity and especially stability of the pepsin
derivative, resulting in rapid loss in activity by a gel induced
denaturation. Observations indicating that too many linkages to the
supporting medium may be harmful to the retention of enzyme activity have
been reported previously for covalently bound proteins by Datta et al (1973)
and Zabriskie et al. (1973). They concluded that higher degrees of
substitution, while advantageous for the creation of a strong interaction
between the gel and the enzyme, might enhance denaturation and thus lead
to lower activity yields.

B. Immobilization of pepsin and chymosin on Phenoxyacetyl cellulose

Phenoxyacetyl cellulose was chosen as support material in this study,
because it has been shown to be a unique medium for immobilizing enzymes
and other proteins (Butler, 1975). Advantages of phenoxyacetyl cellulose
as a support material include (Butler, 1975): (1) hydrophobic binding of
proteins to phenoxyacetyl cellulose is strong and essentially irreversible
under most working conditions (Butler, 1975; Regis Chemical Co.);
(2) enzymes immobilized on phenoxyacetyl cellulose exhibit nearly complete
retention of their catalytic activity; (3) preparation of the adsorbent
(carrier) is extremely facile, versatile, and inexpensive; (4) preparation
of the enzyme-carrier conjugate is convenient, simple and mild;
(5) phenoxyacetyl cellulose contains no charged groups, thus permitting
true hydrophobic enzyme immobilization without interfering ionic effects;
and (6) immobilization of an enzyme on phenoxyacetyl cellulose is
reversible, desorption being accomplished through the use of nonionic
detergents, and this permits reusability of the carrier.

From the difference in absorbance at 280 nm of the enzyme solution
before and after immobilization (adsorption), it was estimated that 4.8 mg
pepsin were adsorbed per g of phenoxyacetyl cellulose; this represents binding efficiency of 80%. Results of pepsin and chymosin immobilized on phenoxyacetyl cellulose are presented in Table 2. As it can be seen, both preparations showed high initial activity on exposure to milk. However, the deactivation rates were very high. The nature of the loss in clotting activity is shown in Figure 1, which presents milk-clotting activities of enzyme-phenoxyacetyl preparations during continuous exposure to skimmilk. It can be seen, that the enzyme inactivation occurred in two stages: the first decay was rapid whereas the second decay was more gradual. Inactivation rates at different points of operation in the reactor are expressed in this study as "D values" and are reported in Table 2. Very low D values (8.5 hr for pepsin and 7.5 hr for chymosin) at 4 hr on the stream indicate that these preparations were very unstable during the first phase of inactivation. Higher D values for both preparations at 14 and 20 hr on the stream indicate that both became progressively more stable.

Decay patterns similar to those observed in this study with pepsin and chymosin (i.e. two phase patterns) have been reported repeatedly for pepsin and calf rennet immobilized on different carriers and used for continuous coagulation of milk (Cheryan et al., 1975b; Cheryan et al., 1976; Taylor et al., 1977). In these studies, the first rapid decay was attributed to deposition of protein and peptides on the immobilized protease. Accumulation of nitrogenous materials and sialic acid paralleled the initial rapid loss in enzymic activity. Both whey proteins and casein appeared to be responsible for loss in enzymic activity. The support materials used in those studies (e.g. porous glass, alumina) were charged having a net positive charge at the pH of the milk which presumably
Fig. 1. Clotting activity of pepsin and chymosin immobilized on phenoxyacetyl cellulose for the continuous coagulation of skimmilk: (●) pepsin; (▲) chymosin.
facilitated the adsorption of milk protein and peptides, which are negatively charged at the pH of milk (6.7). Causes of the second gradual enzymic decay of immobilized proteases remained elusive, although certain whey components could be implicated.

The semilogarithmic plots of activity versus time obtained with pepsin- and chymosin- phenoxyacetyl cellulose preparations suggest that two activity loss mechanisms were involved in, the more rapid one affecting only a certain fraction of the enzyme. A number of possible inactivation causes can be eliminated. Thus, under the conditions of experiment (i.e. operation at 4°C and use of fresh pasteurized milk) microbial growth could not account for the relatively rapid inactivation rates observed. Moreover, inactivation due to adsorption of proteinaceous material on the phenoxyacetyl cellulose can be ruled out, since phenoxyacetyl cellulose is a non-charged carrier and, therefore, does not facilitate electrostatic interactions with the negatively charged milk proteins. One obvious reason responsible for the first rapid decay of enzymic activity seems to be the leakage (desorption) of enzyme from the carrier, since soluble activity, which is an indication of enzyme desorption, was detected in both enzyme preparations (see numbers in parenthesis in Table 2). As it can be seen from Table 2, about 10% of the activity exhibited by the immobilized preparations at 4 and 14 hr on the stream was due to desorbed enzyme. Soluble activities were also observed for milk samples taken at subsequent time intervals (20 and 30 hr on the stream), but these activities were small leading to long clotting times, and thus requiring time-consuming and tedious measurements. That is why these soluble activities were not exactly timed and, therefore, are not included in Table 2. Although enzyme binding to phenoxyacetyl cellulose has been reported to be
irreversible under most working conditions (Butler, 1975; Regis Chemical Co.), it is evident from the results of Table 2 that enzyme was lost from the immobilized systems. According to Pitcher (1980b) the enzyme can be lost from a system because of desorption, severing of chemical bonds or erosion of the support material. A portion of the enzyme leakage observed here may have been due to changes in pH, ionic strength and temperature, since hydrophobic binding is influenced by these factors. For example, as the pH increases and temperature decreases the hydrophobic interaction diminishes. The effect of these changes, if any, is expected to be more profound during the initial stage of the operation. Although phenoxyacetyl cellulose is claimed as a medium being relatively insensitive to such changes (Regis Chemical Co.), it is possible that changes in pH, ionic strength or temperature may lead to the desorption of the weakly (loosely) bound enzyme molecules, since not all of the enzyme molecules are equally well bound to the carrier due to its usually nonhomogeneous surface. Another mechanism by means of which the enzyme has been lost from the phenoxyacetyl cellulose derivatives seems to be the leakage of the ligand from the carrier, since Carr and Bowers (1980) commented that the use of phenoxyacetyl cellulose as a matrix is limited mainly by the relatively rapid degradation of the ester bond (between phenoxyacetyl groups and cellulose) resulting in a slow leakage of the enzyme from the resin. Such leakage of the ligand from the carrier is a very common problem and has been consistently reported for derivatives of cyanogen bromide-activated Sepharose used as carriers for enzyme immobilization (Matsumoto et al., 1979). A third mechanism which may be partly responsible for the observed leakage of enzyme from the carrier is the competitive displacement of the enzyme from the carrier (held through hydrophobic interactions) by certain
milk proteins.

It seems, therefore, that the first rapid decay in activity observed here was probably due to both physical desorption of loosely bound enzyme and slow leakage of the ligand-enzyme complex from the carrier. The second decay of enzymic activity was probably due only to the continued slow leakage of the ligand-enzyme complex from the carrier.

C. Immobilization of pepsin on activated carbon

Activated carbon has a long history as support material in enzyme immobilization and a relatively great number of papers have appeared on its use (Cho and Bailey, 1977, 1978, 1979; Gol'dfel'd et al., 1966; Nelson and Griffin, 1916; Nelson and Hitchcock, 1921; Tosa et al., 1966). Its usefulness is due to the fact that activated carbon has many attractive properties for use as immobilized enzyme support. Activated carbon is cheap, possesses mechanical strength, and can be obtained in several forms including porous structures with a variety of pore size distributions (Cho and Bailey, 1977). Its skeleton density (0.75 g/cm³) is such that activated carbon immobilized enzymes can be fluidized much easier than enzymes immobilized on other inorganic supports such as porous glass of which skeleton density is about 2.4 g/cm³. Furthermore, activated carbon is already employed in numerous food, medical, and fine chemical processing operations, so that the material is relatively familiar in these industries. Questions of possible secondary influences on process performance owing to the enzyme support are therefore minimized, enhancing acceptability of the immobilized enzyme system (Cho and Bailey, 1978). Activated carbon is a highly porous carbonaceous material, prepared by carbonizing and activating organic substances of mainly
vegetable origins. Typically, activations are conducted by chemicals, CO$_2$, O$_2$ or steam at high temperatures. Boehm et al. (1964) detected carboxylic groups, phenolic hydroxyl group, and other oxides on the carbon surfaces.

Two types of activated carbon (untreated and HCl-treated) and two immobilization procedures were investigated in this study. Treatment of activated carbon with HCl was intended to clean the carbon pellets and thus improve access to external and internal surface. This washing step was to be followed by treatment with nitric acid, an oxidizing agent. The motivation for such oxidative pretreatment was derived from the postulated chemistry for diimide-mediated enzyme immobilization (Weetall, 1975). This process involves peptide bond formation between enzyme amino groups and carboxyl groups on the carbon surface. Therefore, by increasing the number of carboxyl groups on the carbon surface, oxidative pretreatment may increase the enzyme loading. It should be noted at this point, however, that treatment of HCl-washed activated carbon with nitric acid had an adverse effect, since it resulted in immediate clotting as the milk came in contact with the nitric acid-treated carbon in the column. This clotting was probably due to residual nitrate ions still existing within the carbon pores, despite the excessive washing with water. Moreover, neutralization of nitric acid-treated carbon before use as a carrier did not have any beneficial effect in avoiding the immediate clotting within the column. As a result, the nitric acid treatment was discontinued and thus is not included in Table 2. Adsorption of pepsin was carried out at pH 1.2, which is close to its isoelectric pH, since it has been repeatedly reported that the maximum adsorption of proteins occurs at their isoelectric point (Armstrong and Chesters, 1964; Brash and Lyman,
1971; McLaren, 1954). Moreover, Taylor et al. (1977) have reported that pepsin was best adsorbed on alumina (a charged carrier) at pH 1.2 and that ionic attraction was not the strong force contributing to the adsorption. Adsorption of pepsin on activated carbon at pH 1.2 is due to hydrophobic interactions between the hydrophobic surface of activated carbon and hydrophobic groups present on the surface of pepsin. The binding efficiency of activated carbon could not be determined due to the black color of the washings after immobilization. Since it has been reported that the enzyme loadings obtained with activated carbon are very similar to those obtained with porous glass (Cho and Bailey, 1977), 100 mg of pepsin were used for immobilization.

Results of pepsin immobilized on activated carbon are presented in Table 2. It is evident that, covalent binding of pepsin on HCl-treated carbon (TAC) produced the catalyst with the highest initial activity, presumably because of the higher enzyme loading achieved by the carbodiimide immobilization process. On the other hand, covalent binding of pepsin on untreated activated carbon (UAC) gave a catalyst which inactivated less rapidly. Adsorption of pepsin on UAC produced initial activity higher than the covalent binding of pepsin on the same type of carbon (probably because of increased enzyme desorption), but inactivated more rapidly.

All enzyme-carriers preparations were periodically sterilized by rinsing with 0.05 M hydrogen peroxide with no loss in enzymatic activity. This, in conjunction with the low temperature (4°C) of operation of the reactor and the use of fresh pasteurized milk, excluded the possible inactivation of the preparations due to microbial growth. Moreover, inactivation due to adsorption of proteinaceous material on the carbon was rejected, because the carbon was negatively charged at the pH of the milk.
and, therefore, repelled rather than adsorbed the negatively charged milk proteins and peptides. The inactivation of all pepsin-activated carbon catalysts was attributed to the desorption of enzyme since soluble activities were detected in all cases. The contribution of soluble enzyme to the clotting activity of each preparation was relatively greater for adsorption than for covalent immobilization (Table 2). In other words, the portion of enzymic activity (exhibited by each preparation) due to desorbed enzyme was greater for the adsorbed than for the covalently bound enzymes probably due to the less stable binding obtained with adsorption. However, desorption of even covalently bound enzymes has been repeatedly reported in the literature (Stanley and Olson, 1974; Taylor et al., 1977). On the other hand, the desorption of adsorbed pepsin was probably due to the increase in the pH (from 1.2 to 6.7), although the preparations were equilibrated at pH 6.7 before being exposed to milk. The adsorption-desorption mechanism of pepsin on activated carbon may be as follows. At pH 1.2 hydrophobic affinity was very strong between pepsin and activated carbon due to the fact that all carboxyl groups were not dissociated. Thus, the enzyme molecules were adsorbed by hydrophobic binding on loci of the carbon each of which included at least one hydrophobic group. As the pH was increased from 1.2 to 6.7, the carboxyl groups of the adsorbing loci in the activated carbon were gradually converted to the dissociated form, simultaneously reducing the hydrophobicity. At pH 6.7, the enzyme was retained on the carbon by the remaining hydrophobic affinity (due to phenolic groups) minus the electrostatic repulsion (produced by the dissociated carboxyl groups). The above adsorption-elution mechanism of enzymes constitutes the principle of "hydrophobic-ionic chromatography" described by Sasaki et al. (1979).
Looking at the Figures 2 and 3 as well as Table 2, it can be seen that the inactivation rates were initially high and subsequently levelled off. These observed differences in the inactivation rates were probably due to the sequence of enzyme desorption. Thus, enzyme molecules adsorbed on the surface may have been desorbed faster than those adsorbed within the pores of the carbon. It also is possible that the strength of adsorption of different enzyme molecules was varying due to nonexistence of homogeneous distribution of hydrophobic binding sites on the carbon surface. This effect could give rise to loosely bound enzyme molecules, which would desorb quickly, and strongly bound enzyme molecules which would desorb less rapidly. Taylor et al. (1977) have also reported that most desorption of pepsin (about 45%), immobilized by physical adsorption on alumina at pH 1.2 and used for continuous coagulation of milk, took place during the first 12 hr on the stream and that after 25 hr slightly more than half (50%) the pepsin had desorbed.

The above assumption of the desorption of adsorbed pepsin because of the increase in the pH of the medium is supported by the findings of Kikawa (1926) who reported that pepsin was best adsorbed on animal charcoal at pH 1 or 2, and that the adsorbed enzyme was leached from the charcoal by a phosphate solution of pH 6.8.

D. Economics of using immobilized proteases for the continuous coagulation of skimmilk

Besides the obvious saving in labor costs and improved efficiency expected when an enzyme is immobilized (due to the conversion of the process from batch into continuous), the major consideration will be the savings in enzyme costs (Cheryan, 1974). The operational stability and half-life
Fig. 2. Clotting activity of pepsin-untreated activated carbon preparations used in the continuous coagulation of skimmilk: (▲) adsorption; (●) covalent binding.
Fig. 3. Clotting activity of pepsin-HCl-treated activated carbon preparations used in the continuous coagulation of skimmilk: (▲) adsorption; (●) covalent binding.
of the immobilized enzyme have a profound importance in this respect, since the potential for reuse of the catalyst cannot be realized unless the immobilized enzyme is sufficiently stable under operational conditions. If the immobilized enzyme must be continually replaced, then little will be gained by the use of an immobilized enzyme process, except an increase in cost (Trevan, 1980). The net outcome of an immobilized enzyme process should be to produce a product which is less expensive than a comparable product produced by a soluble enzyme process.

Table 3 shows the calculation of the economics of using proteases immobilized on hydrophobic supports for the continuous coagulation of skimmilk in cheese manufacture. This economic study was based on determining the amount of enzyme required to coagulate 1 L of skimmilk in 10 min at 30°C, using both the soluble and insoluble (immobilized) form of the same enzyme. Since the secondary phase of coagulation is relatively inactive at the normal pH of milk (6.7), the assays for clotting activity were performed with the pH of the treated milk (effluent) decreased to 6.1, where the coagulation times were much faster. As it can be seen from Table 3, the best enzyme preparation among those tested was attained with phenoxyacetyl cellulose as carrier. The limit of 10 min clotting time at 30°C (considered as the maximum desirable clotting time for evaluation purposes) was reached after 33 hr of continuous operation of the pepsin catalyst at a flow rate of 36 ml/hr. It was calculated that 6.5 and 5.5 L of skimmilk was coagulated with 15 mg pepsin and 300 mg rennin (N.F.), respectively. The clotting efficiencies of these catalysts were 2.7 and 3.1 times lower than those of the soluble forms of pepsin and rennin, respectively. As far as the activated carbon is concerned, the best preparation was obtained with pepsin covalently bound to HCl-
treated activated carbon (TAC). It should be noted here, however, that
the direct comparison of phenoxyacetyl cellulose and activated carbon
preparations in terms of their efficiencies as catalysts for continuous
coaagulation may be inappropriate, since their clotting efficiencies were
calculated on the basis of the amount of enzyme added in the immobilization
step and not on the amount of enzyme bound by the carrier. This is due
to the inability of spectrophotometrical determination of the binding
efficiency of activated carbon as already mentioned because of the black
color of the washings after immobilization. It should be pointed out that
different types of activated carbon exist exhibiting different surface
properties resulting from different preparative methods (Garten and Weiss,
1957). It seems logical, therefore, that if the pepsin binding efficiency
of Darco activated carbon used in this study was lower than the expected
average of 20 mg pepsin/g carbon, then the use of 100 mg pepsin must have
been a waste of enzyme.

There are two major factors that could improve the economics of
using immobilized milk clotting enzymes (Cheryan, 1974). These are:
(1) optimization of the yield or complete immobilization of the enzyme
on each carrier; and (2) dilution of the treated milk as it comes out of
the reactor with fresh untreated milk, which would greatly increase
output (i.e. amount of milk that could be coagulated). Even after taking
both of the above factors into consideration, the data in Table 3 suggest
that the continuous coagulation of skimmilk with proteases immobilized
on the hydrophobic supports studied is not economically attractive.
E. General Discussion and Conclusions

In practice, the use of an immobilized enzyme for continuous coagulation of skimmilk depends on several critical points including separation of the enzymic and clotting phases so that clotting does not occur in the enzyme reactor, high enzymatic activity, sufficiently long retention of enzymatic activity under operating conditions, freedom from microbial growth, and production of a normal product (Ferrier et al., 1972; Taylor et al., 1979). Furthermore, use of a cheap enzyme carrier that would not adsorb deactivating proteinaceous materials, and employment of a mild, simple (one step) and inexpensive immobilization procedure are also important considerations contributing to the economics of an immobilized system.

In this study, both cheap and non-charged (phenoxyacetyl cellulose) or negatively charged (activated carbon) carriers essentially not adsorbing proteinaceous material were used. In addition, the simplest, mildest and cheapest immobilization method available (i.e. adsorption) was mainly employed. Separation of the enzymic and clotting stages was accomplished by using low temperature and milk at its normal pH. All enzyme-carrier preparations exhibited high initial activity. The major problem encountered was the rapid reduction in enzymatic activity. The main reason of this rapid catalyst inactivation appeared to be the loss of enzyme from the carriers, since soluble activity was detected in all enzyme-carrier preparations. The enzyme loss was due to the physical desorption of enzyme from the carrier (activated carbon), mainly caused by the increase in pH, as well as to the relatively rapid leakage of the ligand from the carrier (phenoxyacetyl cellulose). This desorption of the enzyme into the effluent milk, however, should not pose a problem since the soluble form
of the enzyme is inactivated during cheese manufacture (cited in Taylor et al., 1977). The best enzyme preparation (based on the amount of enzyme used in the immobilization step) was obtained with phenoxyacetyl cellulose as the carrier.

According to the data presented in this study it is concluded that continuous coagulation of milk with proteases immobilized on the hydrophobic carriers studied appears to be economically unfeasible. Further studies on other carriers and better (more efficient) immobilization procedures that will give catalysts of greater activity and especially stability are necessary to allow more favorable economic projections. The need for solving the problem created by the shortage of rennet is so acute and the advantages of a continuous coagulation process are so great that any further attempt to assess the economic feasibility of any immobilized enzyme system for the continuous coagulation of milk is believed to be absolutely justifiable.
REFERENCES


Chapter II

A Simple Turbidimetric Method for Determining

the Fat Binding Capacity of Proteins
INTRODUCTION

The ability of proteins to bind fat is a very important functional property for such applications as meat replacers and extenders, principally because it enhances flavor retention and reputedly improves mouthfeel (Kinsella, 1976). The key role(s) of fat in food flavoring has been illustrated by Kinsella (1975) and its capacity to improve flavor carryover in simulated foods during processing is apparent. Soy proteins have been added to ground meats to promote fat absorption or fat binding, and thus decrease cooking losses and maintain dimensional stability in the cooked product (Wolf and Cowan, 1975). Fat separation is a well known major problem in processed meat-in-sauce-or-gravy type products. This problem can be prevented by incorporating into these products (canned or frozen meat/sauce products) a combination of soy protein ingredients (i.e. an extruded soy protein concentrate, a soy protein isolate and lecithin) designed to emulsify, bind, and stabilize fats (Morris, 1980). On the other hand, in some foods such as pancakes and doughnuts, the addition of soy flour helps prevent excessive absorption during frying (Johnson, 1970).

Fat absorption of proteins is usually measured by adding excess liquid fat (oil) to a protein powder, thoroughly mixing and holding, centrifuging and determining the amount of bound or absorbed oil - total minus free (Lin et al., 1974; Wang and Kinsella, 1976). The amount of oil and protein sample, kind of oil, holding and centrifuging conditions, and units of expression have varied slightly from one investigator to another (Hutton and Campbell, 1981).

The mechanism of fat absorption is not clear. However, Wang and
Kinsella (1976) have attributed fat absorption, as assessed by the above method, mostly to physical entrapment of the oil; in support of this a correlation coefficient of 0.95 was found between bulk density and fat absorption by alfalfa leaf proteins. Chemical modification of protein, which increases bulk density concomitantly enhances fat absorption (Franzen, 1975).

The objective of this part of the thesis, was to develop a simple method for determining the ability of proteins to bind fat.
The development of the method and a comparison of the fat binding capacities of several food proteins will be presented.

MATERIALS AND METHODS

A. Materials

Bovine serum albumin (#A-4503), β-lactoglobulin (#L-6879 from milk) and ovalbumin (#A-5503) were all purchased from Sigma Chemical Co., St. Louis, Mo. Soy protein isolate was obtained from General Mills, Inc., Minneapolis, Min. Promine-D was purchased from Central Soya Co., Chicago, Il. Pea protein isolate (M 412-046), Century cultivar field pea, was received from POS Pilot Plant Corp., University of Saskatchewan, Saskatoon, Sask. Rapeseed protein isolate and sunflower protein isolate were prepared by the method of Nakai et al. (1980). Gelatin, Bloom 300, was purchased from United States Biochemical Corp., Cleveland, Oh. Whey protein concentrate (75%) was obtained from Sodispro Technol., St. Hyacinthe, Que. Whole casein was prepared by the following method: approximately 4.5 L of raw milk was heated until it reached a temperature
of 45°C, centrifuged at 4,100 x g for 10 min and cooled 20 min at 20°C. The upper fat layer was discarded and the lower skim milk portion was diluted with water on a 1:1(V/V) basis. The diluted sample was heated to 40°C and the pH was adjusted to 4.6 with 1 N HCl. The mixture was filtered through cheese cloth, the casein precipitate was washed with water and the excess water was squeezed from the cheese cloth. The precipitated casein was dissolved in water and the pH was adjusted to 7.75 so that the casein was totally solubilized. The solution was heated to 40°C and the precipitation with HCl was repeated. The casein precipitate was dissolved in water and the pH was adjusted to 7.0 at which the casein was totally dissolved. The solution was centrifuged at 4,100 x G for 10 min and the supernatant was collected and freeze-dried.

-- Corn oil was from Fisher Scientific Company, Fair Lawn, N.J. Urea, ACS reagent, 99+%, was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wis. Metaphosphoric acid was from J. T. Baker Chemical Co., Phillipsburg, N.J.

B. Methods
(a) Fat binding capacity determination

To 40 mg of freeze-dried protein sample in a 15 ml glass centrifuge tube 1.5 ml of corn oil was added. The contents were stirred and sonicated, with a Braun-Sonic 1510 sonicator (Braun Instruments, San Francisco, Ca) fitted with a needle probe, at 100 watts for 1 min to disperse the protein sample. After holding at room temperature for 30 min the tube was centrifuged at 3,020 x G for 20 min. The free oil was pipetted off and 2 ml of distilled water was added. Oil adhered to the sides of the tube was removed by scraping the sides with a glass rod.
Then, in order to remove any oil that might have been entrapped in the form of film beneath the protein precipitate, the precipitate was gently scraped from the bottom of the tube and any oil found was taken to the top (i.e. surface of water) with the glass rod. Subsequently, 1 ml of 0.1 N metaphosphoric acid (pH 2.1) was added and the tube was centrifuged at 4,340 x g for 15 min. The supernatant was pipetted off. The precipitate was, then, carefully washed with distilled water (3 - 4 ml) without dispersing it. The supernatant was pipetted off. Finally the tube walls were cleaned with a disposable (paper) wiper (to remove any trace of oil, if any existed). 0.3 ml of distilled water was added and mixed well with the glass rod. A digestion medium of 20 ml of 7 M urea in 50% H₂SO₄ was measured into a graduated cylinder. An aliquot of about 2 ml of this digestion medium was added into the tube, the contents were mixed well with a glass rod, and then transferred into an Omni-mixer homogenizing chamber. The centrifuge tube was washed twice with about 2 ml of digestion medium. These washings and the remainder of the digestion medium in the graduated cylinder were poured into the homogenizer chamber. The mixture was homogenized for 30 sec at speed setting 1, and then poured into a 50 ml beaker.

The sample was held for 30 min at room temperature and then the absorbance was taken at 600 nm in a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.), spectrophotometer with a round cuvette against the digestion medium. The absorbance was stable for at least 1 hr. The volume (ml) of oil bound was determined from the standard curve. The protein content of the combined supernatants (#2 and #3) was, subsequently, determined by the Phenol-Biuret method (Brewer et al., 1974) to calculate the amount of protein lost in these supernatants during the handling of the precipitate. The amount of lost protein was converted to the amount of the original sample,
since the protein content of the sample was known, and this value was subtracted from the 40 mg of the starting sample. This calculation gave the amount of oil in ml bound by the corrected amount of protein sample. The fat binding capacity of the sample (expressed as %) was then calculated as the volume of oil in ml bound by 100 g of protein sample.

The standard curve was constructed as follows: To 40 mg of soy protein isolate in a 30 ml beaker, increasing amounts of corn oil were added (0 to 100 μl). While mixing with a glass rod, 0.3 ml of distilled water was added (to facilitate mixing) followed by 20 ml of digestion medium (7 M urea in 50% H$_2$SO$_4$) and further mixing. The mixture was transferred into an Omni-mixer chamber and homogenized for 30 sec at speed setting 1, and then poured into a 50 ml beaker. The sample was held for 30 min at room temperature and the absorbance was then taken at 600 nm in a Spectronic 20 with a round cuvette against the digestion medium.

(b) **Protein (surface) hydrophobicity determination**

Protein surface hydrophobicity was fluorometrically determined according to the method of Kato and Nakai (1980) after slight modification. Each protein sample (2 ml) was serially diluted with 0.01 M phosphate buffer, pH 7.4, to obtain protein concentrations ranging from 0.00156% to 0.05%. Two sets of protein samples were prepared (i.e. two tubes for each protein concentration). Ten μl of cis-parinaric acid solution were added only to one set of tubes. The parinaric acid-protein conjugate was then excited at 325 nm and the relative fluorescence intensity was measured at 420 nm in an Aminco-Bowman spectrofluorometer, using slit width of 0.5 mm. The method was standardized by adjusting the relative fluorescence intensity reading of the fluorometer to 7.4/10 full scale (by turning the
sensitivity knob) when 10 μl of cis-parinaric acid solution was added to 2 ml of decane. Then, the fluorescence readings of the protein samples were taken. The net fluorescence intensity at each protein concentration was determined by subtracting the fluorescence intensity of each sample without cis-parinaric acid from the fluorescence intensity of the corresponding sample containing cis-parinaric acid. The initial slope ($S_0$) of the fluorescence intensity vs. protein concentration plot was used as an index of the protein surface hydrophobicity. The initial slope was determined by linear regression analysis using a Monroe (Orange, N.J.) 1880 programmable calculator.

(c) **Solubility index determination**

Protein samples (1%, w/v, in 0.01 M phosphate buffer pH 7.4) were dispersed by stirring with a magnetic stirrer for 5 min and then blended in a Sorval Omnimixer at speed setting 5 for 1 min. The pH of each dispersion was adjusted to 7.4 by adding 1 N NaOH. For 100% soluble proteins the blending step was eliminated. A portion of each protein suspension was then centrifuged at 27,000 x G for 30 min. Aliquots of the suspension and the supernatant after centrifugation were diluted and the protein contents were determined by the Phenol-Biuret method (Brewer et al., 1974). The percent solubility index ($s$) was taken as the ratio of the protein content of the supernatant to that of the suspension.

(d) **Statistical analysis**

Simple and multiple linear regression analyses were done by using a Monroe 1880 programmable calculator. Backwards stepwise multiple regression analysis and surface visualization plotting were done at the
University of British Columbia using an Amdahl 470 V/8 computer.

Independent variables used in the backwards stepwise regression analysis included surface hydrophobicity ($S_0$), solubility index ($s$), interaction of $S_0$ and $s$, and quadratic powers of $S_0$ and $s$.

RESULTS AND DISCUSSION

A. Fat binding capacity

Wavelength dependence of turbidity

Figure 1 shows the effect of wavelength on absorbance of oil-protein systems. Plot 1 was obtained by mixing 40 mg soy protein isolate (used as a standard protein) with 50 µl corn oil and 20 ml 7 M urea in 50% $H_2SO_4$ (used as a digestion medium) with a glass rod, homogenizing the mixture for 30 sec, holding for 30 min at room temperature and then scanning with a Beckman DB spectrophotometer. Plot 2 was obtained by scanning the same sample with a Spectronic 20 spectrophotometer. Plots 3 and 4 were obtained using the Spectronic 20 instrument when protein or oil was omitted from the mixture, respectively. As it can be seen, the absorbance values read from the Beckman DB spectrophotometer were higher than those measured using the Spectronic 20 spectrophotometer. This is probably due to the different cuvette-to-photodetector distance and thus to the different angle of acceptance of the light by the photodetector as suggested in a study of turbidimetry by Pearse and Kinsella (1978). It is also evident from Figure 1 that as the wavelength was increased the absorbance by all samples decreased. In the present study a wavelength of 600 nm was used because of the negligible absorbance by the protein. Moreover, the
Fig. 1. Effect of wavelength on absorbance.
Spectronic 20 spectrophotometer with a round cuvette was chosen over the Beckman DB spectrophotometer since the latter gave nonreproducible absorbance values. A similar phenomenon was observed by Nakai and Le (1970) who attributed the ability of round cells to give reproducible readings to their focusing effect.

Turbidity dependence on blending time

The effect of blending time during fat binding capacity determination on the absorbance at 600 nm is shown in Figure 2. A gradual rise in absorbance and then attainment of a steady state with increasing blending time was observed. In this study a blending time of 30 sec was chosen.

Standard curve for FBC determination

The standard curve obtained is shown in Figure 3. The regression equation was $Y = 0.008 X + 0.012$, where $Y$ is the absorbance at 600 nm and $X$ is the amount of bound oil in μl. The correlation coefficient ($r$) was 0.9996 and the standard error of estimate ($S_{y.x}$) 0.008.

Comments on the method for FBC determination

A flow diagram of the developed method is shown in Figure 4. The use of 0.1 N metaphosphoric acid was necessary for preventing solubilization, thereby losing soluble proteins (e.g. β-lactoglobulin, BSA, whey, casein) during their resuspension in water (after the first centrifugation). Initially, other protein precipitants (e.g. ethanolic solutions, mercuric chloride, acetate buffer pH 4.6, 8% silicotungstic acid in 1.2 M perchloric acid) were added after protein resuspension to precipitate the solubilized protein. These precipitants were found, however, to be unsuitable since
Fig. 2. Effect of blending time on absorbance at 600 nm.
Fig. 3. Standard curve for fat-binding determination.
40 mg protein sample + 1.5 ml corn oil

- Sonicate for 1 min
- Hold for 30 min @ R.T.
- Centrifuge at 3,020 x g for 20 min

Supernatant #1
(protein ppt.
(free oil)

- Add 2 ml H₂O
- Gently lift ppt.
- Add 1 ml 0.1 N HPO₃
- Centrifuge at 4,340 x g for 15 min

Supernatant #2
(free oil + H₂O)

Supernatant #3
(free oil + H₂O)

- Wash with H₂O
- Add 0.3 M H₂O and mix
- Add 2 ml dig. medium and mix well
- Transfer tube content into homog. chamber
- Wash tube twice with 2 ml dig. med.
- Add 16 ml dig. med.
- Homogenize for 30 sec
- Transfer into beaker and hold for 30 min
- Take A₆₀₀nm
- Calculate F.B.C. (%)

Figure 4 - Flow diagram for the determination of FBC of proteins
the protein precipitate obtained was very firm and not dispersible by the subsequent homogenization step or because of the nonreproducible absorbance reading obtained (silicotungstic acid). Trichloroacetic acid (TCA) in high concentrations (10 - 12%) was found effective in precipitating the soluble proteins tested and, their precipitates were easily dispersed in the digestion medium. However, when 10% TCA was used for FBC determination of relatively insoluble proteins (e.g. soy protein), the protein precipitate obtained was very firm and difficult to disperse. Another problem associated with the use of TCA as a protein precipitant is that TCA possesses a hydrophobic group, and therefore, it may react with hydrophobic sites of the protein thus releasing some of the bound oil. The use of protein precipitants may probably be criticized because many of them (such as TCA, picric acid, salicylsulphonic acid) cause protein denaturation (Perlmann and Herrmann, 1938), which influence the fat binding by proteins. Thus, metaphosphoric acid, a known strong protein precipitant, was used. It was found very efficient in precipitating soluble proteins (causing instantaneous formation of a precipitate), and moreover, all protein precipitates obtained were easily dissolved by the digestion medium. However, the main advantage of metaphosphoric acid as a protein precipitant is the fact that metaphosphoric acid has been repeatedly shown not to cause protein denaturation (Briggs, 1940; Perlman, 1938; Perlmann and Herrmann, 1938). Briggs (1940) concluded that the metaphosphate-protein reaction can be regarded as a complex in which the negative multivalent (polymerized) metaphosphate ion is linked to the positive (amino) groups of the protein by a salt-like bond of very low dissociation tendency (i.e. ionizing capacity). Thus, when the solution containing protein and metaphosphate is dialyzed at pH>7, the metaphosphate
is readily removed and the protein is obtained with all of its original properties unchanged (Briggs, 1940). The protein-metaphosphoric acid complex is also easily solubilized by salt addition (Perlmann and Herrmann, 1938).

After homogenizing and holding the protein sample for 30 min (to allow the air bubbles to rise to the surface), some proteins (e.g. soy protein) formed a thin usually broken foam layer floating on the sample surface. In this case, an aliquot was taken for absorbance measurement by dipping a pasteur pipette through a hole of the foam layer into the sample dispersion. If the foam layer was not well broken, the beaker containing the sample dispersion was slightly inclined. Thus, the layer was moved to a direction opposite to that of the inclination, allowing the taking of an aliquot without disturbing the foam layer. The formation of the foam layer was due to the big volume of the homogenizer's container (100 ml) relative to that of the sample (20 ml) allowing the incorporation of air into the sample. Its formation, therefore, can be avoided by using a small container (e.g. 25 ml).

B. Comparison of the FBC of various proteins

Fat absorption of proteins is affected by the protein source, extent of processing and/or composition of protein, particle size, and temperature (Hutton and Campbell, 1981). The FBC values for some food proteins are given in Table 1. As is seen, soy protein isolate and sunflower protein isolate bound more oil than the other proteins tested. On the other hand, β-lactoglobulin bound the least amount of oil among all the proteins tested. The FBC of the proteins in Table 1 was determined by using the equation derived from the standard curve without any
Table 1 - Relationship between protein hydrophobicity, solubility index, and fat binding capacity of various proteins.

<table>
<thead>
<tr>
<th>Protein Sample</th>
<th>Hydrophobicity ($S_o$)</th>
<th>Solubility index (%)</th>
<th>FBC $^b$ (%) $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>6.0</td>
<td>100.0</td>
<td>37.7 560.0</td>
</tr>
<tr>
<td>Casein</td>
<td>28.0</td>
<td>100.0</td>
<td>10.1 95.0</td>
</tr>
<tr>
<td>Soy protein isolate</td>
<td>95.0</td>
<td>26.4</td>
<td>105.9 161.0</td>
</tr>
<tr>
<td>Promine D</td>
<td>39.0</td>
<td>29.1</td>
<td>85.3 175.0</td>
</tr>
<tr>
<td>Pea protein isolate</td>
<td>66.0</td>
<td>42.6</td>
<td>92.3 145.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>5.0</td>
<td>15.3</td>
<td>19.1 100.0</td>
</tr>
<tr>
<td>Sunflower protein isolate</td>
<td>47.0</td>
<td>31.0</td>
<td>105.8 230.0</td>
</tr>
<tr>
<td>Rapeseed protein isolate</td>
<td>55.0</td>
<td>44.0</td>
<td>66.2 140.0</td>
</tr>
<tr>
<td>Whey protein</td>
<td>182.0</td>
<td>88.7</td>
<td>52.2 220.0</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>325.0</td>
<td>100.0</td>
<td>25.0 340.0</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>426.0</td>
<td>100.0</td>
<td>4.2 210.0</td>
</tr>
</tbody>
</table>

$^a$Average of duplicate determinations

$^b$I : Determined according to the new method described here
II: Determined according to the method of Wang and Kinsella (1976)

$^c$%: ml oil/100 g sample (dry weight)
correction, since the reagent blank (protein plus digestion medium) of different proteins had an absorbance value very close to 0.025 (absorbance of reagent blank of soy protein isolate used as a standard protein for construction of the standard curve). In the case of rapeseed protein isolate and Promine D (another commercial soy protein isolate), however, since their reagent blanks had absorbance values of 0.9 and 0.05, respectively, a correction was made to compensate for these excessive blank (>0.025) absorbances. Thus, 0.065 (=0.09 - 0.025) and 0.025 (=0.05 - 0.025) were subtracted from the absorbances observed for rapeseed and Promine D, respectively, and then, these net absorbance values were entered into the equation of the standard curve for FBC determination.

Table 1 also includes the FBC values of the same food proteins determined by the method of Lin et al. (1974) after its slight modification by Wang and Kinsella (1976). It is noteworthy that ovalbumin and β-lactoglobulin had very high FBC values while the present method yielded considerably lower values.

C. Statistical analysis

Regression analysis was used to quantify the relationship between FBC of 11 food proteins and various independent variables. Simple linear regression analysis showed no significant correlation between protein surface hydrophobicity ($S_o$) and FBC. Multiple linear regression analysis of $S_o$, solubility (s) and FBC also did not show any significant correlation. However, when simple linear regression analysis was applied to correlate $S_o$ and FBC of only 8 proteins of Table 1 (i.e. β-lactoglobulin, BSA and whey protein were excluded), the coefficient of determination was significant ($r^2 = 0.6191$, $p < 0.05$). The regression equation was
'FBC(%) = 22.78 + 0.9976 S_o', and the standard error of estimate (S_{xy}) was 22.21. This correlation can be seen in Figure 5.

Moreover, backwards stepwise regression analysis between FBC and various independent variables showed a highly significant correlation between S_o, interaction of S_o and s (S_o x s), and FBC (R^2 = 0.8017, P < 0.01). A multiple regression model for prediction of S_o and S_o x s effects on FBC is presented in Table 2. FBC was positively affected by S_o whereas the interaction of S_o with solubility had a negative effect on it. The β (normalized coefficient) values in this model suggest that both S_o and S_o x s were equally important in determining the FBC of these proteins. The r^2 (coefficient of determination) and R^2 (coefficient of multiple determination) values indicate the percentage of variation in a dependent variable accounted for by its regression on the independent variable or variables, respectively; the higher the value, the greater the accountability. Comparing simple and multiple regression models in terms of their ability to more accurately predict the FBC of proteins, it is obvious that the latter is the model of choice, since 80.17% of the variation in FBC could be accounted for by the multiple regression model of Table 2 (as opposed to 61.91% of the variation in FBC of only 8 proteins accounted for by the simple regression model). Figure 6 shows the response surface contour of the FBC (of the 11 food proteins of Table 1) as a function of S_o and solubility index. As it can be seen, the iso-response line with the highest FBC value corresponds to relatively medium S_o (75 - 125) and low solubility (20 - 48%) values. As S_o decreased below or increased above these values the FBC decreased. All the above results, therefore, suggest that S_o plays a very important role in the fat binding process.
Fig. 5. Relationship between hydrophobicity ($S_0$) and fat binding capacity of food proteins. 
1, ovalbumin; 2, casein; 3, soy protein isolate; 4, Promine D; 5, pea protein isolate; 6, gelatin; 7, sunflower protein isolate; 8, rapeseed protein isolate.
Table 2 - Multiple regression model for prediction of fat binding capacity of various food proteins.*

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Variable description</th>
<th>Regression coefficient</th>
<th>F-ratio</th>
<th>F-probability</th>
<th>Beta value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat binding capacity</td>
<td>$S_o$</td>
<td>1.381</td>
<td>21.77</td>
<td>0.002</td>
<td>5.034</td>
</tr>
<tr>
<td></td>
<td>$S_o \times s$</td>
<td>-0.014</td>
<td>25.91</td>
<td>0.001</td>
<td>-5.492</td>
</tr>
<tr>
<td>Standard error of estimate</td>
<td>Constant</td>
<td>30.271</td>
<td>8.41</td>
<td>0.020</td>
<td>0.793</td>
</tr>
</tbody>
</table>

(R² = 0.8017; Standard error of estimate = 19.01; F-probability = 0.0015)

* n = 11
Fig. 6. Fat binding capacity response surface contour as a function of hydrophobicity ($S_o$) and solubility index ($s$).
It should be noted that no correlation (simple or multiple of any form) was found for FBC of the same food proteins determined by the method of Wang and Kinsella (1976) with \( S_0 \) or \( S_0 \) and \( s \).

The finding by this study that high protein solubility negatively affected the FBC of proteins has some resemblance with the results of the work of Torgersen and Toledo (1977) who correlated physical properties of proteins with their functional characteristics in comminuted meat systems. They found a significant positive correlation coefficient between solubility and fat binding (fat release on cooking), which meant that the more soluble the protein additives the lesser the fat binding properties of the system to which these protein preparations were used. Moreover, Dippold (1961) reported that a doughnut mix containing 4% soy flour of high solubility (NSI of 80%) absorbed about 50% less fat than the same mix containing 4% soy flour of low solubility (NSI of 60%).

Although the aforementioned studies were conducted on complex food systems and the fat binding capacity determined differently (as fat release or absorption on cooking), and therefore a direct comparison with the finding in this study may be inappropriate, it is likely that high solubility of proteins has an adverse effect on their fat binding capability. One possible reason for the adverse effect of high solubility on the FBC of proteins observed in this study is the conformation of the soluble proteins (BSA, \( \beta \)-lactoglobulin, whey protein) which does not permit their binding sites (hydrophobic side chains) to be sterically available for interaction with the oil (hydrocarbon chains). The explanation is supported by the fact that BSA, \( \beta \)-lactoglobulin (Pham and Nakai, 1981) and whey proteins (Morr, 1979) have mainly \( \alpha \)-helical conformation as opposed to the random or \( \beta \)-pleated sheet conformation of soy protein.
Another reason may be a limited access of oil to hydrophobic sites of soluble (100%) proteins due to the presence of an excessive number of polar groups forming a barrier around the surface hydrophobic groups (of protein).

The finding of this study that with increasing $S_o$ the FBC is increased and subsequently decreased (at high $S_o$ values) may be explained by taking into consideration the suggestion of Wolf and Cowan (1975) that fat absorption may be another aspect of emulsification, since in ground meat products fat absorption or binding appears to involve formation and stabilization of an emulsion. According to Aoki et al. (1981), however, the emulsifying properties of proteins ultimately depend on the suitable balance between hydrophile and lipophile, and do not necessarily increase as the proteins become more lipophilic. These situations are similar to the concept of the required HLB (hydrophile-lipophile balance) values of fats and the HLB values of surfactants for emulsification.

D. Mechanism of fat absorption

Factors affecting the protein-lipid interaction include protein conformation, protein-protein interactions, and the spatial arrangement of the lipid phase resulting from the lipid-lipid interaction (Hutton and Campbell, 1981). Non-covalent bonds, such as hydrophobic, electrostatic, and hydrogen, are the forces involved in the protein-lipid interactions. Hydrogen bonding is of secondary importance in lipid-protein complexes, although it is indirectly important in hydrophobic bonding (Karel, 1973), since in aqueous media the water-water interactions by hydrogen bonding is much stronger than the interaction between water and nonpolar groups, thus giving rise to hydrophobic bonding between nonpolar groups. Electrostatic
attraction can occur between the negatively charged phosphate groups of phospholipids and positively charged protein groups (such as lysyl or guanidyl) or between a positively charged group in the phospholipid (e.g. choline) and a negatively charged amino acid side chain (e.g. aspartyl). A related mode of binding is the formation of salt bridges between a negatively charged amino acid side chain and a negatively charged phosphate group of a phospholipid via divalent calcium or other metal ions (Karel, 1973; Pomeranz, 1973; Ryan, 1977). Hydrophobic bonding is likely to play a major role in stabilizing the interactions of both polar and nonpolar lipids with proteins (Ryan, 1977). Moreover, nonpolar dispersion or Van der Waals forces become important when interacting groups are near (Karel, 1973) and may play a role in attraction between nonpolar groups in systems in which hydrophobic bonds are impossible because of limited water (Pomeranz, 1973).

As with the protein-protein interactions, it is not possible to attribute protein-lipid interactions to any single specific kind of molecular force (Ryan, 1977). However, according to Wall (1979) lipids bind to proteins mainly through association with hydrophobic groups. In the method described in this study electrostatic attraction does not seem to play any role in lipid-protein interaction, since the oil used was refined and so should have a negligible amount of phospholipids. The fact that a highly significant coefficient of determination ($R^2 = 0.8017$) was observed between $S_0$, $S_0 \times s$, and FBC of the food proteins tested suggest that $S_0$ is a major determinant of FBC of proteins.
CONCLUSIONS

A simple turbidimetric method was developed for determining the fat binding capacity (FBC) of various proteins. The turbidity was dependent on wavelength, blending time and volume of oil. A highly significant correlation ($R^2 = 0.8017, P < 0.01$) was found between $S_o, S_o \times s$, and FBC of 11 food proteins tested. Advantages of this method include: (1) the small amount (40 mg) of sample required as compared to 4 g (Sosulski et al., 1976), 1 g (Sathe and Salunkhe, 1981), or 0.5 g (Lin et al., 1974; Wang and Kinsella, 1976) required by the existing methods; and (2) the fat absorption, as determined by the present method, can be attributed to binding of oil by the protein and not to the entrapment.
REFERENCES


Chapter III

Relationships of Hydrophobicity to Emulsifying Properties of Heat Denatured Proteins
INTRODUCTION

To be useful and successful in food applications, proteins in addition to providing essential amino acids, should ideally possess several desirable characteristics referred to as functional properties (Wang and Kinsella, 1976). Moreover, according to Johnson (1970) the functional and physical properties, rather than the nutritional value, of protein in protein-containing products will largely determine their acceptibility as ingredients in prepared foods. Functional properties of proteins connote those physicochemical properties which affect the behaviour of proteins in food systems during preparation, processing, storage and consumption (Kinsella, 1979). These properties reflect the composition and conformation of the proteins, their interactions with other food components, and they are affected by processing treatments and the environment (Kinsella, 1979; Kinsella and Shetty, 1979).

The ability of protein to aid the formation and stabilization of emulsions is critical for many applications in chopped, comminuted meats, cake batters, coffee whiteners, milks, mayonnaise, salad dressings, and frozen desserts. In these products varying emulsifying and stabilizing capacities are required because of the differing composition and stresses to which these products are subjected (Kinsella, 1979). Moreover, the ability of proteins to bind fats is a very important functional property for such applications as meat replacers and extenders, principally because it enhances flavor retention and reputedly improves mouthfeel (Kinsella, 1976). Soy proteins have been added to ground meats to promote fat absorption or fat binding, and thus decrease cooking losses and maintain dimensional stability in the cooked product (Wolf and Cowan, 1975).
To evaluate the emulsifying properties of a protein its solubility profile is usually determined, because of its usefulness as an excellent index of protein functionality (Kinsella, 1976). Good solubility can markedly expand potential applications of a protein (Kinsella, 1976). Denaturation, on the other hand, implicates loss of protein functional properties and is usually measured as a loss of solubility (Nakai and Powrie, 1981). Generally, surfactant properties are related to the aqueous solubility of proteins (Kinsella, 1976). A positive correlation between solubility and the ability of a protein to emulsify and stabilize an emulsion has been reported in many studies (Crenwelge et al., 1974; Pearson et al., 1965; Swift and Sulzbacher, 1963; Volkert and Klein, 1979; Yasumatsu et al., 1972). Many authors point to evidence, however, that emulsifying properties and solubility are not well correlated (Aoki et al., 1980; McWatters and Cherry, 1975; McWatters and Holmes, 1979a, 1979b; Smith et al., 1973; Wang and Kinsella, 1976). Thus, Smith and coworkers (1973) found that no significant correlation existed between solubility and emulsifying capacity or emulsion stability of the proteins studied. Wang and Kinsella (1976) reported that the emulsifying capacity of alfalfa leaf proteins showed no significant correlation with the solubility, since these proteins showed maximum emulsifying capacity at pH 5 where the protein solubility was at the lowest level. The same authors, moreover, suggested that proteins remaining soluble in this pH region had a higher emulsifying capacity than those solubilized above and below this pH region. McWatters and Cherry (1975) and McWatters and Holmes (1979a, 1979b) also found that high levels of nitrogen solubility were not necessarily associated with maximum emulsifying capacity. Moreover, Aoki et al., (1980) reported that the pH-emulsion stability
profile of soy protein (11S protein rich fraction) did not correspond to its pH-solubility profile.

The protein hydrophobicity has been lately receiving much attention since the hydrophobic interactions are considered to play important roles in the functional properties of food proteins (Kato et al., 1981; Kinsella, 1979). Keshavarz and Nakai (1979) reported a significant correlation between surface hydrophobicity (determined by hydrophobic chromatography and hydrophobic partition techniques) and interfacial tension of the proteins studied. Kato and Nakai (1980) subsequently reported that the surface hydrophobicity (determined fluorometrically) showed significant correlations with interfacial tension and emulsifying activity of the proteins studied. Their results suggest that the emulsification of oil with protein can be explained using the concept of protein hydrophobicity. Nakai et al. (1980b) also reported that the effective (surface)hydrophobicity showed good correlations with interfacial tension and emulsifying activity of the plant proteins studied. It is noteworthy that these authors observed a closer correlation of emulsification capacity with hydrophobicity than with solubility. Kato et al. (1981) reported that the emulsifying properties of ovalbumin and lysozyme were markedly improved by partial denaturation resulting from heat- and SDS - treatments, and were linearly correlated with surface hydrophobicity. Moreover, Voutsinas and Nakai (1981), using a new turbidimetric method they developed, found that surface hydrophobicity was correlated with fat binding capacity of the food proteins studied.

While many factors influence the performance of proteins in food systems, heat treatment is one of the most important and is very often used during the processing of protein products. This study was initiated
to elucidate the observation that the emulsifying capacity of soy protein was not adversely affected even by texturization which caused a loss in solubility. The objectives of this part of the thesis, therefore, were to determine the effect of heating on the emulsifying properties of selected food proteins, and, to assess the value of surface hydrophobicity as a predictor of the emulsifying properties of these proteins (because of the aforementioned contradictions among scientists with respect to value of solubility as an index of the emulsifying properties of a protein).

LITERATURE REVIEW

A. Solubility

Solubility is an experimentally measurable property that can yield information about the functional behaviour as well as the physico-chemical nature of the proteins (Shen, 1981). Bigelow (1967) proposed that two structural features, namely charge frequency and hydrophobicity, are the factors which influence protein solubility. The higher the charge frequency and the lower the hydrophobicity, the higher would be the solubility. Solubility is, also, affected by a magnitude of factors, namely, protein source (Sosulski and Bakal, 1969), particle size of the product (Johnson, 1970), processing history, minor and major treatments in its preparation and processing, heating (Kinsella, 1976), protein concentration (Betschart, 1974; Kinsella, 1976), presence of other ingredients such as salts (Hermansson, 1973; Mattil, 1971) and carbohydrates (Tybor et al., 1975), and the experimental conditions of solubility determination, e.g., pH (Hermansson, 1973; Kodagoda et al.,

B. Hydrophobicity

Much effort has been made to quantify the hydrophobic character of proteins due to the importance of hydrophobic interactions for their stability, conformation and function (Bigelow, 1967; Tanford, 1962). Early studies were mainly concerned with the total hydrophobicity of protein (Tanford, 1962), calculated as the sum of the side chain hydrophobicities of all residues (constituent amino acids), or the average hydrophobicity (Bigelow, 1967), calculated from the total hydrophobicity divided by the number of residues. However, as Melander and Horvath (1977) pointed out, protein functions such as solubility and chromatographic behaviour depend on the hydrophobic surface properties, because the nonpolar residues buried in the interior of the native protein are not believed to affect directly these phenomena. Therefore, the above mentioned parameters are not expected to yield information about the surface hydrophobicity, which is likely to be of great biological and technological significance (Melander and Horvath, 1977). In most native proteins, some hydrophobic groups remain exposed at the molecular surface or in crevices (Tanford, 1972), and the hydrophobic side chains occur more frequently on the surface of the protein that had been assumed (Klotz, 1970). Thus, many attempts have lately been made to determine the effective or surface hydrophobicity of proteins that correlates well with the propensity of protein molecule to participate in hydrophobic interactions.

Keshavarz and Nakai (1979) applied hydrophobic affinity
chromatography and hydrophobic partition to determine the effective hydrophobicity of various proteins. However, the former method is time consuming and the latter suffers from the low solubility of some proteins in the phase systems used. Therefore, development of a simple quantitative method for protein effective hydrophobicity determination was highly desirable. Thus, Kato and Nakai (1980) used a fluorescent probe, cis-parinaric acid, to determine the effective hydrophobicity of proteins. cis-Parinaric acid is a natural polyene fatty acid, thus, it can readily simulate natural lipid-protein interacting systems (Nakai and Powrie, 1981). According to Sklar _et al._ (1976), cis-parinaric acid possesses the advantages of the fluorescent probe techniques (i.e. great detection sensitivity, sensitivity of probes to their environment, and the large number of parameters that can be monitored continuously and on a rapid time scale), while minimizing the disadvantages (i.e. perturbations and inability to predict the location of the probe). Since the structure and the conformation of cis-parinaric acid closely resemble those of normally occurring, cis-parinaric acid is expected to cause minimal perturbations in the systems, and moreover, its location and orientation relative to the surroundings is predictable (Sklar _et al._, 1976). cis-Parinaric acid is practically non-fluorescent in water (quantum yield, Q < 0.001) but fluoresces with different quantum yields in organic solvents or aqueous solutions of different proteins. Thus, several lines of evidence support the notion that hydrophobic interactions are responsible for the enhancement of cis-parinaric acid fluorescence. They include: (1) the increases in quantum yield with decreases in the dielectric constant of the solvent (Sklar _et al._, 1977); (2) the high relative fluorescence values observed in solutions of proteins (e.g. bovine serum albumin,
\( \beta \)-lactoglobulin, \( \kappa \)-casein) known to possess hydrophobic binding sites as opposed to low values obtained with solutions of other proteins such as ovalbumin, conalbumin, etc. (Kato and Nakai, 1980); and (3) the increase in the fluorescent intensity observed after denaturation of proteins, e.g. ovalbumin and lysozyme (Kato and Nakai, 1980; Kato et al., 1981).

The good correlation \( r = 0.97 \) observed by Kato and Nakai (1980) between effective hydrophobicity determined fluorometrically and effective hydrophobicity of the same proteins determined by the hydrophobic partition method, suggests that their fluorescence probe method can be reliably used for quantitative estimation of protein effective hydrophobicity. The fluorescence probe method has the following advantages: (1) it is much simpler and quicker than the hydrophobic chromatography and hydrophobic partition techniques reported by Keshavarz and Nakai (1979). Thus, for one analysis of protein effective hydrophobicity, the fluorescence probe method requires only 10 min compared to approximately 2 hr for the hydrophobic partition and 5 hr for the hydrophobic chromatography (due to slow effluent flow rate); and (2) it is one of few methods that can be used for determining the effective hydrophobicity of insoluble proteins.

C. Formation and stabilization of emulsions

An emulsion can be most simply defined as a dispersion of one liquid in another with which it is immiscible. In an emulsion, the dispersed droplets are commonly referred to as the dispersed, discontinuous or internal phase, and the medium in which they are dispersed is the continuous or external phase. The most common method for
preparing an emulsion is by mechanically dispersing one bulk liquid phase in another.

Emulsions of fats and water are thermodynamically unstable because of the positive free energy caused by interfacial tension (Kinsella, 1979). Thus, an emulsion will rapidly separate into two distinct phases upon standing unless a third phase, an adsorbed surfactant, is present in the interface to stabilize it (Morr, 1981). Proteins are examples of hydrocolloids that exhibit unique surfactant properties due to their large molecular weights and their multiplicity of hydrophobic and hydrophilic residues, each of which exhibit a spectrum of affinities for the polar and nonpolar phases in the emulsion system (Morr, 1981). The amino acid composition and sequence, as well as the secondary, tertiary and quaternary structure, are major factors which govern their effectiveness as surfactants (Powrie and Tung, 1976). The major way to stabilize an emulsion is to develop an energy barrier which prevents coalescence (Karel, 1973). According to Karel (1973), the mechanisms of the activation barrier common in foods are:

(1) Film formation at the oil-water interface with strong steric hindrance to coalescence (association of individual droplets). The strength, compactness, and elasticity of interfacial films around droplets greatly influence the stability of an emulsion (Powrie and Tung, 1976). Cumper and Alexander (1950) proposed that film formation by proteins at an interface occurs in three stages: (a) diffusion of protein molecules to the interface where they are adsorbed in the globular form. Yamauchi et al. (1980) considered that the adsorption of protein at first occurs by means of hydrophobic interactions between the protein and a fat surface; (b) surface denaturation by uncoiling of the adsorbed globular
protein; and (c) aggregation of the unrolled polypeptide chain into a coagulum largely devoid of surface activity and consequently forced out of the interface by the spreading pressure of the native protein undergoing surface denaturation. The kinetics of film formation is very much influenced by the composition-conformation of the protein, viscosity of the protein dispersion, pH, ions, temperature and energy input (Tornberg, 1978).

(2) Electrostatic repulsion between charged groups located in the oil-water interface. With regard to oil in water (O/W) emulsions, surface charges can originate from ionization of groups on ionic emulsifiers, adsorption of ions from the aqueous phase on nonionic emulsifier layer, or frictional contact between droplet surfaces and the aqueous medium (Powrie and Tung, 1976).

(3) Formation of hydration layers outside the oil droplets, because water-orienting hydrophilic groups are present at the surface.

(4) Low interfacial tension can stabilize emulsions by allowing large droplet deformations, thus increasing the amount of liquid between collision surfaces.

Viscosity is an important physical property of emulsions in terms of emulsion formation and stability. When the viscosity of the continuous phase is too low, the increased movement of particles (droplets) will cause flocculation and finally coalescence. Emulsions can be stabilized by increasing the viscosity of the continuous phase.

Three different methods have been used in investigating the emulsifying properties of proteins: emulsifying capacity (EC), emulsion stability (ES), and emulsifying activity (EA). EC is usually defined as the volume of oil (ml) that can be emulsified by a standard amount of
protein (g), before phase inversion or collapse of emulsion occurs. There are many factors (i.e. equipment design, shape of container, speed of blending, rate of oil addition, kind of oil, pH of the medium, protein source, protein concentration, salt, etc.) in the determination that can affect the EC of proteins. Thus, EC is not solely a property of the protein under test but rather is a property of the emulsion system, the equipment and method used to produce the emulsion (Tornberg and Hermansson, 1977). Furthermore, in cases where very viscous emulsions are formed, mixing of oil into the emulsion may be inefficient or incomplete and the observed EC value erroneous (Pearse and Kinsella, 1978).

Emulsion stability (ES) refers to the ability of a protein to form an emulsion that remains unchanged for a particular duration, under specific conditions (Kinsella, 1976). ES is commonly measured in terms of the amount of oil and/or cream separating from an emulsion during a certain period of time at a stated temperature and gravitational field (Acton and Saffle, 1970). The time required for a specified degree of breakdown to occur is also used as a measure of stability (Pearson et al, 1965). Several workers centrifuged heated emulsions and expressed the ES in terms of the height of the cream layer as a percentage of the initial height of the emulsion (Wang and Kinsella, 1976; Yasumatsu et al., 1972). However, according to Pearse and Kinsella (1978), the process occurring during the centrifugation of the emulsion may not be characteristic of those occurring in a stored or heated emulsion. Thus, ES measured by the method of Yasumatsu et al. (1972) may not be a valid indication of emulsion stability. Emulsifying activity (EA) reflects the ability of the protein to aid in emulsion formation and stabilization of the newly created emulsion (Kitchener and Musselwhite, 1968). EA is measured by determining
the particle size distribution of the dispersed phase by microscopy, Coulter counting, or spectroturbidity (Walstra et al., 1969). Results from microscopic techniques take much time and show poor reproducibility, while the Coulter counter method is more reproducible (Waniska et al., 1981). The spectroturbidity method is simple, rapid, and theoretically sound (Kerker, 1969) and provides information about the average diameter and particle size distribution.

Because of the problems and shortcomings of many of the traditional approaches and the need for more reliable methods for the quantification of emulsifying properties of proteins by techniques requiring small quantities of protein, Pearse and Kinsella (1978) evaluated the potential of turbidimetry for measuring emulsification. These authors proposed two indexes, emulsifying activity index (EAI) and emulsion stability index (ESI), for the characterization of emulsifying agents, especially proteins. The EAI is related to the interfacial area of the emulsion and has units of area of interface (m$^2$) stabilized by unit weight of protein (g). The EAI is a function of oil volume fraction, protein concentration, and of the type of equipment used to prepare the emulsion. The emulsion breakdown can be monitored by determining the ESI from the decrease in absorbance with time resulting from the irreversible reduction in the interfacial area (brought about by the processes of coalescence and oiling-off). These two indexes (EAI and ESI) are easily measured and, from theoretical and practical considerations, seem more likely to be related to practical performance of products than the EC, ES, and EA tests which are commonly used (Pearse and Kinsella, 1978).
MATERIALS AND METHODS

Materials

Pea protein isolate (a), Century cultivar field pea, was obtained from POS Pilot Plant Corp., University of Saskatchewan, Saskatoon, Sask. Pea protein isolate (b), Pro-Pulse W100, was obtained from Griffith Laboratories Ltd., Scarborough, Ont. Vital wheat gluten, Whetpro 75%, was supplied by Industrial Grain Products Ltd., Thunder Bay, Ont. Canola protein isolate was prepared by the method of Nakai et al. (1980a). The protein content of isolate batches (a) and (b) was 84% and 88%, respectively. Myosin was prepared according to the method of Perry (1955) with slight modification as follows: the pectoralis superficialis and profundus muscles of a freshly slaughtered male chicken (broiler 11 - 12 weeks old) were ground (after being chilled in crushed ice) with a meat grinder and immediately extracted with 3 volumes of cold KCl-potassium phosphate buffer, pH 6.5. The suspension was stirred slowly for 15 min and then allowed to stand at 4°C for 45 min. The extract was subsequently centrifuged for 1 hr at 1,000 x g. Clarification of the crude myosin extract was achieved by gentle filtration of the supernatant through a Whatman No. 4 filter paper. The pH of the filtrate was adjusted to 6.5, if necessary, with NaOH. The crude myosin was then precipitated by the slow addition, with stirring, of 400 ml of crude extract to 3.6 L of distilled deionized water. After overnight settling, the myosin was collected by centrifugation for 1 hr at 12,000 x g. The resultant gel was dissolved in one volume of 0.01 M phosphate buffer pH 7.4 containing 0.6 M NaCl, and the pH was adjusted to 7.4 with NaOH.

All the other materials used in this study were exactly similar to those described in the materials section of the second chapter of this thesis.
Methods

A. Preparation (insolubilization) of protein samples

For hydrophobicity, solubility, emulsifying activity index, emulsion stability index and fat binding capacity determinations, the following protein samples were prepared:

**Bovine Serum Albumin (BSA)**

1% solution in distilled water. Heated: the pH was adjusted to 4.0 and then the solution was heated at 100°C for 5 min and homogenized in an Omni-Mixer (Ivan Sorvall, Norwalk, Conn.) for 1 min at speed setting 1 (lowest speed). Control: not heated.

**β-Lactoglobulin**

1% solution in distilled water. Heated: the pH was adjusted to 1.0, heated at 100°C for 15 min and then homogenized for 1 min at speed setting 1.

**Soy Protein Isolate**

1% aqueous dispersion, stirred on magnetic stirrer for 5 min, and then, homogenized for 1 min at speed setting 5. Samples 1 - 4: pH 5.5, heated at 100°C for 0.25, 0.5, 1.0, and 2.0 min, respectively. Sample 5: pH 5.5, autoclaved at 121°C for 15 min. Sample 6: pH 7.2, autoclaved at 121°C for 15 min.

**Promine-D**

1% dispersion in 0.01 M phosphate buffer, pH 7.4, stirred magnetically for 5 min and homogenized for 1 min at speed setting 5.

**Ovalbumin**

1% aqueous solution. Samples 1 - 4: pH 5.6, heated at 80°C for 1.5, 2.0, 2.5, and 3.0 min, respectively. Sample 5: pH 5.6, heated at 100°C for 5 min. Sample 6: pH 1.0, heated at 100°C for 15 min.
Pea Protein Isolate (a)

1% aqueous dispersion, stirred magnetically for 5 min, and then, homogenized for 1 min at setting 5. Samples 1 - 4: pH 5.8, heated at 80°C for 1, 2, 4 and 7 min, respectively, and then, homogenized for 10 sec.

Pea Protein Isolate (b)

1% dispersion in 0.01 M phosphate buffer, pH 7.4, was magnetically stirred 5 min, and then, homogenized for 1 min at speed setting 5.

Vital Gluten

Samples 1 - 5: 1% dispersions in 0.5, 1.0, 1.5, 1.75 and 2.0 N acetic acid, respectively. Then, the samples were magnetically stirred for 5 min, homogenized 15 sec at speed setting 1, and heated at 100°C for 30 min.

Canola Protein Isolate (a)

1% aqueous dispersion, was stirred for 5 min, and then, homogenized for 1 min at speed setting 5. Samples 1 - 4: pH 5.5, heated at 100°C for 0.5, 1.0, 1.5 and 2.0 min, respectively, and then homogenized for 5 sec at speed setting 1. Sample 5: pH 7.2, autoclaved at 121°C for 10 min.

Canola Protein Isolate (b)

1% dispersion in 0.01 M phosphate buffer, pH 7.4, was stirred for 5 min, and then, homogenized for 1 min at speed setting 5.

Sunflower Protein Isolate

1% dispersion in 0.01 M phosphate buffer, pH 7.4, was stirred for 5 min, and then, homogenized for 1 min at speed setting 5.

Whey Protein

1% solution in 0.03 M CaCl₂. Samples 1 - 3: pH 6.0, heated at 80°C
for 4, 5 and 15 min, respectively. Sample 4: pH 5.8, heated at 80°C for 15 min.

**Whole Casein**

Control: 1% solution in 0.01 M CaCl$_2$, pH 7.4. Samples 1 and 2: 1% solution in 0.01 M CaCl$_2$, pH 7.4, autoclaved at 121°C for 5 and 20 min, respectively. Sample 3: 1% solution in 0.02 M CaCl$_2$, pH 7.4, autoclaved at 121°C for 20 min.

**Gelatin**

Control: 1% dispersion in 0.01 M phosphate buffer, pH 7.4, stirred 5 min, and then, homogenized for 1 min at speed setting 5. Heated: 0.5% dispersion in 0.01 M phosphate buffer, pH 7.4, heated at 75°C for 2.5 min with stirring.

**Myosin**

To form a stock solution for determination of hydrophobicity, solubility, and other functional properties, the original gel was diluted 2X with 0.3 M NaCl in 0.01 M phosphate buffer, pH 7.4. The control sample was unheated and samples 1 and 2 were heated at 75°C for 1 and 5 min, respectively.

Prior to analysis, all heated and unheated (control) protein samples - except Promine-D, pea protein (b), canola protein (b), sunflower protein, gelatin, and myosin - were dialysed against 0.01 M phosphate buffer, pH 7.4, containing 0.02% sodium azide. After hydrophobicity, solubility, EAI and ESI were determined, the protein samples were freeze-dried and subsequently used for fat binding capacity (FBC) determinations.

**B. Solubility index, hydrophobicity and fat binding capacity determinations**

These protein properties were determined as described in the second
chapter of this thesis.

C. Emulsifying activity index (EAI) determination

The EAI was determined by the turbidimetric technique of Pearse and Kinsella (1978). To prepare emulsion, each protein sample was diluted to a concentration of 0.5% with 0.01 M phosphate buffer, pH 7.4. Two ml of corn oil and 6 ml of the diluted protein dispersion were homogenized together in an Omni-mixer with a micro-attachment (Ivan Sorvall, Inc., Norwalk, CN) at speed setting 1 for 1 min at 20°C.

D. Emulsion stability index (ESI) determination

ESI was determined by a modification of the method of Pearse and Kinsella (1978) as follows: the emulsion, prepared as described above (EAI), was held at room temperature and aliquots (0.1 ml) were taken directly from the bottom of the container, containing the emulsion, at different time intervals and diluted to 50 ml (500 x dilution) with 0.01 M phosphate buffer, pH 7.4, containing 0.1% sodium dodecyl sulfate (SDS). The absorbance of diluted emulsions at 500 nm was then recorded with a Beckman DB spectrophotometer. The half-life (min) of the absorbance decay with time, determined graphically, was used as ESI.

E. Viscosity determination

Viscosity measurements of 0.5% protein dispersions at 20°C were made using a Brookfield Synchro-Lectric viscometer, Model LVT fitted with a UL adapter, at 60 rpm.
F. Statistical analysis

Simple and multiple linear regression analyses were used to determine the relationships between hydrophobicity, solubility and emulsifying properties of the protein samples. These analyses were carried out by using a Monroe 1880 programmable calculator. In addition, backwards stepwise multiple regression analyses and surface visualization plotting were carried out using an Amdahl 470 V/8 computer at the University of British Columbia. Five independent variables were used in the initial equation in the backwards stepwise procedure including surface hydrophobicity ($S_o$), solubility index ($s$), interaction of $S_o$ and $s$, and quadratic powers of $S_o$ and $s$. Dependent variables included EAI, ESI, and FBC.

RESULTS AND DISCUSSION

A. Effect of heat treatment on emulsifying properties

Table 1 shows the relationships of hydrophobicity and solubility index of selected proteins with their emulsifying properties. It is evident that, for most proteins under a given set of conditions, protein solubility decreased as heating time increased due to the progressive denaturation of the protein. Gelatin, as expected, was completely solubilized on heating due to the rupture of hydrogen bonds which are responsible for its insolubility. As protein denaturation progressed, as seen by the decrease in protein solubility, the hydrophobicity usually increased. This is due to the gradual exposure of hydrophobic amino acid residues of native proteins (which are usually buried in the interior of the molecules) as a result of the protein unfolding. In the case of
Table 1 - Relationships between protein hydrophobicity, solubility index, emulsifying activity, emulsion stability and fat binding capacity of various proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Hydrophobicity ($S_o$)</th>
<th>Solubility index (%)</th>
<th>EAI ($m^2/g$)</th>
<th>ESI&lt;sup&gt;b&lt;/sup&gt; (min)</th>
<th>FBC(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin - control</td>
<td>325.0</td>
<td>100.0</td>
<td>148</td>
<td>108.50</td>
<td>91.50</td>
</tr>
<tr>
<td>Bovine serum albumin - heated</td>
<td>304.0</td>
<td>26.8</td>
<td>140</td>
<td>90.00</td>
<td>109.00</td>
</tr>
<tr>
<td>3-Lactoglobulin - control</td>
<td>426.0</td>
<td>100.0</td>
<td>96</td>
<td>27.20</td>
<td>37.00</td>
</tr>
<tr>
<td>B-Lactoglobulin - heated</td>
<td>192.0</td>
<td>6.4</td>
<td>51</td>
<td>25.30</td>
<td>27.50</td>
</tr>
<tr>
<td>Soy isolate - control</td>
<td>95.0</td>
<td>26.4</td>
<td>42</td>
<td>6.65</td>
<td>25.20</td>
</tr>
<tr>
<td>- sample 1</td>
<td>97.0</td>
<td>26.4</td>
<td>51</td>
<td>7.30</td>
<td>26.00</td>
</tr>
<tr>
<td>- sample 2</td>
<td>131.0</td>
<td>24.0</td>
<td>56</td>
<td>5.55</td>
<td>17.00</td>
</tr>
<tr>
<td>- sample 3</td>
<td>150.0</td>
<td>14.2</td>
<td>48</td>
<td>1.00</td>
<td>5.13</td>
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<td>8.2</td>
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</tr>
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<td>138.00</td>
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</table>

(continued)
<table>
<thead>
<tr>
<th>Protein</th>
<th>Hydrophobicity ($S_0$)</th>
<th>Solubility index (%)</th>
<th>EAI ($m^2/g$)</th>
<th>$^b_{ESI}$ (min)</th>
<th>FBC(%)</th>
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<tr>
<td>Ovalbumin - sample 6</td>
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<td>61</td>
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<td>Pea isolate (b)</td>
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<td>17.60</td>
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<td>60</td>
<td>0.54</td>
<td>3.76</td>
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<td>- sample 1</td>
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<td>15.9</td>
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<td>0.49</td>
<td>2.80</td>
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<tr>
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<td>50</td>
<td>2.20</td>
<td>8.40</td>
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<td>Canola isolate (b)</td>
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Table 1 - continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>Hydrophobicity ( (S_o) )</th>
<th>Solubility index (%)</th>
<th>EAI ( (m^2/g) )</th>
<th>ESI(^b) (min) I</th>
<th>ESI(^b) (min) II</th>
<th>FBC(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- control</td>
<td>182.0</td>
<td>88.7</td>
<td>87</td>
<td>50.30</td>
<td>87.50</td>
<td>74.5</td>
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<tr>
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<td>75.2</td>
<td>98</td>
<td>61.30</td>
<td>102.00</td>
<td>75.2</td>
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<td>164.0</td>
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<td>108.00</td>
<td>99.4</td>
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<td></td>
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<td>- control</td>
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<td>100.0</td>
<td>58</td>
<td>1.70</td>
<td>39.30</td>
<td>11.3</td>
</tr>
<tr>
<td>- sample 1</td>
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<td>56</td>
<td>9.50</td>
<td>35.00</td>
<td>18.1</td>
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<tr>
<td>- sample 2</td>
<td>21.0</td>
<td>71.5</td>
<td>49</td>
<td>14.75</td>
<td>24.30</td>
<td>16.7</td>
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<tr>
<td>- sample 3</td>
<td>23.0</td>
<td>70.2</td>
<td>50</td>
<td>12.50</td>
<td>16.80</td>
<td>14.0</td>
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<td>Gelatin</td>
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<td></td>
</tr>
<tr>
<td>- control</td>
<td>5.0</td>
<td>15.3</td>
<td>46</td>
<td>4.65</td>
<td>3.10</td>
<td>19.1</td>
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<tr>
<td>- heated</td>
<td>6.0</td>
<td>100.0</td>
<td>59</td>
<td>10.20</td>
<td>9.40</td>
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</tr>
<tr>
<td>Myosin</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>- control</td>
<td>14.0(^c)</td>
<td>100.0(^c)</td>
<td>43(^c)</td>
<td>36.00(^c)</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>- sample 1</td>
<td>44.0(^c)</td>
<td>50.9(^c)</td>
<td>50(^c)</td>
<td>22.40(^c)</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>- sample 2</td>
<td>54.0(^c)</td>
<td>16.5(^c)</td>
<td>48(^c)</td>
<td>10.00(^c)</td>
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</tr>
</tbody>
</table>

\(^a\) average of duplicate determinations

\(^b\) I: 0.1 M NaCl added; II: NaCl not added

\(^c\) 0.3 M NaCl added
whey protein, it can be seen, that excessive heating resulted in a
decrease of hydrophobicity, due probably to participation of some of the
exposed hydrophobic groups in hydrophobic interactions. For casein,
heating did not result in any substantial change of its hydrophobicity
and this was expected, since casein exists in a random coil conformation.
The results of Table 1 also indicate that for samples having the same
solubility, the more hydrophobic the protein, the greater are its
emulsifying properties.

Looking, specifically, at the results of soy protein in Table 1,
it can be seen that, the EAI of all heated samples was slightly greater
than than of the control. The ESI, however, was initially slightly
increased by heating, but subsequently started to decrease as the
solubility progressively decreased; that is, solubility became an
increasingly important factor controlling this property. The higher
EAI values of heated soy protein samples were probably due to their
increased hydrophobicity values. On the other hand, the observed
decrease in ESI upon heating must be due to the decrease in solubility
mainly as a result of protein-protein interaction, and secondarily
because of the increase in hydrophobicity.

The proteins studied here can be broadly divided into 4 categories
according to the effect of heating on their emulsifying properties. The
first category includes BSA, gluten, and whey protein whose one emulsifying
property (EAI) was not substantially affected by heating, whereas the
other (ESI) was improved by heating. The second category includes soy
protein and myosin whose one emulsifying property (EAI) was slightly
improved by heating, whereas the other (ESI) was decreased. The third
category includes β-lactoglobulin, pea, canola, and casein, whose both
emulsifying properties were adversely affected by heating. Finally the fourth category includes ovalbumin and gelatin whose both emulsifying properties were markedly improved upon heating. It is evident, therefore, that heating did not have the same effect on the emulsifying properties of different proteins.

The improvement of emulsifying properties of gelatin upon heating was mainly due to the increase in solubility since its hydrophobicity was not changed by heating. Kato and Nakai (1980), and Kato et al. (1981) reported that the emulsifying properties (EAI and ESI) of ovalbumin and lysozyme were markedly improved upon heating. They also found that this improvement was correlated with the higher hydrophobicities of heat denatured protein samples as compared to those of the native proteins. Their results, moreover, indicated that the more hydrophobic the proteins, the greater the decrease in interfacial tensions and the increase in emulsifying properties. During the handling of the ovalbumin samples we noticed that the apparent viscosities of heat-denatured samples were greater than that of the control. This is evident in Table 2, which shows the apparent viscosity changes of some proteins upon heating. It is suggested, therefore, that higher viscosity appears to be another factor contributing to the great improvement of emulsifying properties of heat denatured ovalbumin samples.

As shown in Table 1, the FBC of different proteins was differently affected by heating. While the FBC of canola (rapeseed) protein was generally not affected by heating, the FBC's of BSA, β-lactoglobulin, ovalbumin, whey protein, and casein were positively affected, and the FBC's of soy and pea proteins were adversely affected by heating.

It is generally accepted that protein denaturation is undesirable
Table 2 - Effect of heating on the apparent viscosity of some proteins at 20°C

<table>
<thead>
<tr>
<th>Protein</th>
<th>Apparent viscosity (Pa.s x 10^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey - control</td>
<td>1.14</td>
</tr>
<tr>
<td>- sample 1</td>
<td>1.14</td>
</tr>
<tr>
<td>- sample 2</td>
<td>1.14</td>
</tr>
<tr>
<td>- sample 3</td>
<td>1.16</td>
</tr>
<tr>
<td>- sample 4</td>
<td>1.20</td>
</tr>
<tr>
<td>BSA - control</td>
<td>1.13</td>
</tr>
<tr>
<td>BSA - heated</td>
<td>1.24</td>
</tr>
<tr>
<td>Ovalbumin - control</td>
<td>1.10</td>
</tr>
<tr>
<td>- sample 1</td>
<td>1.13</td>
</tr>
<tr>
<td>- sample 2</td>
<td>1.13</td>
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<tr>
<td>- sample 3</td>
<td>1.16</td>
</tr>
<tr>
<td>- sample 4</td>
<td>1.17</td>
</tr>
<tr>
<td>- sample 5</td>
<td>1.18</td>
</tr>
<tr>
<td>- sample 6</td>
<td>1.90</td>
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</tbody>
</table>

a0.5% protein in 0.01 M phosphate buffer, pH 7.4
since it adversely affects protein functionality. However, as shown in this study, emulsifying and fat binding properties of some proteins (ovalbumin, whey protein) can be improved by denaturation. According to Morr (1979) denaturation of the whey protein molecule, if produced at the proper stage of the protein concentrate isolation/utilization process, can improve the functionality. The improvement in functionality is probably due to an unfolding of the molecule to expose hydrophobic amino acid residues, thus making the protein more amphiphilic and capable of orienting at the oil-water interface (Morr, 1979). A great improvement of emulsifying properties (EAI and ESI) of ovalbumin and lysozyme by heat denaturation was also reported by Kato and Nakai (1980) and Kato et al. (1981). Moreover, another example of protein denaturation resulting in improvement of functionality reported by Aoki et al. (1981), who determined the effect of alcohol modification of soy protein on its emulsion stabilizing properties. The soy protein was denatured with 50% alcohol (ethanol or n-propanol) by treatment at 35°C for 2 hr. The emulsion stabilizing properties of soy protein modified with ethanol or n-propanol decreased with increasing the solubility, whereas the emulsion stabilizing properties of the unmodified (control) soy protein increased. Aoki et al. (1981) attributed the improved emulsion stability brought about by the alcohol modification of soy protein to the perturbation and unfolding of the hydrophobic interior structure of the native soy protein by alcohol, and to the resulting increase in the exposed hydrophobic amino acid residues. It should be noted, that the increased emulsion stabilizing properties of soy protein between pH 2 and 7, achieved through alcohol modification by Aoki et al. (1981), is very important because soy protein can be expected to play a significant role in the
stabilization of a wide range of food emulsions, all meat emulsions falling well within these pH limits.

B. Statistical Analysis

Regression equations (models) for prediction of the emulsifying and fat binding properties from hydrophobicity and solubility data of heat denatured proteins of Table 1 are presented in Table 3. Simple linear regression analyses showed that the coefficients of determination ($r^2$) between $S_o$ and EAI, and between $S_o$ and ESI (determined without NaCl) were highly significant ($P < 0.001$). No significant correlation was found, however, between solubility and EAI or ESI. Moreover, no correlation was found between $S_o$ or solubility and FBC. Although significant correlations were found between $S_o$ and ESI as well as solubility and ESI determined in the presence of 0.1 M NaCl, however, such correlations are not considered reliable since the effect of 0.1 M NaCl on solubility was not determined. It is well known that even 0.1 M NaCl may positively or negatively affect the solubility of a protein. Sodium chloride was used in the ESI test, to obtain a general idea on the salt sensitivity of the emulsions formed by different proteins. As seen in Table 1, NaCl even at the low concentration used had generally a detrimental effect upon the stability of the emulsions. NaCl exerted its negative effect on emulsion stability probably by reducing the charge on the surface of the particles (oil globules) and by withdrawing water from their hydrated surfaces.

Multiple linear regression analyses (Table 3) between $S_o$, solubility and EAI or ESI, showed highly significant correlations ($R^2$ values were 0.5419 and 0.4336, $P < 0.001$, respectively). In order to determine
<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Regression analysis</th>
<th>Variable description</th>
<th>Regression coefficient</th>
<th>F-ratio</th>
<th>F-probability</th>
<th>t-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>β-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAI (n=52; ( r^2 = 0.468, \ P&lt;0.001; \text{S.E.}^b=18.95) )</td>
<td>Simple linear</td>
<td>Constant</td>
<td>S&lt;sub&gt;o&lt;/sub&gt;</td>
<td>41.162</td>
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<tr>
<td>EAI (n=52; ( R^2 = 0.542, \ P&lt;0.001; \text{S.E.}^b=17.78) )</td>
<td>Multiple linear</td>
<td>Constant</td>
<td>S&lt;sub&gt;o&lt;/sub&gt;</td>
<td>29.283</td>
<td>0.207</td>
<td>7.202***</td>
<td>0.698</td>
</tr>
<tr>
<td>EAI (n=52; ( R^2 = 0.583, \ P&lt;0.001; \text{S.E.}^b=17.40) )</td>
<td>Backwards multiple</td>
<td>Constant</td>
<td>S&lt;sub&gt;o&lt;/sub&gt;</td>
<td>16.877</td>
<td>4.531</td>
<td>0.039</td>
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</tr>
<tr>
<td>ESI (n=49; ( r^2 = 0.377, \ P&lt;0.001; \text{S.E.}^b=30.98) )</td>
<td>Simple linear</td>
<td>Constant</td>
<td>S&lt;sub&gt;o&lt;/sub&gt;</td>
<td>0.880</td>
<td>0.274</td>
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</tr>
<tr>
<td>ESI (n=49; ( R^2 = 0.434, \ P&lt;0.001; \text{S.E.}^b=29.53) )</td>
<td>Multiple linear</td>
<td>Constant</td>
<td>S&lt;sub&gt;o&lt;/sub&gt;</td>
<td>-14.308</td>
<td>-0.007</td>
<td>5.613***</td>
<td>0.623</td>
</tr>
<tr>
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<td>Constant</td>
<td>S&lt;sub&gt;o&lt;/sub&gt;</td>
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(continued)
Table 3 - continued

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<tr>
<th>Dependent Variable</th>
<th>Regression Analysis</th>
<th>Variable Description</th>
<th>Regression Coefficient</th>
<th>F-ratio</th>
<th>F-probability</th>
<th>t-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>β-value</th>
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<tbody>
<tr>
<td>FBC</td>
<td>Backwards</td>
<td>Constant</td>
<td>4.895</td>
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<td>0.691</td>
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<td>1.445</td>
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<td>(n=48; R² = 0.473, P&lt;0.001; S.E. = 20.97)</td>
<td>S</td>
<td>0.451</td>
<td>13.84</td>
<td>0.001</td>
<td>-----</td>
<td>1.602</td>
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<td>11.79</td>
<td>0.001</td>
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</table>

<sup>a</sup>: P<0.05; **: P<0.01; ***: P<0.001

<sup>b</sup> Standard error of estimate
whether both hydrophobicity and solubility had a significant effect on the emulsifying properties of proteins studied, their partial regression coefficients were tested for significance by the Student's t-test. This test showed (Table 3) that both partial regression coefficients were highly significant, and thus, it was concluded that both hydrophobicity and solubility significantly affected the emulsifying properties. Moreover, in order to determine the relative importance of surface hydrophobicity and solubility in estimating the values of emulsifying properties, the β-values (standard partial regression coefficients or normalized coefficients) for hydrophobicity and solubility were calculated (Table 3). It was found that hydrophobicity was almost twice as useful as solubility in estimating or predicting the emulsifying properties of the proteins studied. It should, also, be reported here that multiple linear regression analysis showed no significant correlation between $S_0$, solubility and FBC of the proteins studied.

In an attempt to improve the coefficients of multiple determination ($R^2$) of Table 3, a backwards stepwise multiple regression analysis was applied to the data of Table 1. The multiple regression models, thus obtained, are shown in Table 3. As shown in Table 3, the backwards stepwise multiple regression analysis gave a highly significant correlation between $S_0$, solubility and FBC of the 48 protein samples of Table 1. The relationship, however, was not linear but quadratic. Moreover, comparing the models of Table 3, it is obvious, that the models obtained by stepwise regression analysis have higher $R^2$ values and lower S.E. (standard error of the estimate) values than the multiple and simple regression models. It was, therefore, concluded that these models should be used in predicting the values of emulsifying properties of the
proteins studied. Our discussion, therefore, will be confined within these models.

The EAI of the proteins studied was significantly affected by $S_o$, solubility and the square of solubility (Table 3). As it is known, $R^2$ values indicate the percentage of variation in a dependent variable accounted for by its regression on the independent variables. The higher the value, the greater the accountability. The $R^2$ value for EAI was 0.583, indicating that 58.3% of the variability in EAI of the protein studied could be accounted for by the 3 independent variables stated above. Comparison of the $\beta$-values of these variables indicated that the most important independent variable in the model was solubility. In this model, the statistical significance of the square of solubility index indicates that as the value of solubility index increases, the effect of that variable declines, i.e., the response of EAI to increasing levels of solubility may be depicted as a curvilinear graph rather than a straight line. Response surface plots (contours) were generated by computer to aid in visualizing the effects of $S_o$ and solubility on the functional properties studied. As it can be seen in Figure 1, regardless of the solubility, as hydrophobicity was increased the EAI was initially increased and then decreased. Moreover, at low and medium hydrophobicity values, increasing solubility levels increased the EAI. However, at very high $S_o$ values, solubility did not appear to play a significant role in the ability of a protein to form an oil-in-water emulsion.

The ESI of the proteins studied was significantly affected by $S_o$, solubility index, the interaction of $S_o$ and solubility index, and the square of solubility index (Table 3). The $R^2$ value of the model was 0.5842, indicating that 58.42% of the variability in ESI could be
Fig. 1. Emulsifying activity index response surface contour as a function of hydrophobicity \( (S_o) \) and solubility index \( (s) \).
accounted for by the aforementioned 4 independent variables. A comparison of the β-values of these variables indicated that the most important independent variable in the model was solubility. Figure 2 shows the ESI contour plot as a function of $S_o$ and solubility index. As shown, regardless of the solubility, as $S_o$ was increased the ESI was initially increased and then decreased. At low and medium $S_o$ values, increasing solubility index levels increased ESI. However, at very high $S_o$ values, solubility index did not appear to be important for the stability of emulsion.

The FBC of the proteins studied was significantly affected by $S_o$, solubility index, and the square powers of these two variables (Table 3). The $R^2$ of the model was 0.473 ($P < 0.001$), indicating that 47.3% of the variability in FBC could be accounted for by the 4 independent variables. Moreover, a comparison of the β-values of these variables indicated that the most important independent variable in the model was the square of solubility index. The statistical significance of quadratic powers of $S_o$ and solubility in the FBC model of Table 3, indicates that as the value of $S_o$ or solubility index is increased, their effects on FBC decline (i.e. the response of FBC to increasing levels of $S_o$ or solubility can be depicted as a curvilinear graph).

The finding of this study that with increasing $S_o$ the emulsifying properties were initially increased and then decreased can be explained by taking into account the fact that the emulsifying properties of proteins ultimately depend on the suitable balance between the hydrophile and lipophile, and do not necessarily increase as the proteins become more lipophilic (Aoki et al., 1981). Thus, as Aoki et al. (1981) reported, the excessive denaturation of the soy protein by n-propanol resulted in
Fig. 2. Emulsion stability index response surface contour as a function of hydrophobicity ($S_o$) and solubility index (s).
a lower emulsion stabilizing properties (as compared to the moderate
denaturation by ethanol). Needless to say, there exist many complicated
factors, e.g., molecular size, molecular flexibility, charge, etc.,
besides the balance of hydrophile and lipophile which participates in
determining the emulsifying properties of proteins (Aoki et al., 1981).

Wolf and Cowan (1975) reported that in ground meat products fat
absorption or binding appeared to involve formation and stabilization of
an emulsion. Thus, they suggested that fat absorption may simply be
another aspect of emulsification. This suggestion helps to explain the
observation in this study that hydrophobicity had a curvilinear effect
on FBC, by utilizing the concept of hydrophile-lipophile balance discussed
above. Voutsinas and Nakai (1981) also observed that with increasing
S₀ the FBC was initially increased and then decreased.

The findings of this study concerning the relationship between
solubility and emulsifying properties are contrary to the general belief
that solubility is the best index of protein emulsifying properties, that
is, the higher the solubility of a protein the better its emulsifying
properties. These findings, however, are in agreement with the results
of Smith et al. (1973), McWatters and Cherry (1975), Wang and Kinsella
(1976), McWatters and Holmes (1979a, 1979b), and Aoki et al. (1980), who
reported that emulsifying properties of proteins cannot be predicted
solely on the basis of protein solubility level.

Pearson et al. (1965) reported that only that fraction of protein
which is soluble can function as an effective emulsifying agent. Moreover,
Franzen and Kinsella (1976) suggested that, as a protein becomes more
soluble, it forms layers around the fat droplet to facilitate association
with the aqueous phase. Granular, insoluble proteins, however, separate
from the oil phase or just float on the oil surface where they remain inert and contribute little toward emulsification. Similarly, soluble proteins enclose the fat globule and render the emulsion more stable to heat treatment. Also, according to Bull (1972), the surface activity of a protein is a function of the ease with which the protein can migrate to, adsorb at, unfold, and rearrange at an interface. Therefore, solubility in the aqueous phase, is closely related to surface activity of the proteins (Kinsella, 1979). Many authors, on the other hand, have reported that high levels of solubility were not necessarily associated with maximum emulsifying properties (Aoki et al., 1980; McWatters and Cherry, 1975; McWatters and Holmes, 1979a, 1979b; Smith et al., 1973; Wang and Kinsella, 1976). Flint and Johnson (1981) in an interesting study evaluated the film formation by soy protein (isolate) at an oil-water interface for the pH range 1 - 10. Definite films were seen at all pH values below that of the isoelectric point (~4.6) of the protein and up to pH 6.5. At pH 5.4, despite the low solubility strong film formation was found to occur. However, beyond this point the strength of film formation gradually decreased until at an upper limit of pH 7.5 the presence of an interfacial layer could barely be seen. The marked pH dependence of film formation on the alkaline side of the isoelectric point was attributed by Flint and Johnson (1981) to the fact that with increasing pH the protein becomes more soluble in the aqueous phase and consequently less likely to be brought out of solution (coagulated) at the phase boundary. The ability of soy protein to form a film even at very low pHs, where the solubility (of the soy protein) is high, suggested that this phenomenon may not be linked solely to solubility but also to the availability of
lipophilic groups for binding at the oil-water interface. Changes in the conformation of the proteins present may occur at acid pHs which enhance the combination of the protein and oil leading to the formation of interfacial film described (Flint and Johnson, 1981).

The finding of this study that high solubility had a negative effect on FBC is in agreement with the results of Voutsinas and Nakai (1981) who attributed the low FBC of soluble proteins to their conformation (mainly helical) which may not permit their binding sites to be sterically available for interaction with oil or to the limited access of oil (hydrocarbon chains) to the protein binding sites due to a possible barrier around them formed by the excessive number of protein polar groups.

CONCLUSIONS

The emulsifying properties of the food proteins studied here were differently affected by heating. Thus, for some proteins heating did not have a substantial effect on one emulsifying property but it affected positively or negatively the other properties, whereas for other proteins heating had a positive or negative effect on all emulsifying properties. It was, therefore, demonstrated that heat-denaturation is not always accompanied by loss of emulsifying properties. This is particularly true for protein whose $S_o$ was greatly increased upon heating. On the other hand, for proteins of low $S_o$ whose $S_o$ was slightly increased by heating, their emulsifying properties generally were initially increased but subsequently decreased, since the decreasing
solubility became an increasingly important factor controlling their emulsifying properties.

Results of this study indicate that the emulsifying properties of the proteins studied (native and heat denatured) could well be predicted solely on the basis of protein $S_o$ level but not on the basis of protein solubility level. It was, therefore, demonstrated that surface hydrophobicity is a very important property governing protein functionality and can be used as a reliable predictor of emulsifying properties. It was also shown that both hydrophobicity and solubility data should be taken into consideration in order to explain and more accurately predict the emulsifying and fat binding properties of the heat denatured proteins.
REFERENCES


Chapter IV

Relationships Between Protein Hydrophobicity and Thermal Properties of Food Proteins
INTRODUCTION

The utilization of proteins as food ingredients is largely determined by their functional properties such as emulsifying activity, emulsion stability, foaming capacity, water-holding capacity, fat absorption or fat binding, and thermal properties (i.e. thickening, coagulation and gelation).

Viscosity changes can be used to evaluate the thickening power of proteins, a property of practical interest in fluid foods, e.g., soups, beverages, batters, etc. (Kinsella, 1976). The ability of protein to form a gel and provide a structural matrix for holding water, sugars, and food ingredients is useful in food applications and in new product development, because it provides an added dimension to protein functionality (Kinsella, 1976, 1979). Moreover, the gelling properties of protein contribute to texture in ground meat— and simulated ground meat— products such as frankfurters and luncheon meats, and give chewiness to the products. Because the gelling ability is very important in the manufacture of processed meats, the use of many nonmeat proteins in processed meat products depends upon their compatibility with meat proteins, but mostly upon their capacity to form gels, under normal processing conditions (Kinsella, 1976). Wheat gluten forms a gel when heated and may be used in fish— and meat— based products as a gelling/structural agent (Kinsella, 1976). Both whey and soy proteins form gels, when dispersions of 8% or more are heated and cooled (Hermansson, 1972, 1975; Hermansson and Akesson, 1975). Thus, these are suitable functional proteins for processed meats.

Coagulation and curd formation is an important functional property
of proteins, e.g., soy protein and casein, which form tofu (soy-curd) and cheese, respectively (Kinsella, 1976). Egg albumin is a key ingredient in many food products because of its ability to coagulate upon heating (Shimada and Matsushita, 1980a). Coagulated proteins may provide body to a good product. The cells of bread possess coagulated proteins (Powrie and Nakai, 1981).

With the recent increase of interest in the food uses of edible proteins, the desirability of quantitative information on their functional properties has become more apparent. The objectives of the present study, therefore, were to evaluate the thermal properties of selected food proteins and to assess the value of hydrophobicity as a predictor of these functional properties.

LITERATURE REVIEW

The terms gelation and coagulation are not very clearly defined (Schmidt, 1981). Gelation may be theoretically defined as a protein aggregation phenomenon in which polymer-polymer and polymer-solvent interactions are so balanced that a well ordered tertiary network or matrix is formed (Schmidt, 1981). This semi-elastic matrix is capable of immobilizing or entrapping large amounts of water in addition to other food components. On the other hand, coagulation is a random protein aggregation in which polymer-polymer interactions are favored over polymer-solvents reactions, resulting in a less elastic, less hydrated structure than that of a protein gel. Empirically, there is unavoidable overlap in this terminology. At the macroscopic level, it may be difficult to
differentiate between a highly solvated coagulum (or coagel) from a true protein gel (Schmidt, 1981).

It has long been recognised (Ferry, 1948; Circle et al, 1964; Catsimpooolas and Meyer, 1970) that a gel can be made from many native proteins by heating them in concentrated aqueous solution at suitable pH and ionic strength. The mechanism suggested by Ferry in 1948 is still the most generally accepted heat-induced protein gelation mechanism. This mechanism is a two-stage process and involves an initial denaturation of native protein into unfolded polypeptides (first step), which then gradually associate to form the gel network under appropriate conditions (second step). For a given rate of denaturation, the smaller the attractive forces between chains of denatured protein, the slower the second step of the gelation process will be; accordingly, the higher will be the concentration of free denatured protein that accumulates as an intermediary in the course of gelation. The higher the concentration of these long-chain molecules, the finer the gel network should be. Since the temperature coefficients of denaturation are enormous, it is to be expected that a temperature increase would accelerate the first step far more than the second, leading to a finer structure (Ferry, 1948). However, conditions of the cooling step, which is usually required to permit gelation, also affect physical characteristics of gels (Kinsella, 1976). Upon cooling, the uncoiled polypeptides associate to form the network. In complex globular protein systems aggregation may occur more randomly and simultaneously with the initial step (Schmidt, 1981). Thus, Tombs (1970, 1974) concluded from aggregation studies that the higher the randomness of aggregation the more likely it is that a coagel is obtained instead of a gel.
In general sense there are two basic types of heat-induced gel structures, namely reversible and irreversible, depending on the conditions involved (Schmidt, 1981). In the reversible gelation, a sol or progel condition can be obtained upon heating which is usually accompanied by increased viscosity. This progel "sets" to form a gel upon cooling. This type of gel can usually be melted to reform the progel upon subsequent heating suggesting that the aggregation step is reversible. Gelatin gels may also be characterized as reversible gels (Stainsby, 1977). Irreversible gels will soften or shrink with subsequent heating, but melting or reversion to the progel does not occur under practical conditions (Schmidt, 1981).

In contrast to gelatin gels, whose stability is unaffected and most of whose properties are only slightly changed by wide variations of concentration, pH, and salt content, denatured protein gels can be formed only under highly specific conditions with just the right balance of attractive and repulsive forces between polypeptide chains (Ferry, 1948). Thus, the method of preparation of protein, its concentration, pH, temperature and duration of heating, cooling conditions, the presence of salts, thiols, sulfite, and/or lipids all influence the properties of the gels formed (Circle et al. 1964; Catsimpoolas and Meyer, 1970).

In addition to effects of the size, shape and arrangement of the primary protein strands comprising the gel network, the characteristics of protein gels are affected by intra- and inter-strand cross-linking. Protein gels may be cross-linked by specific bonding at specific sites on the protein strands or by nonspecific bonding occurring along the protein strand. Hydrogen bonding, disulfide bridging and hydrophobic attractions play major roles in cross-linking and stabilizing the structure of a
protein gel or coagulum (Schmidt, 1981). The nature and degree of cross-linking would vary with the type of protein and gelation environment (Schmidt, 1981).

MATERIALS AND METHODS

Materials

HCl-solubilized gluten was prepared according to the method of Wu et al. (1976) as follows: a 5% dispersion of vital gluten (Whetpro 75%) in 0.05 N HCl was autoclaved at 121°C for 15 min. The pH was adjusted to 7.0 and the sample was dialyzed 2 days against running water, one day against distilled water, and then, freeze-dried. The protein content of the dry product as determined by the micro-Kjeldahl method was 74.5%. All other proteins and reagents were the same as those used in chapters II and III of this thesis.

Methods

A. Thickening determination

Aqueous dispersions of 8% (W/W) protein were prepared for selected food proteins by stirring for 5 min on a magnetic stirrer and then homogenizing in a Sorval Omni-mixer at speed setting 1 for 1 min. The air was removed from the slurries under vacuum. The experiment was conducted at pH 7.0, the adjustment being made with 1 N NaOH. Two different models of Brookfield (Cooksville, Ont.) Synchro-Lectric viscometers were used to measure the viscosity of protein dispersions. Thus, a model LVT, fitted with a UL adapter and operated at 6 rpm, was used
for measuring the viscosity of the unheated protein dispersions at 24°C. A model RVT, fitted with a jacketed cylinder attachment (small sample adapter) and a spindle No. SC4-21, was used for measuring the viscosity of the heated samples at 90°C as follows: the protein dispersion was transferred to the small sample adapter, heated to 90°C by a circulating bath, and when the temperature of the dispersion reached the 90°C mark, the viscometer was turned on and continuously operated at 100 rpm. Viscosity readings were taken at one min intervals for 5 min. The difference between final (heated for 5 min at 90°C) and initial (unheated, 24°C) viscosity indicated the viscosity gained or lost during the heating process and was used as a measure of the thickening power of the protein.

B. Heat coagulation determination

Heat coagulability was determined according to the method of Balmaceda et al. (1976) as follows: a 5% (W/W) protein dispersion in water was prepared by stirring for 5 min on a magnetic stirrer. The pH was adjusted to 7.0 with 1 N NaOH and the dispersion was then centrifuged at 27,000 x g for 30 min. The protein content of the supernatant was determined by the Phenol-Biuret method (Brewer et al. 1974). A portion (10 ml) of the supernatant was heated in a centrifuge bottle (hermetically closed) at 98 - 100°C for 30 min and then cooled to room temperature. The heated sample was subsequently centrifuged at 27,000 x g for 15 min, filtered through Whatman No. 2 filter paper, and the protein content of the filtrate was determined. The heat coagulability (HC) of the sample was calculated from the following equation:

\[
HC = \frac{\text{Protein cont. supernatant (\%)} - \text{protein cont. filtrate (\%)} \times 100}{\text{Protein cont. supernatant (\%)}}
\]
C. Gelation measurement

Aqueous dispersions of 10% (W/W) protein were prepared for selected food proteins by stirring on a magnetic stirrer for 5 min. The pH was adjusted to 7.0, and an aliquot (15 ml) of each dispersion was transferred into a closed container, heated at 100°C for 40 min and then cooled in an ice-bath to room temperature. The gelling ability of each protein was qualitatively determined by visually judging the characteristics of the final product and assigning to it a value corresponding to a rating scale of -4 to +3. The rating scale was as follows: -4, smooth liquid; -3, slight feathering (slightly granular liquid); -2, moderate feathering; -1, syneresis (after inversion of the container the structure collapsed); 0, viscous gel-like semi-liquid; +1, soft gel, +2, medium gel; and +3, firm gel.

D. Hydrophobicity determination

Surface hydrophobicity (S_o) was determined as described in Chapter II of this thesis. The hydrophobicity of the same proteins was also determined fluorometrically with cis-parinaric acid as a probe, after the protein dispersions were heated at 100°C for 10 min in the presence of 1.5% sodium dodecyl sulfate (Townsend, 1982). This hydrophobicity was designated as S_e and it was shown by Townsend (1982) to be linearly correlated with the average hydrophobicity values of Bigelow (1967) for various proteins. Thus, in this study S_e will be used as an index of the average hydrophobicity of proteins.
E. Determination of sulfhydryl and disulfide groups of proteins

The sulfhydryl (free and buried SH) and total sulfhydryl (SH + reduced SS) groups of the proteins were determined according to the method of Beveridge et al. (1974) after slight modification as follows: (1) soy protein isolate, Promine-D, pea protein isolate (a), canola isolate (b), sunflower isolate, HCl-solubilized gluten. A 75 mg sample was suspended in 1 ml of Tris-glycine buffer, pH 8.0, containing 0.12% EDTA, denoted as Tris-Gly; 4.7 g of guanidine hydrochloride was added, and the volume made to 10 ml. For SH determination, to 1 ml of this solution was added 4 ml of 8 M urea in Tris-Gly and then 0.05 ml of Ellman's reagent (4 mg of 5, 5' - dithiobis - 2 - nitrobenzoic acid in 1 ml Tris-Gly) was added. For total SH (SH + reduced SS) determination, to 1 ml of the protein solution was added 0.05 ml of 2-mercaptoethanol and 4 ml of 8 M urea in Tris-Gly, and the mixture was incubated for 1 hr at 25°C. After an additional 1 hr incubation with 10 ml of 12% TCA, the tubes were centrifuged at 5,000 x g for 10 min. The precipitate was twice resuspended in 5 ml of 12% TCA and centrifuged to remove 2-mercaptoethanol. The precipitate was dissolved in 10 ml of 8 M urea in Tris-Gly and the color was developed with 0.04 ml of Ellman's reagent. (2) for casein and whey proteins. For SH determination, 10 mg of sample was dissolved in 5 ml of 8 M urea in Tris-Gly, and then, 0.04 ml of Ellman's reagent was added. For total SH determination, 10 mg of sample was dissolved in 5 ml of 10 M urea in Tris-Gly. Then, 0.1 ml of 2-mercaptoethanol was added and mixed with a Vortex. The mixture was held at 25°C for 1 hr. After precipitation and washing of protein as for the other proteins above (1), the precipitate was dissolved in 10 ml of 8 M urea in Tris-Gly. An aliquot of 3 ml was taken and 0.05 ml of Ellman's reagent was added to it for color development.
Absorbance was measured at 412 nm on a Beckman DB spectrophotometer. The amount of SH and total SH was calculated from the following equation:

$$\mu M \text{ SH/g} = 73.53 \frac{A_{412}}{C} \cdot D$$

where $A_{412}$ = the absorbance at 412 nm;

$C$ = the sample concentration in mg solids/ml; and $D$ = the dilution factor.

F. Statistical analysis

Statistical analysis of the data obtained in this study was done as described in chapter III of this thesis. Five independent variables were used in the initial equation in the backwards stepwise multiple regression analysis including hydrophobicity ($S_o$ or $S_e$), sulfhydryls (SH or SH + reduced SS), interaction of hydrophobicity and sulfhydryls, and the quadratic powers of hydrophobicity and sulfhydryls. It should be mentioned that the models for the prediction of the thermal properties as well as the functional properties studied in Chapters II and III of this thesis were selected on the basis of the statistical significance of F-probabilities of the partial regression coefficients.

RESULTS AND DISCUSSION

Table 1 shows the relationships of hydrophobicities and sulfhydryls of various proteins with their thermal properties. As can be seen, the proteins studied exhibited different behaviour upon heating. Thickening of proteins was measured as the increase in apparent viscosity of protein dispersions upon heating. Among the proteins studied whole casein and Promine-D showed some thinning (decrease in viscosity) upon heating,
Table 1 - Relationships between protein hydrophobicities, sulfhydryls, sulfhydryls + reduced disulfides and thermal properties of various proteins.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Protein</th>
<th>Surface hydrophobicity ($S_0$)</th>
<th>Average hydrophobicity ($S_e$)</th>
<th>SH ($\mu$M/g prot.)</th>
<th>SH+ r.SS ($\mu$M/g prot.)</th>
<th>Thickening app. viscosity (Pa.s $\times 10^{-3}$)</th>
<th>Heat coagulation (%)</th>
<th>Gelation rating\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy isolate</td>
<td>95</td>
<td>822</td>
<td>3.2</td>
<td>56.1</td>
<td>12.5</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Promine-D</td>
<td>39</td>
<td>927</td>
<td>3.2</td>
<td>57.7</td>
<td>-26.0</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Pea isolate</td>
<td>66</td>
<td>277</td>
<td>12.1</td>
<td>54.8</td>
<td>2.2</td>
<td>0.0</td>
<td>-4</td>
</tr>
<tr>
<td>Canola isolate</td>
<td>65</td>
<td>950</td>
<td>7.4</td>
<td>90.2</td>
<td>32.2</td>
<td>47.7</td>
<td>-1</td>
</tr>
<tr>
<td>Sunflower isolate</td>
<td>47</td>
<td>597</td>
<td>6.5</td>
<td>126.9</td>
<td>84.8</td>
<td>22.6</td>
<td>-1</td>
</tr>
<tr>
<td>Whole casein</td>
<td>28</td>
<td>725</td>
<td>1.4</td>
<td>3.5</td>
<td>-0.8</td>
<td>4.8</td>
<td>-3</td>
</tr>
<tr>
<td>Whey protein</td>
<td>182</td>
<td>387</td>
<td>20.2</td>
<td>248.4</td>
<td>105.1</td>
<td>9.9</td>
<td>+1</td>
</tr>
<tr>
<td>HCl-solubilized gluten</td>
<td>17</td>
<td>349</td>
<td>2.8</td>
<td>101.9</td>
<td>6.2</td>
<td>0.0</td>
<td>-4</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Average of duplicate determinations

\textsuperscript{b}For gelation rating scale see Materials and Methods
whereas all others showed different degrees of thickening, with whey protein exhibiting the greatest thickening power.

Heat coagulation was determined as % loss in solubility of a protein after heating (98 - 100°C for 30 min). As seen from Table 1, soy isolate, Promine-D, pea isolate and HCl-solubilized gluten did not show any coagulation on heating, whereas canola isolate, sunflower isolate, whole casein, and whey protein showed different degrees of coagulation, with canola isolate exhibiting the greatest coagulability. The observation that whole casein exhibited a small coagulation was unexpected, since it is well known that casein is very resistant towards heat coagulation. However, protein solubility is affected by a magnitude of factors, such as conditions of solubility determination, processing history, the presence of other ingredients in the sample, etc. (Kinsella, 1976), and this can account for the observed coagulation of casein.

The gelling ability of proteins is usually determined quantitatively by measuring the viscosity of the gel with Helipath viscometers (Circle et al., 1964; Catsimpoolas and Meyer, 1970, 1971; Hermansson, 1972; Hermansson and Akesson, 1975). However, Fleming et al. (1975) reported that viscosity was not directly associated with the gelation property; a soybean concentrate (Isopro) showed a high viscosity after heating but remained granular and pourable. Similarly, the high viscosity of the sunflower concentrates studied did not indicate gel formation since the products formed an irregular structure with a foamy appearance. On the other hand, the importance of qualitative evaluation of protein gel systems
has been lately discussed by Schmidt (1981). For the aforementioned reasons the gelling ability of the proteins in this study was qualitatively evaluated by visually observing the characteristics of the final product. As seen from Table 1, whey was the only protein that showed a true gel structure upon heating and cooling. Soy isolate and Promine-D formed a viscous gel-like semi-liquid (not a self-supported gel). All other proteins showed either some degree of coagulation (feathering or syneresis) or remained as smooth liquids (pea isolate and HCl-solubilized gluten).

The results of this study can not easily be compared with results of other studies due to lack of consistent methodology. For example, protein concentration, temperature and duration of heating, cooling conditions (mainly duration of cooling), pH, etc., vary considerably among different studies. For instance, some studies have been conducted at the natural pH of each product (Sosulski et al., 1976). It should be noted here, however, that when during preliminary studies heat coagulation was conducted at the natural pH of each product, all products exhibited different degrees of coagulation as opposed to the results of Table 1. This is, of course, due to the smaller electrostatic repulsions between protein molecules at pHs lower than 7.0 (but above the isoelectric point). Thus, the attractive forces generated between protein molecules by thermal unfolding predominated over the repulsive forces (due to the protein charge) resulting in coagulum formation. It can be mentioned, however, that Hermansson (1972) reported that gelation occurred for Promine-D and whey protein concentrate (WPC) but not for caseinate (10% protein dispersions). She also observed that the gel strength of Promine-D increased to a maximum (corresponding to 80°C) and then decreased as the temperature was raised above this point (in contrast to the gel strength
Multiple regression models for prediction of hydrophobicity, sulfhydryls, and sulfhydryls + reduced disulfides effects on thermal functional properties studied are presented in Table 2. As shown, two models were obtained for prediction of the thickening power of proteins. In the first model, thickening was significantly affected by $S_e$ (average hydrophobicity), the interaction of $S_e$ and sulfhydryls, and the square of $S_e$. The $R^2$ value of this model was 0.961, indicating that 96.1% of the variability in thickening could be accounted for by the aforementioned 3 independent variables. A comparison of the $\beta$-values of these variables indicated that $S_e$ was the primary determinant of thickening. This model also indicates that $S_e$ had a curvilinear effect on thickening, that is, as the value of $S_e$ increased the thickening was initially increased and then decreased. This trend can also be seen in Figure 1, which shows the thickening response surface contour as a function of $S_e$ and SH content. As shown, regardless of the SH content, the response of thickening to increasing levels of $S_e$ increased and then decreased. Moreover, it is evident that at low $S_e$ values, increasing SH content increased the thickening power, but at medium and high $S_e$ values, SH content did not seem to significantly affect thickening. In the second model (Table 2) thickening was significantly affected by $S_e$, the sum of sulfhydryls and reduced disulfides, the interaction as well as the square powers of these two independent variables. The $R^2$ value of this model was 0.995. Comparing both regression models of Table 2, it seems that the latter is most reliable in predicting the thickening power of proteins, since its $R^2$ and S.E. values are higher and smaller, respectively.

The heat coagulability of proteins was significantly affected by the
Table 2 - Multiple regression models for prediction of thermal properties of various proteins

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Variable description</th>
<th>Regression coefficient</th>
<th>F-ratio</th>
<th>F-probability</th>
<th>β-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickening</td>
<td>Constant</td>
<td>-202.675</td>
<td>33.04</td>
<td>0.005</td>
<td>4.461</td>
</tr>
<tr>
<td></td>
<td>$S_e$</td>
<td>0.754</td>
<td>0.75</td>
<td>0.016</td>
<td>1.016</td>
</tr>
<tr>
<td></td>
<td>$S_e \times SH$</td>
<td>0.016</td>
<td>71.96</td>
<td>0.001</td>
<td>0.867</td>
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<tr>
<td></td>
<td>$S_e^2$</td>
<td>-0.001</td>
<td>38.98</td>
<td>0.003</td>
<td>-4.857</td>
</tr>
<tr>
<td></td>
<td>$S_e \times (SH+ r.SS)$</td>
<td>-0.001</td>
<td>38.98</td>
<td>0.003</td>
<td>-4.857</td>
</tr>
<tr>
<td></td>
<td>$(SH+ r.SS)^2$</td>
<td>0.004</td>
<td>33.49</td>
<td>0.029</td>
<td>1.906</td>
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</tr>
<tr>
<td></td>
<td>$SH$</td>
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<td>6.443</td>
<td>0.050</td>
<td>-0.930</td>
</tr>
<tr>
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<td>$S_e \times SH$</td>
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<td>13.870</td>
<td>0.014</td>
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<td>Gelation</td>
<td>Constant</td>
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<td>11.48</td>
<td>0.020</td>
<td>0.895</td>
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<tr>
<td></td>
<td>$S_e$</td>
<td>0.006</td>
<td>11.48</td>
<td>0.020</td>
<td>0.895</td>
</tr>
<tr>
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<td>$(SH)^2$</td>
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<tr>
<td>Gelation</td>
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<td>0.013</td>
<td>0.809</td>
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<td>$SH+ r.SS$</td>
<td>0.022</td>
<td>15.33</td>
<td>0.011</td>
<td>0.834</td>
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</table>

$^a$Standard error of estimate
Fig. 1. Thickening response surface contour as a function of $S_e$ and SH content.
SH content and the interaction of $S_e$ and SH content (Table 2). The $R^2$ value of the model was 0.740, indicating that 74.0% of the variability in heat coagulation could be accounted for by these two variables. A comparison of the $\beta$-values of these variables indicated that the interaction of $S_e$ and SH content was the most important variable in the model. Figure 2 shows the heat coagulation response surface contour as a function of $S_e$ and SH content. As shown in this Figure, regardless of the SH content, as $S_e$ increased the heat coagulability was initially increased and then decreased. The region of the maximum response (heat coagulation) was localized in the region defined by relatively high level of $S_e$ and relatively low levels of SH content. It should be noted that no significant correlation was found between $S_e$, SH + reduced SS and heat coagulation.

Two models were obtained for prediction of the gelling ability of proteins (Table 2). In the first model, gelation was significantly affected by $S_e$ and the square of sulfhydryls. The $\beta$-values of this model suggested that both independent variables were equally important in determining the gelling ability of the proteins studied. In this model, the statistical significance of the square of sulfhydryl content indicates that the response of gelation to increasing levels of SH can be depicted as a curvilinear graph rather than a straight line. In the second model, gelation was significantly affected by $S_e$ and the sum of sulfhydryls and reduced disulfides. It is obvious from Table 2, that the relationship between $S_e$, SH + reduced SS and gelation was linear. Both independent variables of this model were equally important for determining the gelling ability of proteins. Comparing both regression models, it seems that the latter is most reliable in predicting gelability since its $R^2$ and
Fig. 2. Heat coagulation response surface contour as a function of $S_e$ and SH content.
S.E. values were higher and smaller, respectively.

It should be noted, that as expected, no correlation (simple or multiple linear) was found between surface hydrophobicity ($S_o$) and any of the thermal properties studied.

The results of this study concerning the importance of $S_e$, SH and SS contents for the thermal properties of proteins are in agreement with the results of most previous studies attempting to identify the types of bonds responsible for protein gelation or coagulation (Circle et al., 1964; Furukawa et al., 1979; Fukushima, 1980; Hillier et al., 1980; Itoh et al., 1980a, 1980b; Shimada and Matsushita, 1980a, 1980b, 1981).

Circle et al. (1964) reported that the specific disulfide-reducing agents, sodium sulfite and cysteine, profoundly decreased the viscosity of both unheated and heated 10% dispersions of soy isolate, and prevented gelation. This finding was interpreted as being indicative of participation of disulfide bonds (through sulphydryl-disulfide chain reaction) in the gelation process. Furukawa et al. (1979) demonstrated that the gel network of isolated soy protein was formed through cross-linking by disulfide, hydrogen and hydrophobic bonds. The findings of Circle et al. (1964) and Furukawa et al. (1979) are in contrast to the finding of Catsimpoolas and Meyer (1970) who reported that the bonds involved in the sol-progel and gel transitions appeared to be primarily of noncovalent nature (i.e. hydrogen, hydrophobic and ionic bonds). Hillier et al. (1980) attributed the heat-induced gelation of whey powders to disulfide cross-linking. They showed conclusively that the rate of gelling (taken as an index of the gelling ability of a protein preparation) depended on the sulphydryl content of the whey powders, and that it was the amount of total SH groups that was important, not the free SH; this was expected,
since at 80°C, the standard temperature used in measuring gelling time, there could be little difference in the reactivity of free and masked SH groups (Hillier et al., 1980). The same authors, however, found a poor correlation between the rate of gelling and total SH content and suggested there were important differences, in addition to SH content, between the whey powders which affected their rate of gelling and their appearance after gelation. Itoh et al. (1980a, 1980b) reported that in the formation of polymeric protein molecules during the heat gelation of carp actomyosin SH groups were involved (through formation of SS bonds). According to Schmidt (1981) hydrophobic interactions are important to dissociative-associative reactions which initiate the gelation process. These attractions could also be involved in layering or thickening of the gel network strands upon cooling which results in improved strength and stability.

According to Fukushima (1980) the tofu-gel (a gel, although is also referred to as curd, prepared by coagulation of soybean milk with calcium salt) made from 7S globulin is mostly stabilized by hydrophobic bonds, while the tofu-gel made from 11S globulin is stabilized by both disulfide bonds through sulphydryl-disulfide interchange reaction and hydrophobic bonds. This is the reason why 7S tofu-gel is soft, while 11S tofu-gel is remarkably harder (Saio et al. 1971). Shimada and Matsushita (1980a) based on turbidity studies suggested that the thermocoagulable matrix formation of egg albumin was due to disulfide cross-linking (by sulphydryl-disulfide exchange) and hydrophobic interactions. The same authors (Shimada and Matsushita, 1980b, 1981), subsequently concluded that the mechanism of protein thermocoagulation is largely dependent on hydrophobic interactions among proteins.
CONCLUSIONS

Results of this study indicate that the thermal properties of the proteins studied could not be explained solely on the basis of average hydrophobicity or sulfhydryls (SH or the sum of SH and reduced SS). However, it was demonstrated that these functional properties could be reliably predicted on the basis of both average hydrophobicity and sulfhydryls. It was, thus, concluded that these variables are the main determinants of the thermal properties studied.
REFERENCES


GENERAL CONCLUSIONS

The results of the study on hydrophobic immobilization of proteases included in the first chapter of this thesis indicated that all immobilized enzyme preparations exhibited high initial activity. The major problem encountered was the rapid reduction in enzymatic activity due to the loss of enzyme from the hydrophobic carriers. An economic study showed that continuous coagulation of skimmilk with proteolytic enzymes immobilized on the hydrophobic carriers employed was unlikely to be feasible. Therefore, this study was discontinued and our interest was then shifted to the relationship of protein hydrophobicity to functionality.

In the second chapter a new, simple turbidimetric method was developed for determining the ability of protein to bind fat, since fat absorption or fat binding, as assessed by the existing methods, was actually a measure of oil which was physically entrapped into protein tested. The results in this study showed that the turbidity due to oil was dependent on the wavelength used for turbidity measurement, blending time and volume of oil. The fat binding capacity of 11 food proteins tested was positively affected by surface hydrophobicity and negatively by an interaction of surface hydrophobicity and solubility.

According to the results obtained in the third chapter, heating did not always deteriorate the emulsifying properties of the proteins. Instead, for proteins, of which surface hydrophobicity was markedly increased upon heating, their emulsifying properties were substantially improved. This improvement was attributed to the greater surface hydrophobicity as well as to increased viscosity of these heat denatured
protein samples. However, for proteins with low surface hydrophobicity, of which hydrophobicity was only slightly increased by heating, their emulsifying properties were generally adversely affected by heating probably due to decrease in solubility. Simple linear regression analysis of the obtained data revealed that surface hydrophobicity, but not solubility, could be used as a reliable predictor of the emulsifying properties of heat denatured proteins. It is, therefore, possible that surface hydrophobicity is a very important property governing protein functionality. However, backwards stepwise multiple regression analysis showed that both surface hydrophobicity and solubility should be taken into account in predicting the emulsifying and fat binding properties of heat denatured proteins.

Regression analysis of the data obtained in the fourth chapter showed that average and not the surface hydrophobicity of proteins was important for heat gelation, coagulation and thickening. Simple linear regression analysis demonstrated that neither average hydrophobicity nor sulfhydryl content could by itself explain these thermal properties. However, when the average hydrophobicity and sulfhydryl content were both used in multiple regression analysis, the thermal properties of proteins could be accurately predicted.