Enzymatic hydrolysis of corn gluten proteins

by

Julie Elizabeth Hardwick

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NOMENCLATURE

A	Arbitrary constant
A_s	Surface sites of insoluble substrate
B	Volume of base consumed during hydrolysis
e_0	Initial enzyme concentration
E	Enzyme concentration
E_a	Number of moles of enzyme adsorbed in equations (11) and (14) or activation energy in equation (20)
h	Hydrolysis equivalents, defined as equivalents of peptide bonds per g
h_{tot}	Total number of peptide bonds in a protein, same units as h
I	Insoluble substrate concentration
IE	Insoluble substrate concentration, resistant to hydrolysis
k, k', k_1, k_2	Reaction rate constants
k_d	Kinetic constant for enzyme denaturation
k_i	Kinetic constant for enzyme inactivation
k_I	Reaction rate constant for insoluble pool I
$\bar{k}_I$	Mean reaction rate constant for insoluble pool I
k_s	Reaction rate constant for soluble pool S
$\bar{k}_s$	Mean reaction rate constant for soluble pool S
K	Partition coefficient between phases I and II
K_m	Michaelis-Menten constant
K_I	Michaelis-Menten constant for inhibition
K_p	Michaelis-Menten constant for product inhibition
$K_{mI'mP'mS}$	Michaelis-Menten constants for pools I, P, and S, respectively, used in modelling (Table 5)

MP	Mass of protein
N_b	Normality of base in protein hydrolysis experiments
P	Concentration of soluble low molecular weight peptides, not precipitated by TCA
P_j, P_n	Soluble peptides of different molecular weight
R	Gas constant
r_I, r_S	Rate of hydrolysis of substrates I and S, respectively
S	Substrate species, protein or high molecular weight peptides, precipitated by TCA
S_i, S_{i-j}	Substrates of different molecular weight
S_0	Initial substrate concentration
t	Time
T	Temperature, degrees Kelvin or Celsius
v	Reaction rate in kinetic expression
v_m	Maximum rate of reaction
V_I	Volume of phase I, soluble phase

Greek letters

σ_S, σ_I	Standard deviation for rate constants k_S and k_I, respectively, used in the kinetic models

INTRODUCTION

Enzymatic hydrolysis has been used to favorably modify the functional properties of a protein, such that the hydrolysate could be incorporated into foods for its flavor, functional, and nutritional value.

Corn gluten meal contains approximately 60% protein. The major drawback of using corn gluten meal in foods is that it is very insoluble both in water and dilute alkaline solutions (Ofelt and Evans, 1949, Russell and Tsao, 1982). If corn gluten meal is dispersed in water, without agitation, it quickly separates out into solid and liquid phases. The object of enzymatic hydrolysis is to produce soluble protein hydrolysates. The hydrolysates should exhibit either a bland or pleasant flavor, in order that the nutritional value of the hydrolysates can be used in food products (Kinsella, 1978). Corn gluten meal has been hydrolyzed using alkaline microbial proteases (Adler-Nissen, 1977). The hydrolysate has not yet been commercially incorporated into foods.

The purpose of this study is to develop a kinetic model to describe the mechanism for the enzymatic hydrolysis of corn gluten meal, using an alkaline protease. If the mechanism is more clearly understood, problems encountered in the hydrolysis should be identified. It may then be possible to develop a more efficient process for the hydrolysis, resulting in a higher yield of soluble protein without the production of bitter flavors.

In order to check the validity of different models, experimental data were generated. Corn gluten meal was hydrolyzed batchwise in a baffled, stirred reactor at different enzyme concentrations for a fixed pH and

temperature. The effect of size reduction on the hydrolysis was studied. Size reduction increases the surface to volume ratio, which theoretically could increase the rate of hydrolysis.

A series of compartmental models were studied. These included a linear model, a Freundlich adsorption model (McLaren, 1963, Archer et al., 1973) and Michaelis-Menten models with and without the effects of product inhibition (Constantinides and Adu-Amankwa, 1980, O'Meara and Munro, 1985). These models were solved iteratively using available software (IMSL, 1987). A graphical method for two simultaneous reactions was also considered (Mihalyi and Harrington, 1959).

Calculated values of soluble protein with time were generated from parameters, estimated by fitting a kinetic model to experimental data. The different kinetic models were evaluated by comparing the fit of the calculated values to the experimental data.

LITERATURE REVIEW

Hydrolysis of Proteins

Proteins consist of many amino acids joined together by peptide linkages. They have a molecular weight range from 1200 to several million. The hydrolysis of the peptide linkages breaks the protein into smaller peptides. If the hydrolysis is carried far enough, the protein may be broken down into amino acids.

There are three common methods of hydrolysis. These are acid hydrolysis, alkaline hydrolysis and enzymatic hydrolysis.

Acid hydrolysis

Sulfuric acid and hydrochloric acid are applied the most frequently in partial acid hydrolysis; acetic acid is also used. Dilute or concentrated acids may be used; a shorter time is required to achieve the same degree of hydrolysis with a more concentrated acid (English and Grulke, 1934, Light, 1967). The time of hydrolysis is also reduced if the reaction is carried out at elevated temperature. For example, if the hydrolysis is carried out using 5.7N hydrochloric acid at 37°C, the time required for the hydrolysis is 3 days. In contrast, using the same 5.7N hydrochloric acid at a temperature of 100°C, the time of the hydrolysis is 20-30 minutes (Light, 1967, Ricks et al., 1977).

The reaction conditions chosen for acid hydrolysis depend very much on the raw materials. If the hydrolysate is to be used in foods, the protein content and the flavor are of major importance, so the hydrolysis may not be as extreme or for as long (Prendergast, 1974). Acid

hydrolysis may also be used to determine the protein content of foods; in this case the hydrolysis is carried to completion, using 6N hydrochloric acid at 137°C and for a time period varying between 12 and 96 hours (Davies and Thomas, 1973).

For food use the cost of hydrolysis must be relatively low and the hydrolysate should produce flavors which enhance or intensify naturally occurring flavors. Prendergast (1974) reported that sulfuric acid produces a salt-free product but with rather a harsh flavor. In addition, large volumes of calcium sulfate must be filtered off after neutralization, which results in a low yield and a relatively costly product. Sulfuric acid is consequently used mostly in the production of dietary and special products. Hydrochloric acid is generally preferred, but the hydrolysate always contains sodium or potassium chlorine, which are formed when the acids are neutralized.

Alkaline hydrolysis

Alkaline hydrolysis does not produce pleasant flavors, but it can be used for analysis to determine amino acid content. Some amino acids such as cystine and arginine are completely destroyed. Tryptophan, which is destroyed by total acid hydrolysis, can be completely recovered by total alkaline hydrolysis (Andrews and Baldar, 1985).

Total hydrolysis is carried out using 4.2N sodium hydroxide or barium hydroxide; the time of hydrolysis depends on which amino acids are of interest. Low recoveries of all amino acids, except tryptophan, are observed for a hydrolysis time between 1 and 10 hours. If the hydrolysis is continued for 16 hours, 85-90% recovery of cystine, tyrosine, and

glutamine, and 95-100% recovery of proline, valine, leucine and tryptophan are found (Davies and Thomas, 1973).

Enzymatic hydrolysis

Enzymatic hydrolysis is generally slow, quite expensive, and rarely goes to completion. Native proteins are globular and the peptide bonds are to a certain extent not accessible for enzymatic attack. The reaction is also slow because it is carried out at the lower temperatures tolerated by the enzyme.

Proteins for use in foods are hydrolyzed enzymatically to improve their functionality. The functionality may be altered by many different types of enzymes, but mostly proteases are used (Adler-Nissen, 1976).

The rate of proteolysis depends not only on the primary structure of the protein, but also on the secondary and tertiary structure. The folding of the protein in some systems may reduce the accessibility of some bonds, which may be easily split when the chain is unfolded.

The hydrolysis of insulin chains, for example, involves mostly the primary structure, and therefore depends mainly on the kinetic properties of the protease (Ronca et al., 1975). The digestion of myosin by trypsin results in the cleavage of only 59% of the susceptible bonds, suggesting that the core is resistant, or extremely slowly digested (Mihalyi and Harrington, 1959). If a protein is very unsoluble due to its globular structure, peptide bonds may be cleaved at a slow rate until inner parts of the protein molecule become accessible to the enzyme by prior cleavages.

The hydrolysis of soybean protein by Penicillium duponti (Constantinides and Adu-Amankwa, 1980) involves the cleavage of the insoluble protein and partially soluble aggregates. The partially soluble polypeptides are hydrolyzed in a fast reaction to smaller soluble peptides, whereas the more compact protein core is solubilized by the adsorbed enzyme in a slower reaction.

The rate of production of soluble soybean protein starts to decrease. However, the substrate concentration is still high enough to rule out substrate exhaustion. The solubilization is not greatly increased on further addition of enzyme, but it is increased if the suspending solution is removed, and the hydrolysis continued by resuspension of the solids. This effect can be attributed to the presence of product inhibition (Constantinides and Adu-Amankwa, 1980).

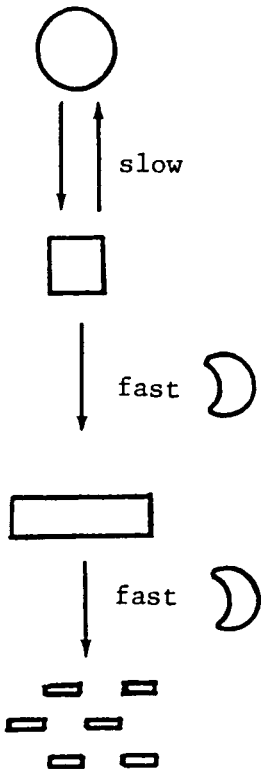
Mechanism Linderstrom-Lang proposed that the hydrolysis of a protein may be described by two types of reaction sequences. These are termed the "one by one" and the "zipper" mechanisms. Figure 1 shows the basic ideas behind the two mechanisms (Adler-Nissen, 1976).

The "one by one" reaction is so called because the enzyme hydrolyzes one chain at a time. This would result if bond accessibility increased as cleavage progressed. If the first "denaturation" step, represented as a reversible step in Figure 1, is rate determining, then only native protein and end products are detected (Adler-Nissen, 1985).

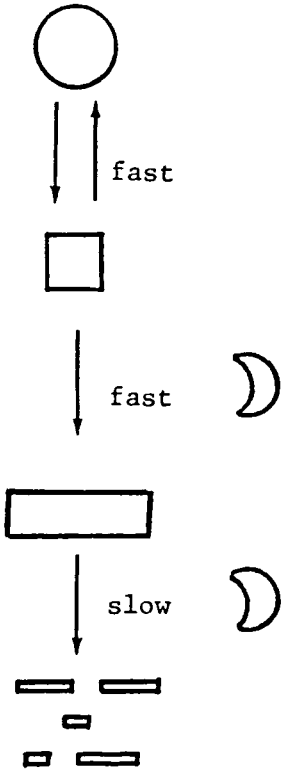
In contrast, the "zipper" reaction applies when the native protein is easily denatured and a large number of bonds are exposed. The enzyme can cleave at several different sites, but if the rate of cleavage is

Figure 1. Linderstrom-Lang one-by-one and zipper type models. Native protein is denoted by circles, denatured protein by rectangles, peptides by small rectangles of different length according to the chain length. The crescents symbolize enzymatic attack (Adler-Nissen, 1976)

one-by-one type



zipper type



slow, there will be a build up of intermediate products, as the end products are formed slowly.

Most hydrolysis reactions will be a combination of the two mechanisms. One mechanism may be favored over another depending on the nature and concentration of the substrate and the enzyme; the pH and temperature may also play a part (Adler-Nissen, 1976).

Uses of hydrolysates In enzymatic hydrolysis, the major problem is excessive hydrolysis, resulting in the formation of "bitter peptides". A bitter taste is unacceptable if the hydrolysates are to be used in foods. Bitter peptides may be avoided by controlling the degree of hydrolysis and consequently reducing the formation of low molecular weight peptides.

Gelatin and egg albumin are used as foaming agents in desserts, but not for flavoring since these hydrolysates exhibit a moderately bland flavor (Prendergast, 1974).

Soy protein is used as an ingredient in low pH foods, but it has to be made more palatable and its solubility profile has to be improved. As a result of a 10% degree of hydrolysis, soy protein has been 65-75% solubilized at pH 8 and 50°C (Olsen and Adler-Nissen, 1979b). Soy protein hydrolysates may be applied to soft drinks to increase their nutritional value (Adler-Nissen, 1977).

Other food applications have been in the area of dietary disorders, for example when insufficient pancreatic enzymes are present. Enzymatic hydrolysates have been used in the treatment of cystic fibrosis. Protein is supplied in the pre-digested form because the small intestine is

impaired. An example of such an application is the use of skim milk and casein (Clegg, 1977, Adler-Nissen, 1977).

As a consequence of the successful application of some food hydrolysates, there is motivation to investigate other protein rich sources, provided bitterness can be avoided during the hydrolysis, e.g. fish protein hydrolysates.

The hydrolysis of corn gluten meal, a concentrate of corn protein is a good protein source, but its properties are not ideal for this purpose. Adler-Nissen (1978a) reports the successful modification of corn gluten meal using Alcalase 0.6L, without creating bitter peptides. The hydrolysis was, however, performed on corn gluten meal from only one supplier. It should therefore not be eliminated that some conclusions may be specific to the particular corn gluten meal used.

The highest substrate concentration used was 8% w/w protein due to a large (more than ten times) increase in viscosity during the course of the hydrolysis. Higher protein concentrations could not be used due to possible agitation problems. There is no report on the use of the hydrolysate as a protein additive in foods.

pH Stat Hydrolysis Method

Principle

The degree of hydrolysis, which is actually the percentage of peptide bonds cleaved, may be measured by monitoring the base addition required to maintain pH during the hydrolysis (Adler-Nissen, 1985). The breakage of a peptide bond during hydrolysis can be illustrated by the following equation:

$$-CHR'-CO-NH-CHR'' + H_2O \xrightleftharpoons{\text{Enzyme}} -CHR'COOH + NH_2CHR'' \quad (1)$$

The free carboxyl and free amino groups may or may not be ionized, depending on the pH and to some extent the temperature of the reaction. At 25°C, the pK of the carboxyl group in a polypeptide is between 3.1 and 3.6, and the pK of the amino group is between 7.5 and 7.8 (Adler-Nissen, 1985). If a reaction proceeds at pH 8 and 25°C, the carboxyl group will be fully dissociated and the amino group will be partially protonated (Eriksen, 1982a). Hence hydrolysis results in release of protons where the stoichiometry depends on the degree of protonation of the α amino group. The protein exchange is shown in equation (2)

$$-CHR'-COOH + NH_2-CHR'' \rightarrow -CHR'-COO^- + NH_3^+-CHR'' \quad (2)$$

The degree of protonation of the α amino group can be expressed in terms of the pK of the amino group at a particular pH and temperature, and the pH of the system (equation (3))

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}} \quad (3)$$

The degree of dissociation α, increases with increasing temperature, the pKa of the amino group at pH 8 and 25°C is 7.7, but at 50°C the pKa drops to 7.1 (Eriksen, 1982a, Adler-Nissen, 1982).

A protein is made up of a certain number of peptide bonds which theoretically could all be cleaved to give 100% degree of hydrolysis. The total number of peptide bonds in a particular protein may be calculated

from the amino acid composition in mmols of the individual amino acids per g of protein (Adler-Nissen, 1985). The total number of peptide bonds is denoted by h_{tot}.

The pH stat meter is used to determine the actual number of peptide bonds per g which are cleaved during hydrolysis. This is denoted by h, and the degree of hydrolysis is defined as

$$DH = \frac{h}{h_{tot}} \times 100\% \qquad (4)$$

When hydrolysis is performed at neutral or mildly alkaline conditions, the degree of hydrolysis (DH) can be monitored continuously. Without the use of the pH stat technique, the pH drops as hydrolysis proceeds, shown in equation (2). The pH stat technique involves the addition of base, such as sodium hydroxide, to maintain a constant pH. The consumption of base is proportional to the hydrolysis equivalents, h. h is given by (Eriksen, 1982b)

$$h = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \qquad (5)$$

Operation

The pH stat meter requires the pH electrode to be immersed in the reaction system. The pH electrode is connected to a pH meter, which in turn is connected to an adjustable controller.

A motor-driven burette is responsible for the delivery of the titrant, either acid or base. One end of the burette must also be immersed into the reaction system.

The controller may be set to maintain a particular pH value. If the pH deviates from the desired value, titrant is added until the original pH is restored (Jacobsen et al., 1957). The consumption of base can be plotted with time by a recorder.

The controller starts the motor-driven burette when the output potential of the pH meter deviates from the desired value. It stops when the pH is brought back to the original value.

The controller is a proportional controller. The advantage to this is that near the set point, smaller amounts of titrant are added, and addition stops when the set point is reached.

Carbon dioxide from the atmosphere can introduce some error into the measurement of degree of hydrolysis when the pH stat is operating at alkaline pH. Adler-Nissen (1985) showed that, for a 1 liter charge operating at pH 9 and 50°C, less than 0.08 mmoles of carbon dioxide are absorbed over a four hour experiment. It is therefore assumed that the carbon dioxide uptake is negligible relative to the amount of titrant consumed during the hydrolysis. Carbon dioxide uptake can be avoided if an inert gas such as nitrogen is flushed through the system.

Corn Gluten Meal

Corn gluten meal is a by-product of the corn wet milling process. In corn wet milling, after steeping and grinding, the lighter germ may be collected off the top of a hydrocyclone separator. Starch, protein, and fiber are collected off the bottom. Fiber is further separated by grinding

and screening. Finally, the denser starch is separated from the gluten meal by hydrocyclone separation (Hoseney, 1986).

In the dried form, corn gluten meal is a very valuable animal feed, as it provides a high level of rumen bypass protein (Hoseney, 1986, Buck et al., 1987).

Corn gluten meal is bright yellow due to the presence of xanthophylls; it is used as a pigment source for poultry, imparting a bright yellow color to their skin (Buck et al., 1987).

Composition and properties

Corn gluten meal on a dry basis contains typically 60% protein, 15-18% fat, 20-25% carbohydrate, 3.5% fiber, and 1-2% ash (Buck et al., 1987). Corn gluten protein may also be broken down into further protein fractions. The major protein fractions are 68% zein and 28% glutelin, with only 1.2% globulins (Neumann and Wall, 1984, Buck et al., 1987).

The different classes of protein are defined according to their solubilities. The most abundant protein, zein, is soluble in relatively strong alcohol, such as 70% ethanol, or in dilute alkaline solutions. It is insoluble in water or solutions of neutral inorganic salts (Russell and Tsao, 1982, Neumann and Wall, 1984).

Glutelin, the next most abundant protein fraction, is defined as being readily soluble in dilute sodium or potassium hydroxide solutions, but insoluble in water, saline solutions, or alcohol. Globulins are soluble in saline solution (Russell and Tsao, 1982).

The solubility in different solutions may be used to sequentially fractionate the different proteins. Firstly, saline solution would remove

the globulins, zein may then be removed using 70% ethanol and lastly, the glutelin is removed by 0.1N sodium hydroxide (Neumann and Wall, 1984).

Most experimental work has been focused on zein, mainly due to the highly hydrophobic nature of corn proteins in simple water/alcohol systems. Solubility studies show that in a 70% ethanol/30% water mixture, the solubility of zein is still low at 15 mg protein/ml (Augustine and Baianu, 1987). Zein is also dispersible in sodium or potassium hydroxides within the narrow pH range of 11.3 to 12.7 (Ofelt and Evans, 1949). The dispersion is a result of the formation of the alkaline salt of tyrosine, caused by a dissociation of the phenolic-hydroxyl groups.

Gel filtration shows that zein proteins are monomers and dimers of similar sub-units. Molecular weights of 22000 and 45000 were found for the monomers and dimers, respectively (Ganchev and Ivanov, 1969b).

Uses of corn gluten meal

Corn gluten meal is an important protein source for animals. It would be desirable to use it for human nutrition since some fractions are rich in sulfur amino acids (Sternberg et al., 1979).

Corn gluten meal and soy protein have complementary amino acid compositions, such that an optimal protein content for foods can be obtained by blending the two. The gluten meal is upgraded for human use to give maize protein concentrate (MPC). The maize protein concentrate is a bland, light colored material containing approximately 70% protein and under 0.6% fat, the solubility of which is even less than the native protein. Maize protein concentrate has been used as a stabilizer in peanut butter. It can be substituted at levels of 10-15% in breakfast

cereal (Sternberg et al., 1979). Soy protein and maize protein isolate can be coextruded and substituted at varying concentrations in soy flour products (Buck et al., 1987).

The major drawback of using corn gluten meal in human food is its insolubility in water. The functionality of corn gluten meal can be modified by enzymatic hydrolysis using Alcalase 0.6L, such that its water absorption capability is significantly improved and it remains a one phase system if left suspended in an aqueous medium (Adler-Nissen, 1978a).

Hydrolysis Models

The Michaelis-Menten approach to enzyme kinetics has been widely applied. Kinetic constants in food protein hydrolysis experiments are evaluated using different approaches for soluble enzyme and insoluble substrate systems.

A kinetic model provides a mechanistic understanding of enzymatic hydrolysis. With macro-molecular substrates, a complete model of the system can be very involved. In most cases, a few valid assumptions will yield a more manageable model, which will still represent the mechanism adequately for the purpose of reactor design.

A statistical model using empirical rate equations may also be applied to soya isolate. Although, information on the mechanism is lost, the precision is very good for the optimization and design of hydrolytic processes.

Kinetic models

Different kinetic models have been applied to soluble enzyme and insoluble substrate systems. The enzymatic hydrolysis of soybean protein (Constantinides and Adu-Amankwa, 1980) and lean meat protein (O'Meara and Munro, 1985) was modelled using a Michaelis-Menten product inhibition model. Alternatively, the adsorption of the enzyme onto the substrate (Freundlich adsorption method) followed by the subsequent hydrolysis was used to model the enzymatic hydrolysis of fish protein hydrolysate (Archer et al., 1973). The hydrolysis of oxidized chains of insulin A and B has been modelled as two simultaneous parallel reactions (Montali et al., 1980). It is not apparent which kinetic model might exhibit a good fit to the experimental data until the system is carefully studied.

Michaelis-Menten model Insoluble substrate systems may be modelled using mechanisms similar to those used for the hydrolysis of soluble substrates. Such models follow the traditional Michaelis-Menten format, shown below

$$v = \frac{dP}{dt} = \frac{v_m \cdot S}{K_m + S} \tag{6}$$

The model used by Constantinides and Adu-Amankwa (1980) modifies the Michaelis-Menten equation to account for product inhibition and may be represented by the following mechanism

$$E + S_i \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES_i \xrightarrow{k_2} S_{i-j} + P_j + E \tag{7}$$

$$E + P_n \underset{k_{-n}}{\overset{k_n}{\rightleftarrows}} EP_n \tag{8}$$

$$E \xrightarrow{k_I} I \tag{9}$$

Equation (7) represents a multistep process where intermediates S are generated and further degraded. Equation (8) represents the product inhibition, where a proportion of the product competitively ties up the enzyme. Equation (9) represents the inactivation of the enzyme. S_{i-j} represents all the "intermediates", of varying molecular weights, in the hydrolysis. In the same manner, P_j represents a group of smaller, but varying molecular weight peptides.

Similar mechanistic models were used for the enzymatic hydrolysis of insoluble lean meat protein using the protease Alcalase 0.6L (O'Meara and Munro, 1985) and also the hydrolysis of cellulose by Trichoderma viride (Howell and Stuck, 1975). The model for all three systems may be represented by the following equation

$$\frac{dP}{dt} = \frac{k_j E_0 (S_0 - \Sigma P)\ e^{-k_i t}}{K_m + (S_0 - \Sigma P) + (K_m/K_P)\Sigma P} \qquad (10)$$

The global constants k_j, k_i, K_m, and K_P were evaluated mathematically using the Marquardt nonlinear regression iterative technique to fit the model to experimental data (Constantinides and Adu-Amankwa, 1980, O'Meara and Munro, 1985). The rate equation is integrated using a Runge-Kutta numerical integration algorithm. The global constants in the hydrolysis of cellulose by Trichoderma viride (Howell and Stuck, 1975) were evaluated by a graphical method.

Freundlich adsorption model The kinetic model considers firstly the adsorption of the enzyme on the insoluble substrate, which is evaluated using the Freundlich adsorption isotherm. The enzyme adsorption was then combined with a kinetic expression for the hydrolysis. The enzymatic

hydrolysis of fish protein concentrate, starch, and cellulose (Archer et al., 1973, McLaren, 1963) have all been modelled in this manner.

The Freundlich adsorption isotherm considers the distribution of the solute between the soluble phase I and the insoluble phase II. For an insoluble substrate with surface A_s onto which the enzyme can bind (McLaren, 1963, Archer et al., 1973),

$$\frac{E_a}{A_s} = K\left[\frac{E_I}{V_I}\right]^n = KE^n \tag{11}$$

If hydrolysis takes place on the surface of the substrate, the power n should be 2/3 or close to it. The value of n is less than 2/3 if the substrate has a high internal surface area, where additional hydrolysis occurs (McLaren, 1963).

The mechanism for the adsorption to solid substrate and formation of soluble protein is

$$E + A_s \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA_s \xrightarrow{k_3} P + E \tag{12}$$

The rate of product formation is given by the expression

$$\frac{dP}{dt} = k_3(EA_s) \tag{13}$$

If the assumption is made that all the adsorbed enzyme is in the form of the enzyme-substrate complex,

$$(EA_s) = \frac{E_a}{V_I} \tag{14}$$

The resulting rate equation for formation of products using the Freundlich adsorption isotherm (equation (11)) may be expressed as

$$\frac{dP}{dt} = \frac{k_3 K A_s E^n}{V_I} \tag{15}$$

If A_s and V_I are assumed to be constant, the initial rate of hydrolysis becomes a function of enzyme concentration only. The enzyme can occur as free enzyme in solution or as bound enzyme, as shown below.

$$E_0 = (E) + (EA_s) \tag{16}$$

The final kinetic expression for product formation becomes

$$\frac{dP}{dt} = k'[E_0 - (EA_s)]^n \tag{17}$$

The values of constants k' and n may be evaluated by plotting log(dP/dt) versus log E (Archer et al., 1973, McLaren, 1963).

If the surface area of the substrate is small, for example in the digestion of gelatin gel with trypsin, the enzyme in the enzyme-substrate complex is assumed to be negligible, and the enzyme concentration E in equation (17) is replaced by E_0, the total amount of enzyme in the system (McLaren, 1963).

Simultaneous first-order reactions An alternative method which was applied to the hydrolysis of oxidized chains of insulin A and B was to consider two simultaneous first-order reactions (Mihalyi and Harrington, 1959, Ronca et al., 1975). The enzyme subtilisin Carlsberg was used for the hydrolysis; this enzyme has a very wide specificity. The kinetics

of the reaction were followed using the pH stat technique, which unfortunately cannot detect the separate reactions. The sum of both reactions with time was therefore measured. The existence of two simultaneous reactions was identified by kinetic analysis.

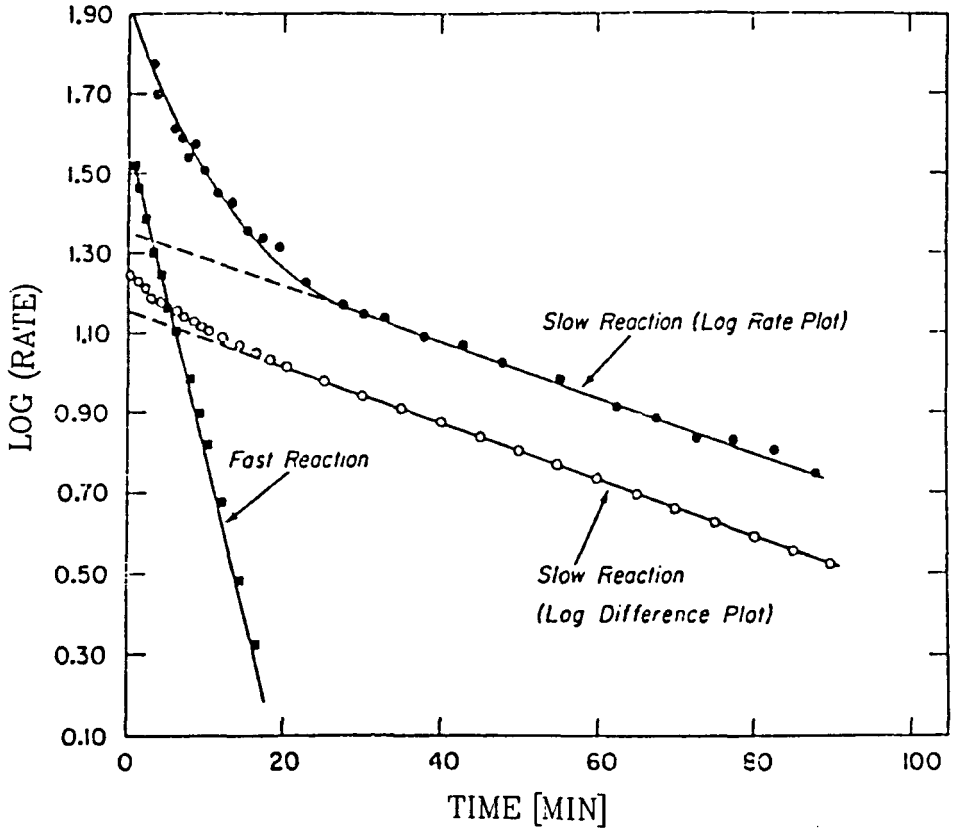
The two simultaneous reactions consist of a fast and a slow reaction where the proteolysis of one class of peptide bonds is a fast reaction. These bonds are identified as $CyS(O_3H)$-Ser and Leu-Tyr. The other class of bonds are cleaved more slowly according to a first-order reaction. The cleavage sites in this case are Leu-Glu, Asn-Tyr, and Try-$Cys(O_3H)$ (Montali et al., 1980). Other slowly cleaved bonds are also identified as Glu-Gln (Ronca et al., 1975). Ronca et al. (1975) claim that the limited proteolysis of other sites is also possible, due to the broad specicity of subtilisin Carlsberg.

The rate constants were evaluated by plotting the natural log of the proteolysis rate against time. The proteolysis rate is given by the rate of base consumption (dP/dt) measured by the pH stat. It is generally concluded that the fast reaction is complete before a significant fraction of the slow reaction has occurred (Mihalyi and Harrington, 1959). The slow reaction is shown by the last part of the plot, which is linear (Figure 2). An expression for each reaction is obtained using the first-order rate law

$$\frac{dP}{dt} = -\frac{dS}{dt} = kS \tag{18}$$

This equation can be integrated using the boundary condition that S is S_0, the initial substrate concentration, at time zero, which leads to

Figure 2. Graphical analysis of typical pH stat curve. Logarithm of the rate of base uptake versus time. Straight lines correspond to isolated slow (O) and fast (□) reactions. Simultaneous fast and slow reaction denoted by ● (Mihalyi and Harrington, 1959)


1.90
1.70
1.50
1.30
1.10
0.90
0.70
0.50
0.30
0.10
LOG (RATE)
0
20
40
60
80
100
TIME [MIN]
Slow Reaction (Log Rate Plot)
Fast Reaction
Slow Reaction
(Log Difference Plot)

$$\ln \frac{dP}{dt} = \ln(kS_0) - kt \qquad (19)$$

The slope of the linear part is -k and the intercept is $\ln(kS_0)$, from which S_0 may be found. S_0 depends on the number of bonds split and the degree of dissociation. At pH values greater than 9.0, it corresponds to the number of bonds split.

The first part of the curve is the sum of the fast and slow reactions (Figure 2). The k value and S_0 for the fast reaction are evaluated by a second semi-log plot of proteolysis rate versus time. The data for the fast reaction may be plotted after the rate of the slow reaction rate has been subtracted from the combined rate (Ronca et al., 1975, Archer et al., 1973, Mihalyi and Godfrey, 1962).

The hydrolysis of fish protein concentrate can be modelled as two first-order reactions. The fast reaction corresponds to bound enzyme cleaving accessible polypeptide chains. The slow reaction is the hydrolysis of the more compact core protein. Archer et al. (1973) conclude that the rate controlling process for fish protein concentrate is the diffusion of the enzyme onto the surface active sites and the compact core.

Statistical model

The statistical model (Adler-Nissen, 1985) gives no information on the mechanism of the hydrolysis, but serves as an empirical comparison between hydrolysis curves for the purpose of optimization of the process.

The statistical model compares an experimental hydrolysis curve with a standard, defined hydrolysis curve. Scaling factors are used to correct

the time scale for any differences between the experimental curve and the standard curve. Differences between the curves could be a result of a different hydrolysis temperature or a different enzyme concentration, for example. Statistical techniques are used to compare hydrolysis curves, since experimental error as well as reaction conditions would imply differences between the two hydrolysis curves.

The statistical analysis model relies on the fact that only one enzyme is present, and that the substrate concentration is high enough to ensure that the enzyme is saturated with substrate throughout the course of the hydrolysis (Adler-Nissen, 1985).

The model has been applied to the hydrolysis of soya isolate using Alcalase. It would not, however, be applicable to corn gluten meal, where the enzyme may not be saturated with substrate throughout the course of the hydrolysis. The applicability of this model was therefore not pursued.

Enzyme Reactions

Enzyme properties

Enzymes are specific biological catalysts. The same thermodynamic and kinetic constants can be applied as with any catalytic reaction, so that the rate at which an enzyme reaction proceeds is altered, but not the final position of the equilibrium. The substrate to undergo reaction has to bind to the active site of the enzyme, which forms an enzyme-substrate complex.

The activity and stability of the enzyme depend very much on the surroundings. The rate of hydrolysis is dependent on the enzyme

concentration and, in addition, the ionic strength, pH, temperature, and concentration of substrate and inhibitors (Wiseman, 1985). The effects of these parameters on the rate of hydrolysis are illustrated in the Michaelis-Menten equation, the model for product inhibition, and the Freundlich adsorption model, equations (6), (10), and (17), respectively.

Also fluid forces and chemical agents can effect an enzyme's activity. Any one of these factors may effect the shape or ionization state of the enzyme.

Enzyme concentration The choice of enzyme concentration in an industrial process is mostly an economic consideration of how much enzyme is required to generate the products at a satisfactory rate. In general, the amount of enzyme required relative to the substrate is small (Wiseman, 1985).

Substrate concentration A high substrate concentration tends to stabilize enzymes, even under nonideal conditions, permitting operation at higher temperature and lower enzyme dosage. In food protein hydrolysis, the substrate concentration is generally less than 10% by weight (equation (10)) (Boyce, 1986).

Effect of temperature The rate of reaction increases with increasing temperature according to the Arhenius equation. The rule of thumb is that for every 10°C increase in reaction temperature, the enzyme will react twice as fast.

$$k = A \exp(-E_a/RT) \tag{20}$$

However, enzyme stability is also influenced by the temperature, and thermal denaturation can occur (Wiseman, 1985, Bailey and Ollis, 1977).

The high activation energy for enzyme denaturation makes the enzyme quite sensitive to small changes in temperature. Denaturation has been described by a first order decay law

$$E(t) = E(0) \exp(-k_d t) \tag{21}$$

Effect of pH At the optimum pH, the maximum possible amount of enzyme is in the active form. Enzymes possess ionizable groups, whose charge varies with a pH change, which in turn alters the conformation of the enzyme and consequently the binding of the substrate at the active site (Wiseman, 1985). The optimum operational pH often is a compromise between the effects on enzyme activity and enzyme stability (Boyce, 1986).

Enzyme inhibition Enzymes can be inhibited in different ways. Enzyme inhibition can be categorized into irreversible and reversible inhibition.

Irreversible inhibition involves the destruction or modification of one or more functional groups of the enzyme, resulting in the loss of some or all of the enzyme activity (Lehninger, 1972, Wiseman, 1985).

The most common types of reversible inhibition are competitive and non-competitive. Competitive inhibition involves competition between inhibitor and the normal substrate molecules for the binding site of the enzyme. Competitive inhibition can generally be reduced by increasing the substrate concentration (Lehninger, 1972). Competitive inhibition is described by equation (22)

$$V = \frac{v_m \cdot S}{S + K_m(1 + I/K_I)} \tag{22}$$

Non-competitive inhibition involves the inhibitor binding at some other site on the enzyme. Inhibition cannot be reduced by increasing the substrate concentration. The general rate equation for non-competitive inhibition is given by

$$V = \frac{v_m \cdot S}{(1 + I/K_I)(S + K_m)} \tag{23}$$

Effect of mechanical forces Mechanical forces, including viscous shear and interfacial tension, can disrupt the conformation (denature) of the enzyme. The fragility of the enzyme may impose a limit on the type of agitation in an enzyme reactor. Agitation increases the substrate mass transfer rate, but it can cause enzyme denaturation (Bailey and Ollis, 1977).

Class of proteases

The general name applied to enzymes used to hydrolyze proteins is protease. Proteases can be further divided into endopeptidases and exopeptidases. Both classes hydrolyze the peptide linkage. Endopeptidases act upon peptide linkages that are not near the end of the substrate molecule, whereas exopeptidases act only on peptide linkages that are at the end of the peptide chain (Laidler, 1958).

All proteases of significant industrial importance are endopeptidases rather than exopeptidases. Endopeptidases can be grouped according to the biochemical nature of the active site. These groups are serine proteases, thiol proteases, carboxyl proteases and metalloproteases (Moo-Young, 1985, Rehm and Reed, 1987). A general overview of the class, source, and uses for the important commercial enzymes is shown in Table 1.

Serine proteases The serine proteases have serine and histidine at the active site. Two sub-groups of the serine proteases are trypsin-like proteases and alkaline proteases. The serine proteases are active in the neutral to alkaline pH range, with pH optima between 8 and 11 (Rehm and Reed, 1987). The alkaline proteases have, in general, a much broader specificity to the substrate than the trypsin-like enzymes. The alkaline proteases are all specific to aromatic or hydrophobic amino acids at the carboxyl side of the splitting point (Moo-Young, 1987).

Thiol proteases Thiol proteases have cysteine at their active site. They all exhibit a broad pH activity, with their optima around neutrality. The commercial thiol proteases are plant proteases such as papain and bromelain (Rehm and Reed, 1987).

Carboxyl proteases Carboxyl proteases are widely distributed in animal cells, molds, and yeast. They exhibit their optima in the acid pH range and many of them have aspartate residues at their active site. They show specificity towards aromatic or bulky amino acid residues at both sides of their splitting points.

Table 1. Classification of commercial proteases and their uses (Rehm and Reid, 1987)

Class	Source	Use
Serine proteases (alkaline)	Animal pancreas -	
	trypsin	Pharmaceutical
	chymotrypsin	
	Microbial (*Bacillus* spp.)	Detergents
	subtilisins	Food
Thiol proteases	Plant -	
	papain (papaya)	Meat tenderizing
	bromelain (pineapple)	Beer haze prevention
	ficin (fig latex)	
Carboxyl proteases (acid)	Animal -	
	rennets (calf stomach)	Milk clotting in cheese
	pepsin (pig stomach)	manufacture
	Microbial -	
	acid proteases (*Aspergillus* and *Rhizopus*)	Baking and other food uses
	microbial rennets (Mucor)	
Metalloproteases (neutral)	Microbial	
	neutral proteases	Aspartame manufacture
	Bacillus and *Aspergillus*	Food, brewing

The carboxyl proteases are divided into two sub-groups according to their catalytic activity. One group resembles rennin and the other group resembles pepsin. Pepsin-like proteases have more acidic optima than the rennin-like proteases.

Metalloproteases They all have a bound metal ion in the active site that is involved in the catalytic mechanism. Consequently, they are inhibited by chelating agents such as ethylene-diamine tetraacetic acid (EDTA).

The metalloproteases are sub-divided into neutral and alkaline proteases. Enzymes of the neutral group usually have zinc at their active site, and are specific towards hydrophobic amino acids on the amino side of the peptide bond to be cleaved. Members of the alkaline group are very similar to those of the neutral group, other than their pH optima.

Functional Properties

The functional properties of a food are defined as any properties that effect the utilization of a food other than its nutritional properties (Cherry, 1981). Certain functional properties of a food can be favorably modified via enzymatic hydrolysis. Three factors which are very important in assessing the functional value of a food are the solubility, the flavor, and the viscosity.

Solubility

The solubility is a "hydrophilic" functional property; its measurement generally is the first study to be made when more complicated functional properties are to be investigated. A systematic study of the solubility

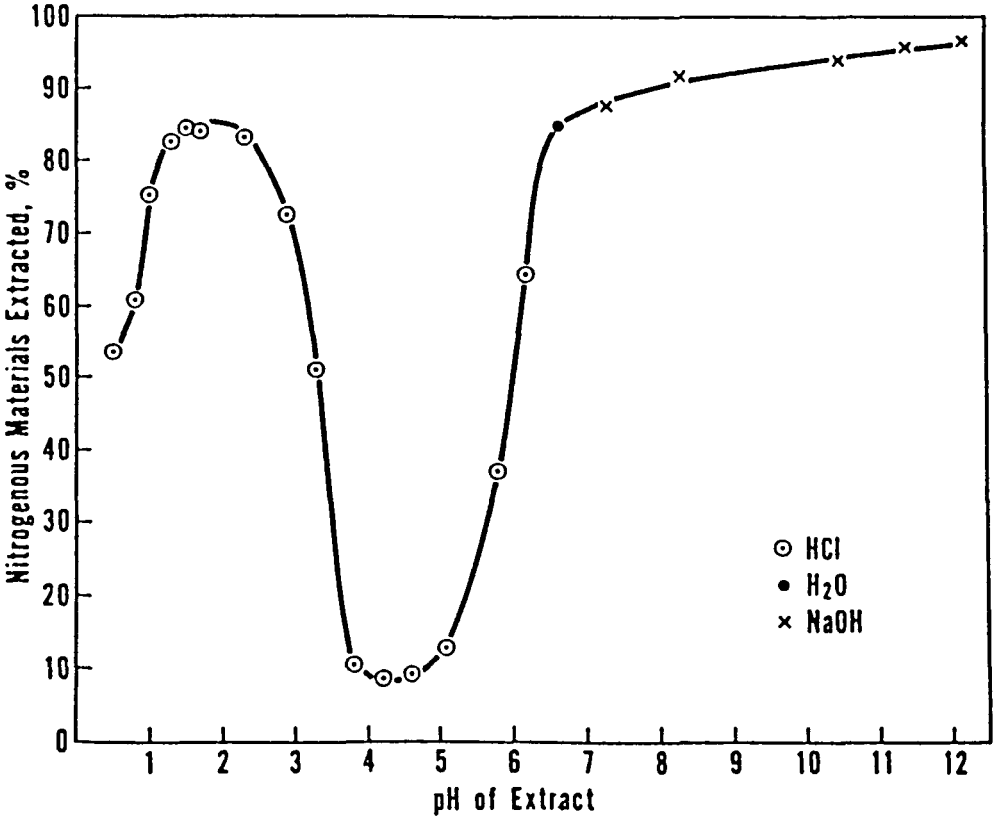
properties should be made under a variety of ionic environments. The solubility at different pHs gives a good indication of the potential uses of the protein, as well as indicating the inapplicability for some other uses (Mattil, 1971).

Most foods contain a variety of ions, either natural or added. The solubility of any protein can be varied by altering the ionic environment. The nitrogen solubility index is generally used to indicate solubility. It involves dispersing a sample of protein in the presence of a salt such as NaCl or $CaCl_2$ (Wolf, 1970). The solubility is studied through the full pH range. Samples are analyzed for soluble protein (Mattil, 1971, Adler-Nissen, 1985).

A typical solubility study for defatted soybean meal is shown in Figure 3 (Wolf, 1970). The region of insolubility around pH 4-5 is the isoelectric region, which according to Wolf can be eliminated if the molecular weight of the protein can be reduced, for example by enzymatic hydrolysis. Ideally, for a wide range of applicability, the solubility should be increased through as wide a pH range as possible.

The solubility of soy protein isolate can be increased even for short hydrolysis times using Alcalase or Neutrase (Olsen and Adler-Nissen, 1979a). The solubility curves are relatively flat, reflecting the hydrolysate product distribution. The hydrolysate contains fairly small, highly soluble peptides and nearly insoluble denatured protein (Olsen and Adler-Nissen, 1979a).

Figure 3. Extractability of proteins in defatted soybean meal as a function of pH (Wolf, 1970)


Nitrogenous Materials Extracted, %
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
pH of Extract
HCl
H2O
NaOH

Flavor

Flavor is an organoleptic property, which is a property attributed to one of the sense organs. The flavor is very important if a protein such as soy protein or corn gluten meal is to be incorporated into foods.

Proteins in general have no intrinsic flavor, but they can potentially modify flavor according to their capacity to bind flavors or off-flavors. Flavors can be created during cooking or released during enzymatic hydrolysis (Kinsella, 1979).

Bitterness is thought to be related to hydrophobicity of peptides, so the higher the content of hydrophobic amino acids in the protein, the greater the tendency to produce bitterness when the protein is hydrolyzed (Olsen and Adler-Nissen, 1979a).

There are various approaches to removing off-flavors. One approach is to add a desirable flavor, to mask the impact of off-flavors.

Taste evaluations of a particular product should be carried out by a trained panel. Taste tests for bitterness involve use of two samples and four bitter tasting standards, which are a mixture of sample and increasing amounts of quinine hydrochloride. A certain concentration of quinine hydrochloride is taken as the threshold value. Samples are then assigned a score on a scale of one to five, with one representing no flavor and five, a strong bitter flavor (Adler-Nissen, 1985, Olsen and Adler-Nissen, 1979a).

Viscosity

Viscosity is a functional property arising from the ability of proteins and other macromolecules to form temporary intermolecular cross-links (Cherry, 1981). The viscosity of protein dispersions is mostly influenced by the molecular properties of the protein such as molecular weight, hydration, and the shape of the molecule. Rheological properties are a function of shear rate, protein concentration, temperature, pH, and ionic strength (Kinsella, 1979).

In enzymatic hydrolysis, smaller molecular weight peptides are produced from a higher molecular weight protein. A reduction in molecular weight would generally bring about a decrease in the apparent viscosity. Olsen and Adler-Nissen (1979a) have shown that this occurs for the enzymatic hydrolysis of soy protein. However, when corn gluten meal is subjected to enzymatic hydrolysis, an increase in viscosity by more than ten times results. The hydrolysis may expose sites on the surface which can hydrogen bond, resulting in a strengthened cohesion between particles.

MATERIALS AND METHODS

Materials

Corn gluten meal

The protein content of the corn gluten meal (Grain Processing, Muscatine, IA) was found to be 60.61%, using the micro-Kjeldahl assay (AOAC, 1970). An 8% weight protein slurry was made up with deionized water (after Adler-Nissen, 1977).

Enzyme preparation

Four different proteases were used. The enzyme which gave the highest yield of soluble protein and the highest degree of hydrolysis was then screened further.

P4032 protease P4032 protease (Sigma Chemical Company, St. Louis, MO) was a microbial alkaline protease from Aspergillus oryzae. The required mass of enzyme was dissolved in 5 ml of deionized water for ease of addition to the reactor. The enzyme solution was made up immediately before addition, because it is unstable without the presence of substrate and may deactivate.

Trypsin (T8128) Trypsin (T8128) (Sigma Chemical Company, St. Louis, MO) was a crude preparation from porcine pancreas. The trypsin (T8128) solution was prepared in the same manner as P4032 protease.

Alcalase 2.4L Alcalase 2.4L (Novo Laboratories, Inc., Wilton, CT) is a food grade preparation of subtilisin Carlsberg in the liquid form. The enzyme was diluted by one to ten to reduce its viscosity for ease of addition to the reactor. The dilution was made directly before addition

for the same reasons as with P4032 protease, to reduce any chances of deactivation in the absence of substrate.

Different batches of Alcalase 2.4L were compared by using the Anson method of activity determination (Anson, 1939, Novo, 1978), except the color development between batches was compared directly. The Anson method compares the color development of an enzyme sample to 1 milli-equivalent of tryosine per minute (1 Anson unit).

Neutrase 0.5L Neutrase 0.5L (Novo Laboratories, Inc., Wilton, CT) is obtained by fermentation of Bacillus subtilis. The enzyme solution was diluted by one to ten in the same manner as Alcalase 2.4L to reduce the viscosity.

Analyses

Kjeldahl nitrogen assay

Nitrogen content was measured by the Kjeldahl assay. The amount of protein in the corn gluten meal samples was obtained by multiplying the nitrogen content by the Kjeldahl conversion factor (6.25) for corn gluten proteins (Adler-Nissen, 1985).

Approximately 0.1 g of protein was digested, distilled (Labconco Micro Digestion unit and Micro Kjeldahl unit, Fisher Scientific Co., Itasca, IL), and titrated according to the AOAC method (1970).

All analyses were performed in duplicate. The accuracy of the method was checked using ammonium sulfate standards (Ma and Zuazaga, 1942).

Biuret protein assay

The soluble protein content was determined by the biuret method (Doumas, 1975). The assay uses the absorbance of a complex formed between the peptide bonds and copper ions in the biuret reagent. The absorbance of the samples was measured at 540 nm. Standard solutions of bovine serum albumin (Sigma Chemical Co., St. Louis, MO) were used to generate a standard curve. Protein samples were analyzed in a 1 to 40 mg/ml protein range.

Trichloroacetic acid precipitation

Addition of 5 ml of 2.4N trichloroacetic acid (TCA) was added to 10 ml of protein solution and mixed on a vortex mixer, resulting in the precipitation of proteins and higher molecular weight peptides (Alder-Nissen, 1985). After centrifugation (2.7×10^3 g for 30 minutes), the concentration of soluble smaller molecular weight peptides was determined by the biuret assay (Doumas, 1975).

Viscosity

The viscosity of the corn gluten slurry after hydrolysis was measured with a Haake RV-12 viscometer (Haake Inc., Saddle Brook, NJ). The viscometer was set to increase the shear rate from 0 to 1200 sec^{-1} over a three minute period.

Sieve analysis

A 30 g sample was placed in the top sieve in a series of six 8" sieves and bottom pan (U.S. Standard Testing Sieves A.S.T.M. Ell Spec, W.S. Tyler Inc., OH) and shaken in a sieve shaker for 30 minutes (Willis, 1981).

The weight of each sieve and the pan was recorded before and after shaking to obtain the size distribution. Less than 5% of the sample should be in the top sieve and the pan for a good size distribution (Willis, 1981).

B.E.T. surface analysis

B.E.T. analysis was used to determine the total surface area of approximately 1 g of corn gluten meal (Accusorb 2100E physical adsorption analyzer, Micromeritics, Norcross, GA) at a saturation temperature of 77.5K.

Krypton gas was used as the adsorbate. B.E.T. analysis relates the volume of adsorbate removed on adsorption to the surface area of the sample onto which adsorption occurs (McClellan and Hamsberger, 1967).

Experimental Procedure

Three areas of experimental work were implemented to study the enzymatic hydrolysis of corn gluten meal. Screening runs were performed to find the enzyme which resulted in the highest yield. This was followed by determination of the pH and temperature that gave a good rate of reaction as well as stable conditions for the enzyme. The effect of size reduction on the hydrolysis of the corn gluten over a range of enzyme concentrations was ascertained. Finally, a series of experiments was conducted to study the degree of hydrolysis and soluble protein with time. This data was used to develop a kinetic model for the hydrolysis.

Screening experiments

The four enzymes, P4032 protease, trypsin (T8128), Alcalase 2.4L, and Neutrase 0.5L, were used to hydrolyze corn gluten meal. Literature values

(shown in Table 2) for the temperature and pH were used. The effects of pH and temperature on the hydrolysis were studied using Alcalase 2.4L.

Enzyme screening runs The screening experiments were conducted with a 500 g charge of 8% weight protein slurry in a 825 ml baffled reactor. The original pH of the corn gluten meal slurry was between 4.3 and 4.6. The speed of agitation was adjusted to 450 rpm (Stir-pak stirrer, Cole Palmer Instrument Co., Chicago, IL), and the slurry was allowed to reach the correct temperature (Immersion Circulator Model 73, Fisher Scientific Co., Itasca, IL), after which the pH was adjusted to 7, 8, or 9, depending on the optimum pH of the particular enzyme shown in Table 2. The amount of enzyme used in all cases (after Ganchev and Ivanov, 1969a) was 0.1% weight of the substrate protein. The relative activities could not be compared, because the enzymes were standardized in different activity units.

The pH dropped during the course of the hydrolysis due to cleavage. At fifteen minute intervals, the pH was brought to the initial value manually using standardized sodium hydroxide.

After four hours of hydrolysis a 50 ml sample was taken. The hydrolysis was stopped by lowering the pH to 4.2 using concentrated hydrochloric acid. The sample was centrifuged (Sorvall RC-5 refrigerated centrifuge, Du Pont Co., Wilmington, DE) at 27×10^3 g for 30 minutes. The resulting supernatant was analyzed for protein content using the biuret assay (Doumas, 1975). The degree of hydrolysis was calculated from the consumption of sodium hydroxide during the course of the reaction (after Adler-Nissen, 1985).

Table 2. Literature values of optimum pH and temperature for different proteases (Godfrey and Reichelt, 1983)

Enzyme	pH	Temperature
P4032 protease (from Asp. oryzae)	7	50°C
Pancreatic trypsin	8	45°C
Alcalase 2.4L	9	50°C
Neutrase 0.5L	9	50°C

Choice of reaction conditions These experiments were devised to determine the most favorable pH, temperature, and concentration range for Alcalase 2.4L.

The enzyme dosage was studied using an 800 g (775 ml) charge of corn gluten slurry in the same baffled reactor. The reactor contents were allowed to reach a temperature of 50°C, then the pH stat meter (Computer Aided Titrimeter (CAT system), Fisher Scientific Co., Itasca, IL) was started and the pH raised to 9. The reactor and pH stat configuration are shown in Figure 4. The first aliquot, 0.05 AU/L (0.02 g/L) was added with a Pipetman pipette (Rainin Instrument Co., Woburn, MA).

Sodium hydroxide consumption was monitored with time. After 15 minutes, the base addition rate was linear and a second equal aliquot of Alcalase was added. This procedure was repeated for a total of four aliquots of enzyme. The total hydrolysis time was one hour. The three experiments shown in Table 3 were performed until a range of enzyme concentrations between 0.05 AU/L (0.02 g/L) and 0.875 AU/L (0.35 g/L) had been studied. The rate of hydrolysis was calculated at each enzyme concentration, from which the most favorable range of enzyme concentration was determined.

The effect of temperature and pH at an intermediate concentration of Alcalase 2.4L (0.375 AU/L) were examined in the same manner--by examining a region of constant rate of hydrolysis. The pH and temperature values studied are shown in Table 4.

Figure 4. Reactor and pH stat configuration used in protein hydrolysis experiments. The reactor was supported from beneath. A stirring motor with variable speed was used

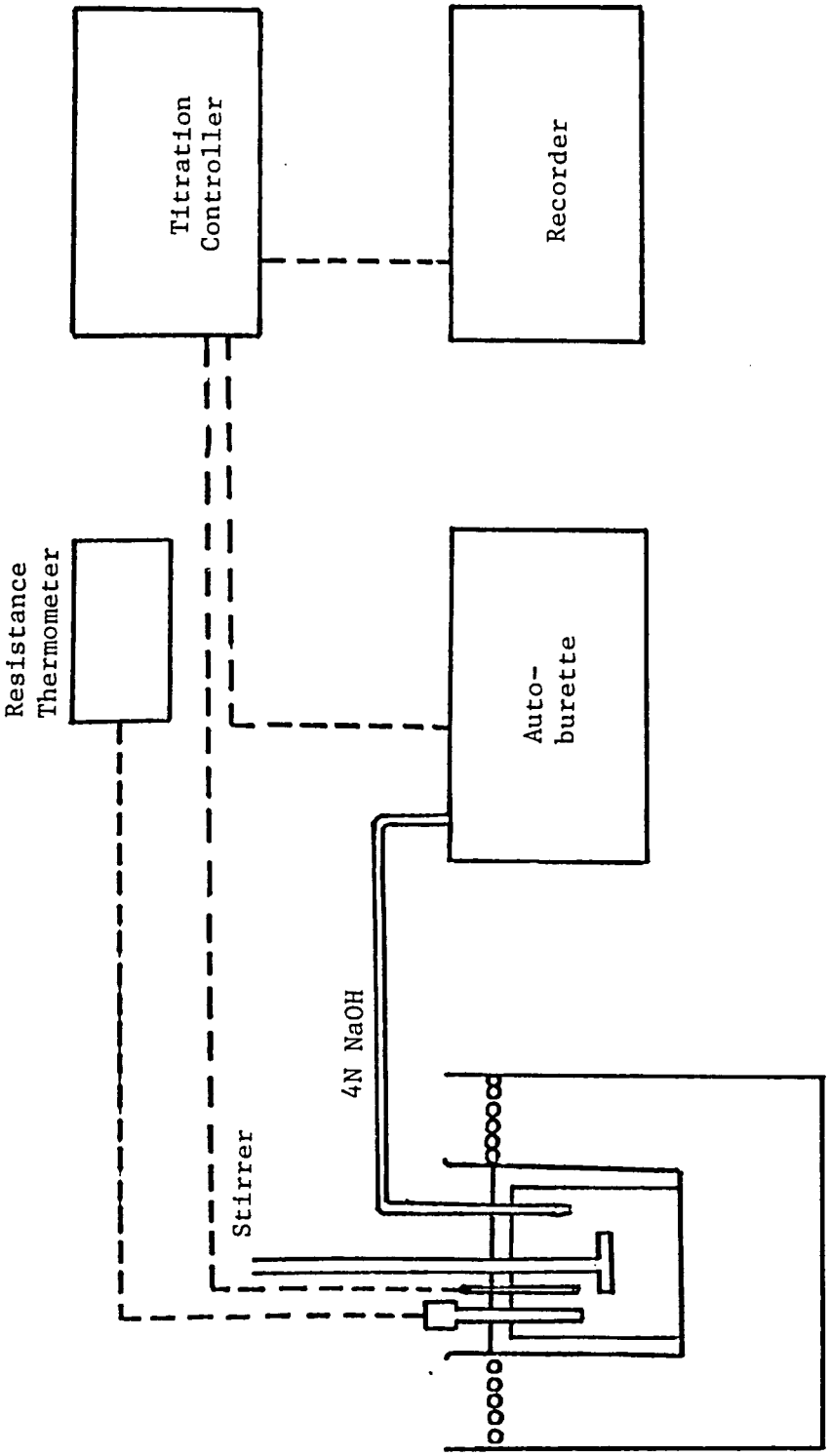

Titration Controller
Recorder
Resistance Thermometer
Auto-burette
4N NaOH
Stirrer

Table 3. Screening runs performed to investigate the effect of Alcalase 2.4L dosage (0.05 AU/L - (0.875 AU/L) on the rate of hydrolysis

Enzyme dosage (Au/L)		
Run 1	Run 2	Run 3
0.05	0.25	0.5
0.1	0.30	0.625
0.15	0.35	0.75
0.2	0.4	0.875

Table 4. Investigation of pH and temperature at an Alcalase 2.4L concentration of 0.375 AU/L. X denotes parameter investigated

pH	Temperature (°C)		
	40	50	60
9	X	X	X
10		X	
11		X	

Design experiments

Design experiments were carried out at pH 9 and 50°C in the concentration range 0.375 AU/L (0.15 g/L) and 0.75 AU/L (0.31 g/L) Alcalase 2.4L. To gain an insight into the effect of size reduction on the rate of hydrolysis, experiments were performed using the above conditions with and without size reduction.

The dry corn gluten meal was pulverized using a rotating hammer mill (Micro Switch, Freeport, IL). The corn gluten meal was passed through the grinder three times to ensure a narrow size distribution. A sieve analysis (U.S. Standard Testing Sieves, A.S.T.M. E11 Spec, W.S. Tyler Inc., OH) was performed on 30 g of unground and 30 g of ground sample. The sieve sizes used for the unground samples were between 1400 μm (12 mesh) and 45 μm (350 mesh), whereas for the ground sample, the size range was between 1180 μm (14 mesh) and 38 μm (400 mesh).

All the design runs were based on an 800 g charge to the reactor, containing 8% protein by weight. The corn gluten slurry was agitated at 450 rpm for one hour at pH 9 and 50°C to allow for the limited dissolution of the corn gluten meal without the action of the enzyme. After 1 hour the base consumption was typically 30-50% of that after enzyme addition. The enzymatic hydrolysis followed, beginning with taking a 15 ml sample and adding the required amount of enzyme. The titrator was zeroed at this point.

The hydrolysis time was four hours, during which 15 ml samples were taken at two and four hours. Concentrated hydrochloric acid (0.15 ml) was added to the samples to deactivate the enzyme.

The samples were centrifuged (27 x 10^3 g for 30 minutes) and the supernatants were analyzed for protein content by the Kjeldahl (AOAC, 1970) and biuret assays (Doumas, 1975). At the end of the hydrolysis, an extra sample was taken and the viscosity determined.

Modelling experiments

In order to understand the mechanism of the corn gluten meal hydrolysis, a series of modelling experiments was performed. To do this, the soluble protein and the degree of hydrolysis were followed with time. The range of enzyme concentrations studied was 0.375 AU/L (0.15 g/L) to 0.75 AU/L (0.31 g/L) at pH 9 and 50°C.

The experimental procedure was the same as for the design runs, except for sampling and analysis. Ten ml samples were taken every 10 minutes for the first hour, every 15 minutes for the second hour, and every 30 minutes for the remaining two hours of the four hour hydrolysis. The enzyme was deactivated by placing the sample in a water bath at 90°C for 15 minutes.

At four hours, sampling was stopped and a second aliquot of Alcalase 2.4L was added. The hydrolysis was continued for another hour. The consumption of sodium hydroxide was observed after the addition of the second aliquot of enzyme. This simple procedure tests for the possibility of limited hydrolysis due to the presence of insufficient enzyme to saturate the substrate. The samples were centrifuged and the supernatants were assayed for soluble protein. Precipitation with trichloroacetic acid (TCA) was then carried out on the supernatants. After addition of TCA, the samples were centrifuged at 27 x 10^3 g for 30 minutes. The

supernatants were analyzed for soluble protein. TCA precipitates all but the low molecular weight peptides. The degree of hydrolysis was calculated from the values of base consumption.

Model Development

The kinetic models considered were all based on three compartments. I represents insoluble corn gluten remaining during the course of hydrolysis. The compartments S and P both represent soluble material. S is a group of high molecular weight protein/peptides, precipitated by trichloroacetic acid (TCA), and P is a group of low molecular weight peptides, not precipitated by TCA.

The three compartments and the rates connected with them are shown in Figure 5. The rate expressions r_I and r_S may be written as

$$r_I = -\frac{dI}{dt} = \frac{d(S + P)}{dt} \tag{24}$$

$$r_S = -\frac{dS}{dt} = \frac{dP}{dt} \tag{25}$$

Various kinetic expressions for both r_I and r_S were tested in different combinations with each other, as shown in Table 5.

The linear/linear model was used as the basic starting point for a compartmental model. The Michaelis-Menten approach was the next progression from the linear model. Both an insoluble substrate (I) and a soluble substrate (S) were involved in this model. The estimation of four constants k_I, k_S, K_{mI}, and K_{mS} resulted.

Product inhibition was evident when soybean protein and lean meat protein were enzymatically hydrolyzed, as mentioned earlier (Enzymatic

Figure 5. Illustration of different compartments used in the kinetic modelling of the hydrolysis

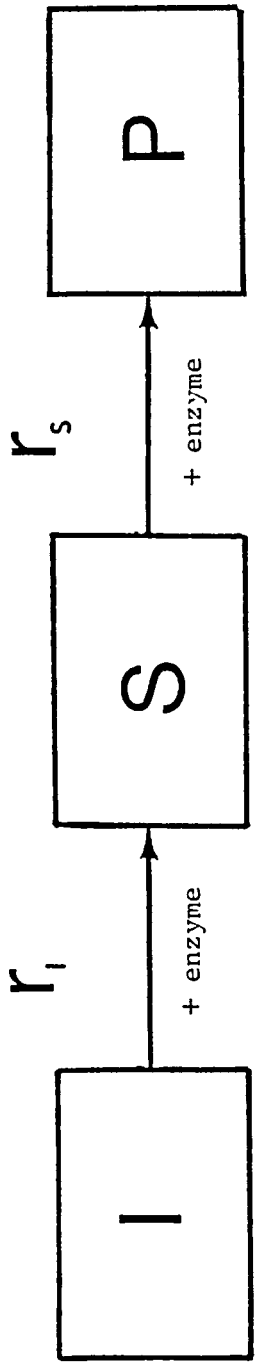

P
S
I
r_s
r_i
+ enzyme
+ enzyme

Table 5. Kinetic expressions used to model the rates of r_I and r_S. $\leftrightarrow$ denotes the combination of r_I and r_S was studied

Model	Insoluble substrate r_I		Soluble substrate r_S
Linear/linear	$k_I e_0(I - IE)$	$\leftrightarrow$	$k_S e_0 S$
Michaelis-Menten/ Michaelis-Menten	$\frac{k_I e_0(I - IE)}{1 + (I/K_{mI}) + (S/K_{mS})}$	$\leftrightarrow$	$\frac{k_S e_0 S}{1 + (I/K_{mI}) + (S/K_{mS})}$
Insoluble substrate-Freundlich adsorption/ linear	$k_I (e_0(I - IE))^{2/3}$	$\leftrightarrow$	$k_s e_0 S$
Simplified product inhibition/product inhibition	$\frac{k_I e_0(I - IE)}{1 + (P/K_{mP})}$	$\leftrightarrow$	$\frac{k_S e_0 S}{1 + (P/K_{mP})}$

Hydrolysis section of Literature Review). It was therefore studied in a model for the hydrolysis of corn gluten meal.

The idea of limited surface sites for enzyme binding (with an excess of enzyme in solution) was used to describe the corn gluten hydrolysis. The adsorption of the enzyme was described by the Freundlich adsorption isotherm, followed by subsequent hydrolysis. The assumption was made that the relative amount of enzyme bound to the substrate was small compared with the free enzyme in solution (McLaren, 1963).

The constants in each compartmental model were evaluated by finding the values giving the least squares fit to the experimental data of I, (S + P), and P versus time. Minimization was done using the non-linear iterative technique of Levenburg-Marquardt (I.M.S.L., 1987). The rate equations were integrated using the Gear method of numerical integration (I.M.S.L., 1987). Calculated values of soluble and insoluble protein were compared with the experimental values by way of the parameters estimated from each compartmental model.

The possibility of two simultaneous reactions was also investigated, where one reaction would be a fast first-order reaction and the second a slow first order reaction. The kinetic expressions for two simultaneous reactions would be the linear expressions for r_I and r_S shown in Table 5. A plot of log(rate) versus time was made as discussed earlier (Kinetic Models section of Literature Review) (after Mihalyi and Godfrey, 1962). The rate of hydrolysis (dh/dt) was obtained from the pH stat values of base consumption with time. The number of peptide bonds cleaved (h) was obtained using equation (5). These values were first smoothed, and then

the rate (dh/dt) was calculated using Lagrange's forward, central, and backward difference formulas for equally spaced points (Perry and Chilton, 1973). If two simultaneous reactions do occur, one fast and one slow, the plot should resemble Figure 2.

RESULTS AND DISCUSSION

Evaluation of Experimental Conditions (Screening Experiments)

Comparison of enzymes

The degree of hydrolysis and the concentration of soluble protein obtained, for each enzyme studied, is shown in Table 6. Trypsin produced the highest yields using the same concentration (0.08 g/L) for each enzyme. For a higher concentration of Alcalase 2.4L (0.19 g/L), the amount of soluble protein was greater than for trypsin, with a lower degree of hydrolysis.

The main objectives of the hydrolysis were to obtain as high a concentration of soluble protein as possible. At the same time, too high a degree of hydrolysis should be avoided, due to the formation of bitter flavors (Olsen and Adler-Nissen, 1979a). The ratio of the protein concentration to the degree of hydrolysis should therefore be as large as possible. These ratios, shown in Table 6, indicate that Alcalase 2.4L would be more suited towards the objectives of the hydrolysis than would trypsin.

Enzymes normally are compared on the basis of their activity units. However, of all the enzymes used, only Alcalase 2.4L and Neutrase 0.5L were defined in the same activity units. Table 6 shows the protein concentration and the degree of hydrolysis for corresponding activities of Alcalase 2.4L (0.02 g/L) and Neutrase 0.5L (0.08 g/L). Alcalase 2.4L gave slightly better yields than Neutrase 0.5L. At higher enzyme concentrations, according to the literature (Godfrey and Reichelt, 1983), even higher yields were obtained with Alcalase 2.4L compared to

Table 6. Comparison of the degree of hydrolysis and the soluble protein concentration obtained by the proteases P4032 protease, trypsin (T8128), Alcalase 2.4L, and Neutrase 0.5L

Protease	Enzyme concentration [g/L]	Degree of hydrolysis (%)	Soluble protein concentration [mg/mL]	Ratio $\frac{\text{protein concentration}}{\text{degree of hydrolysis}}$
P4032 Protease	0.08	4.19	6.97	1.66
Trypsin (T8128)	0.08	10.5	18.10	1.72
Alcalase 2.4L	0.08[a]	4.4	8.62	1.96
	0.08	5.46	11.94	2.19
	0.19	8.10	21.68	2.68
Neutrase 0.5L	0.08[a]	3.04	7.43	2.44

[a]Concentrations have comparable activities. Alcalase 2.4L has an activity of 2.4 Anson units/g and Neutrase 0.5L, 0.5 Anson units/g.

Neutrase 0.5L. On this basis, Alcalase 2.4L was chosen to study other experimental parameters affecting the hydrolysis.

Effect of enzyme dosage

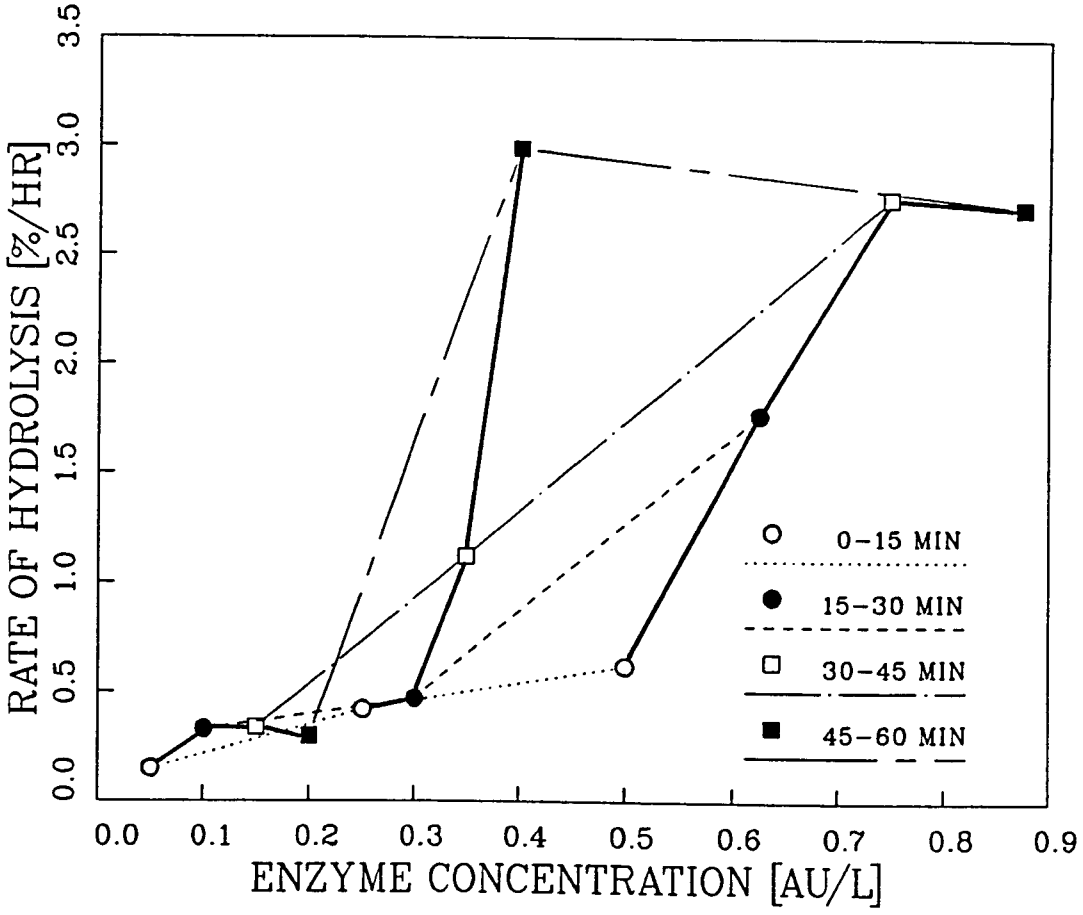
The solid lines in Figure 6 show the effect of enzyme dosage on the rate of hydrolysis. The rate of hydrolysis not only increased with enzyme dosage, but also with time. Corresponding time points in Figure 6 are joined by dotted lines. The rates of hydrolysis were corrected by subtracting the control values (no enzyme) for the same time period. In general, the rate of hydrolysis was greater at enzyme concentrations of 0.25 Anson units per liter (AU/L) and above. At lower enzyme concentrations (0.05 AU/L - 0.2 AU/L), the rate of hydrolysis was slow and showed no detectable increase with increasing enzyme concentration.

At the higher concentrations (0.5 AU/L - 0.875 AU/L), the rate of hydrolysis still increased after the addition of each aliquot of enzyme. The fact the rate still increased with enzyme dosage illustrated that the reaction wasn't substrate limited.

At the low enzyme concentrations (0.05 AU/L - 0.2 AU/L), the rate of hydrolysis (corrected for the control) was 0.15%/hr at 0.05 AU/L (0 to 15 minutes) and increased to 0.3%/hr at 0.2 AU/L (45 to 60 minutes). The enzyme at these low concentrations does not cause a significant increase in the rate of hydrolysis compared with the control (no enzyme). The control resulted in an "apparent" rate of hydrolysis which was 0.63%/hr (0 to 15 minutes) falling to 0.25%/hr (45 to 60 minutes).

The main objective of the screening runs was to verify that the enzyme concentrations recommended in the literature were reasonable for

Figure 6. Effect of enzyme concentration in Anson units per liter (AU/L) on the rate of hydrolysis in %/hr. —— connects points from the same experiment over time. Broken lines correct for possible increased accessibility by connecting data points obtained at the same time of hydrolysis in each experiment. Rates are corrected using control rates (no enzyme) over the same time period


RATE OF HYDROLYSIS [%/HR]
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
ENZYME CONCENTRATION [AU/L]
0-15 MIN
15-30 MIN
30-45 MIN
45-60 MIN

the hydrolysis. At the highest enzyme concentrations (0.5 AU/L - 0.875 AU/L), the hydrolysis should be controlled to avoid bitter flavors (Adler-Nissen, 1985). At the lower concentrations (0.05 AU/L - 0.2 AU/L), it is likely that the functional properties of the hydrolysate weren't sufficiently modified. A typical range of enzyme concentrations used in the literature was 0.12 AU/L to 0.48 AU/L Alcalase 0.6L, in overall agreement with the experimental results.

Effect of pH

Table 7 shows the average rate of hydrolysis for consecutive changes in pH during the same experiment. The rate of hydrolysis increased by three times when the pH was subsequently increased from pH 9 to pH 10. However, later results (see Figure 9) showed that the rate at this enzyme concentration (0.375 AU/L) would also have doubled over a similar time period if the pH had been maintained at pH 9. On this basis, the increase in the average rate of hydrolysis which can be attributed to the change in the pH from 9 to 10 was 0.81%/hr.

Table 8 shows the degree of hydrolysis and the soluble protein concentration at pH 9 and 10 after two hours of hydrolysis. The ratio of protein concentration to the degree of hydrolysis was consistently larger at pH 9. As mentioned earlier, provided the yield of soluble protein is acceptable, this ratio should be as large as possible. Another advantage of working at pH 9 is that the enzyme is stable (Table 7), while at pH 10, measurable inactivation of the enzyme occurs. In view of the experimental results and the values recommended in the literature, pH 9 was taken to be the "standard" pH for further hydrolyses.

Table 7. Experimental results showing the effect of pH on the average rate of hydrolysis in %/hr. Average rates of hydrolysis were calculated after consecutive changes in pH during one experiment. Literature values of how the % relative activity of the enzyme varies with pH

pH	Average rate of hydrolysis[a] (%/hr)	% relative activity[b]
9	0.96[c]	100
10	2.73[d]	95
11	1.63[e]	85

[a]Enzyme concentration was 0.375 AU/L.

[b]Activity measured after one hour's hydrolysis (Novo, 1984a).

[c]Rate measured over a linear period between 65 minutes and 80 minutes.

[d]Rate measured over a linear period between 90 minutes and 120 minutes.

[e]Rate measured over a linear period between 130 and 160 minutes.

Table 8. Effect of pH on the rate of hydrolysis and the soluble protein concentration at three levels of Alcalase dosage (0.375 Anson/L, 0.5 Anson/L, and 0.75 Anson/L). Hydrolysis time: 2 hours at 50°C

Enzyme concentration (Au/L)	pH	Degree of hydrolysis (%)	Soluble protein concentration (mg/mL)	Ratio $\frac{\text{protein concentration}}{\text{degree of hydrolysis}}$
0.375	9	2.39	9.96	4.17
0.375	10	2.50	9.47	3.79
0.50	9	3.23	13.06	4.04
0.50	10	3.52	11.87	3.37
0.625	9	4.57	17.88	3.91
0.625	10	4.89	18.17	3.71

Effect of temperature

The effect of temperature on the rate of hydrolysis is shown in Table 9. There was a gradual increase in the rate of hydrolysis with increasing temperature, but the effect of time must also be taken into consideration. The rate of hydrolysis at 50°C and 60°C was measured over a 30 minute period. Later results (see Figure 9) showed that the rate at this enzyme concentration increased if the temperature remained at 50°C. The effect of temperature on the rate of hydrolysis was therefore assumed to be negligible.

Table 9 also shows literature values for the percentage residual and percentage relative activities of Alcalase at different temperatures (Novo, 1984a). A temperature of 50°C was used in all further hydrolyses.

Hydrolysis Effects in Combination with Size Reduction (Design Experiments)

The rate of hydrolysis and the percentage of soluble protein increased with increasing enzyme concentration, reflected in Figures 7 and 8, respectively. For all enzyme concentrations, the rate of hydrolysis (shown in Figure 9) accelerated, followed by a decline during the later period of the hydrolysis. At the lowest enzyme concentration (0.375 AU/L), the rate of hydrolysis proceeded more slowly than the higher enzyme concentrations. The initial acceleration therefore occurred later in the hydrolysis, and consequently so did the decline in the rate. Initially the most accessible bonds would be cleaved, and as a result of these cleavages further accessible bonds would be exposed. It is this process that caused the acceleration in the rate of hydrolysis. The

Table 9. Experimental results showing the effect of temperature in degrees Celsius on the average rate of hydrolysis (%/hr). Average rates of hydrolysis were calculated after consecutive changes in pH during one experiment. Literature values of how the % residual and relative activity of the enzyme vary with temperature

Temperature (°C)	Average rate of hydrolysis[a] (%/hr)	% Residual activity[b]	% Relative activity[b]
40	0.83[c]	100	50
50	1.05[d]	100	78
60	1.24[e]	~70	100

[a]Enzyme concentration was 0.375 AU/L.

[b]Activity measured over one hour's hydrolysis (Novo, 1984a).

[c]Rate measured over a linear period between 0 and 15 minutes.

[d]Rate measured over a linear period between 45 and 75 minutes.

[e]Rate measured over a linear period between 105 and 135 minutes.

Figure 7. Variation with enzyme concentration (AU/L) of % degree of hydrolysis after 2 and 4 hours of hydrolysis. Error bars represent high and low values of two observations. Reaction conditions: pH 9, 50°C

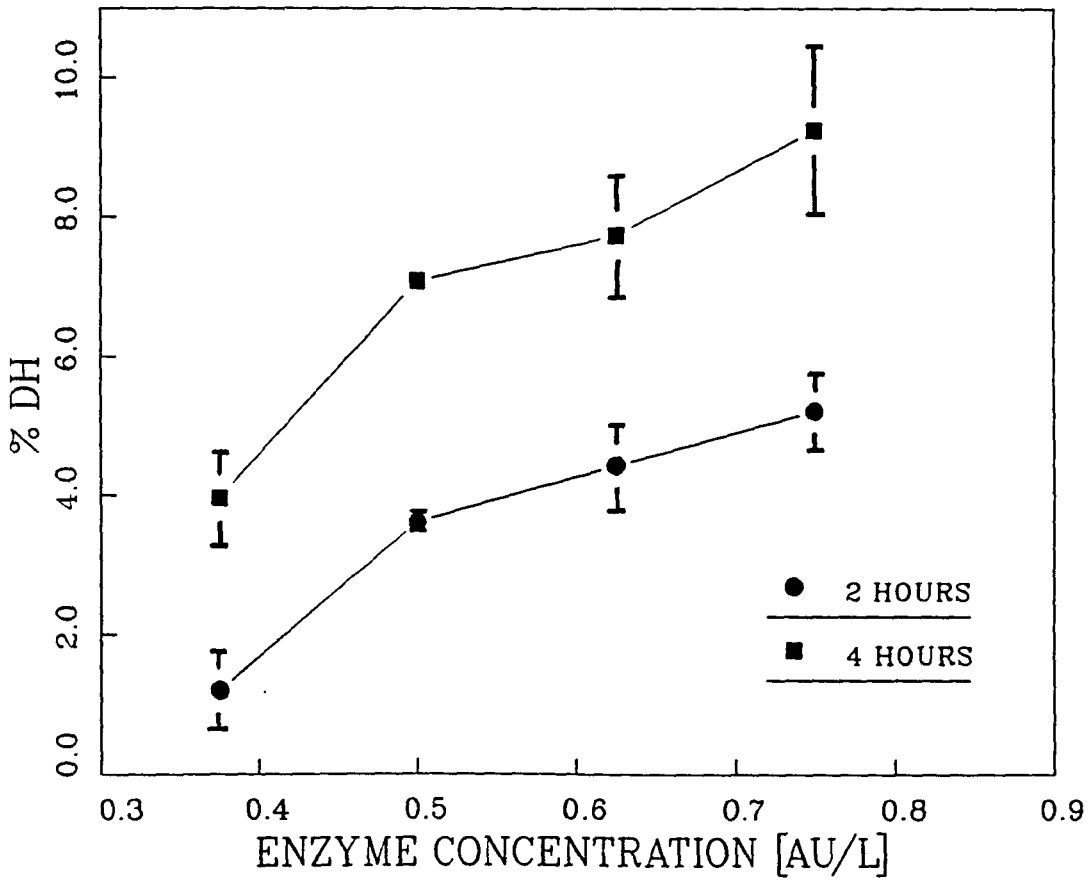

% DH
0.0
2.0
4.0
6.0
8.0
10.0
0.3
0.4
0.5
0.6
0.7
0.8
0.9
ENZYME CONCENTRATION [AU/L]
2 HOURS
4 HOURS

Figure 8. Variation with enzyme concentration (AU/L) of soluble protein (mg protein/ml) after 2 and 4 hours of hydrolysis. Error bars represent high and low values of two observations. Reaction conditions: pH 9, 50°C

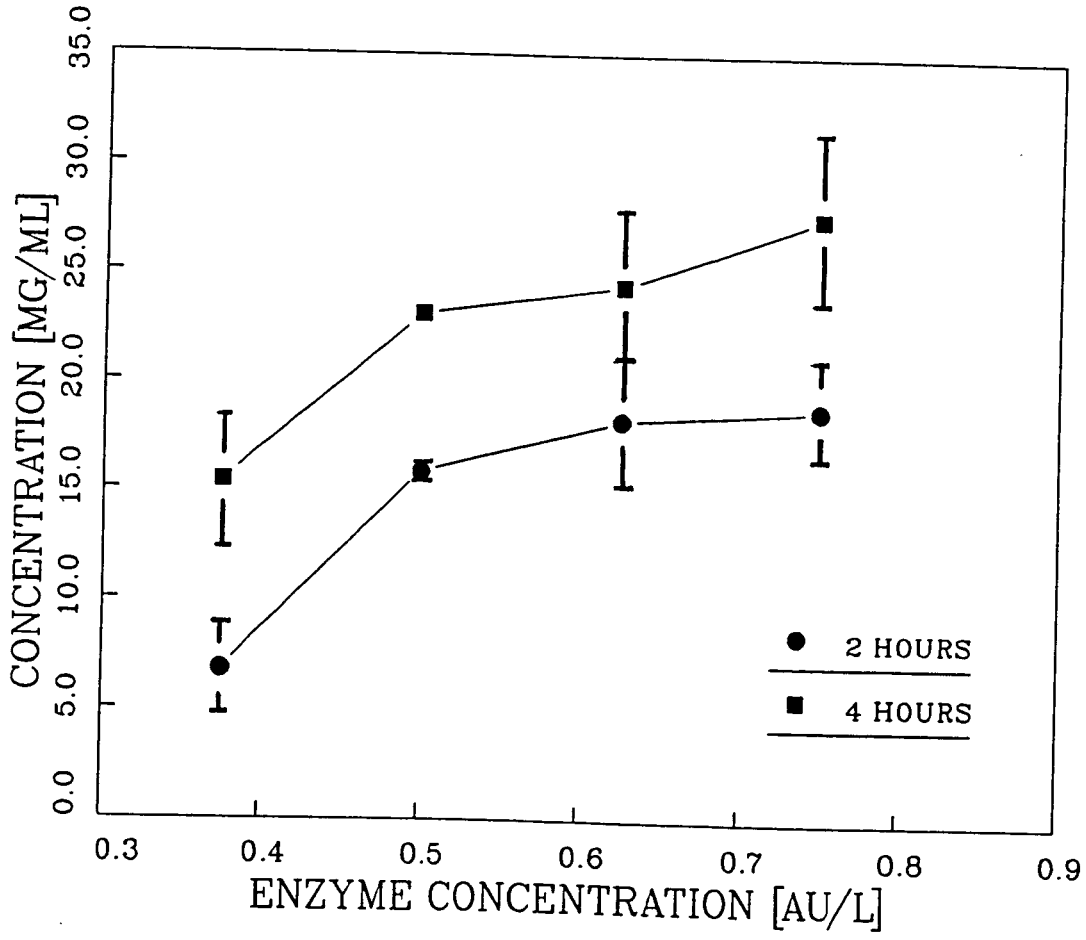
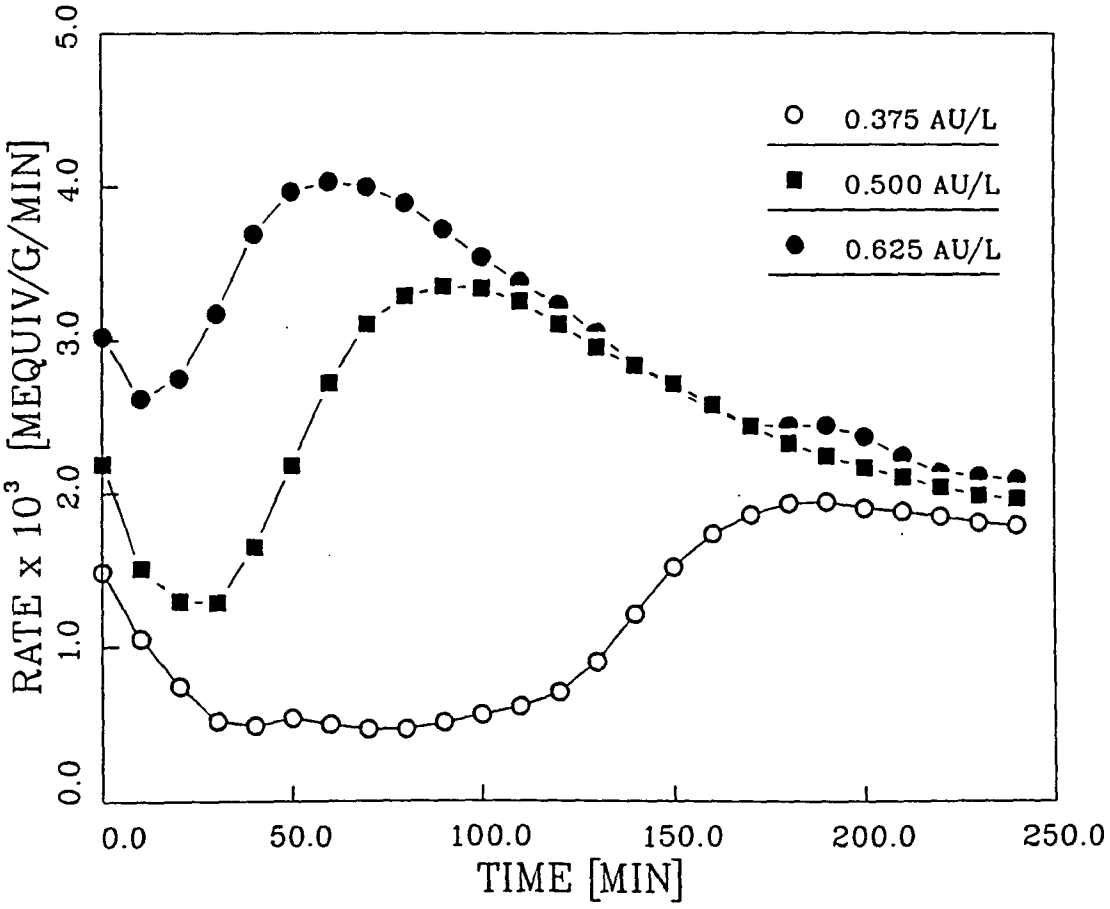

CONCENTRATION [MG/ML]
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
0.3
0.4
0.5
0.6
0.7
0.8
0.9
ENZYME CONCENTRATION [AU/L]
2 HOURS
4 HOURS

Figure 9. Variation in the rate of hydrolysis (mequiv/g/min) with time (min) at three levels of enzyme concentration (AU/L). Smoothed values of h were used to calculate the rate - dh/dt. Reaction conditions: pH 9, 50°C


0.375 AU/L
0.500 AU/L
0.625 AU/L
RATE x 10^3 [MEQUIV/G/MIN]
TIME [MIN]
0.0
1.0
2.0
3.0
4.0
5.0
0.0
50.0
100.0
150.0
200.0
250.0

lower the concentration of enzyme, the longer it takes to reach the same extent of cleavage at higher enzyme concentrations.

The eventual decline could be attributed to either product inhibition, a reduction in the soluble pool, or a reduction in susceptible bonds. Further experimentation would be required to ascertain which of the above is actually occurring.

The size of unground corn gluten meal was found to be mostly (74.56%) between 355 μm (44 mesh) and 180 μm (85 mesh). Pulverizing the corn gluten reduced 84.59% of the sample to a size between 75 μm (200 mesh) and 38 μm (400 mesh). There was an average error of ±0.6% associated with the degree of hydrolysis in Figure 7. The difference in the degree of hydrolysis between non-reduced and size reduced corn (Figure 10) was not significant, according to a t test at the 90% level. Similar conclusions can be made using the error introduced in the values of soluble protein concentration (Figure 8). The differences in the soluble protein between non-reduced and size reduced corn (Figure 11) were not significant at the 90% level (t test). On the basis of the experimental error, size reduction appeared to have no effect on the rate of hydrolysis--the number of available surface sites did not limit the rate of reaction.

If the corn gluten meal has a very high internal surface area, the effect of size reduction would be negligible. B.E.T. analysis was used to find the total surface area of the samples. The theoretical surface area was calculated by assuming that the corn gluten meal consists of spheres (no internal surface area) in the size range measured. The actual measured surface area compared to these theoretical values

Figure 10. Difference between the degree of hydrolysis for non-reduced (NR) and size reduced (SR) corn gluten meal (NR-SR) versus enzyme concentration (AU/L). 90% confidence interval is shown. Reaction conditions: pH 9, 50°C

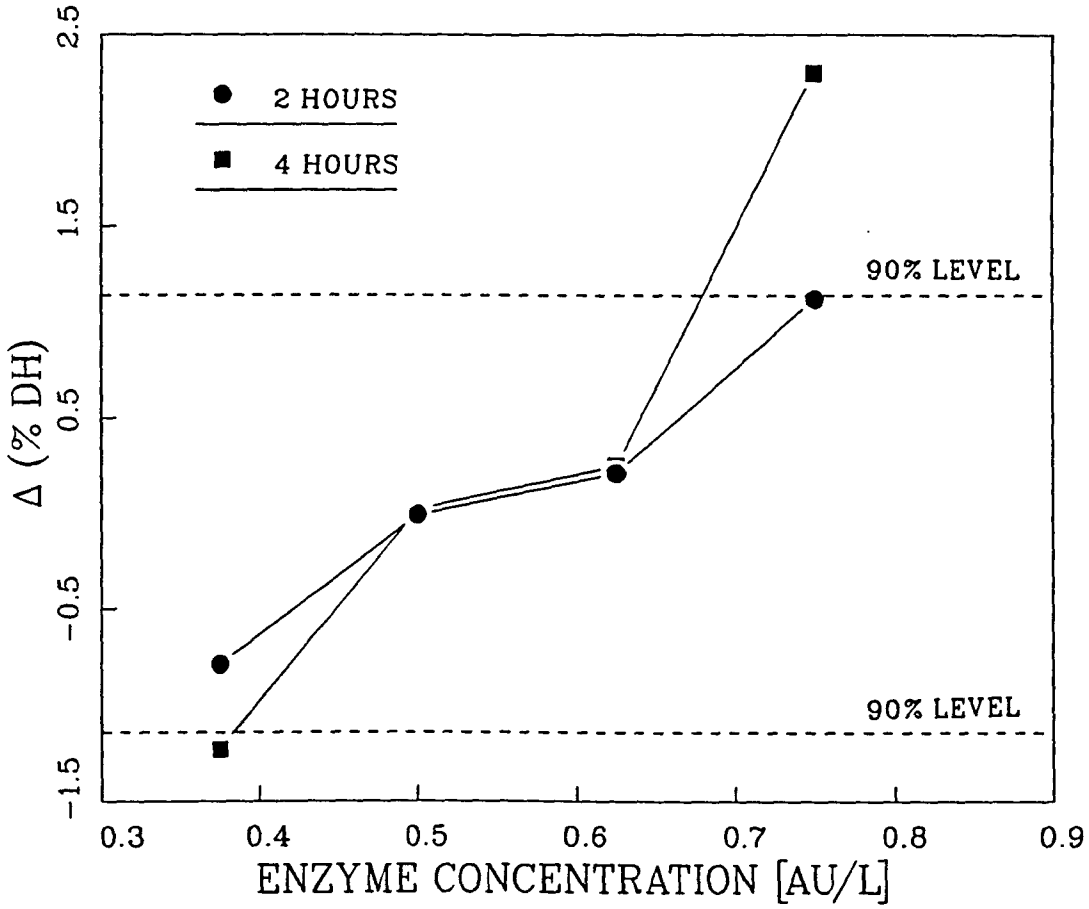
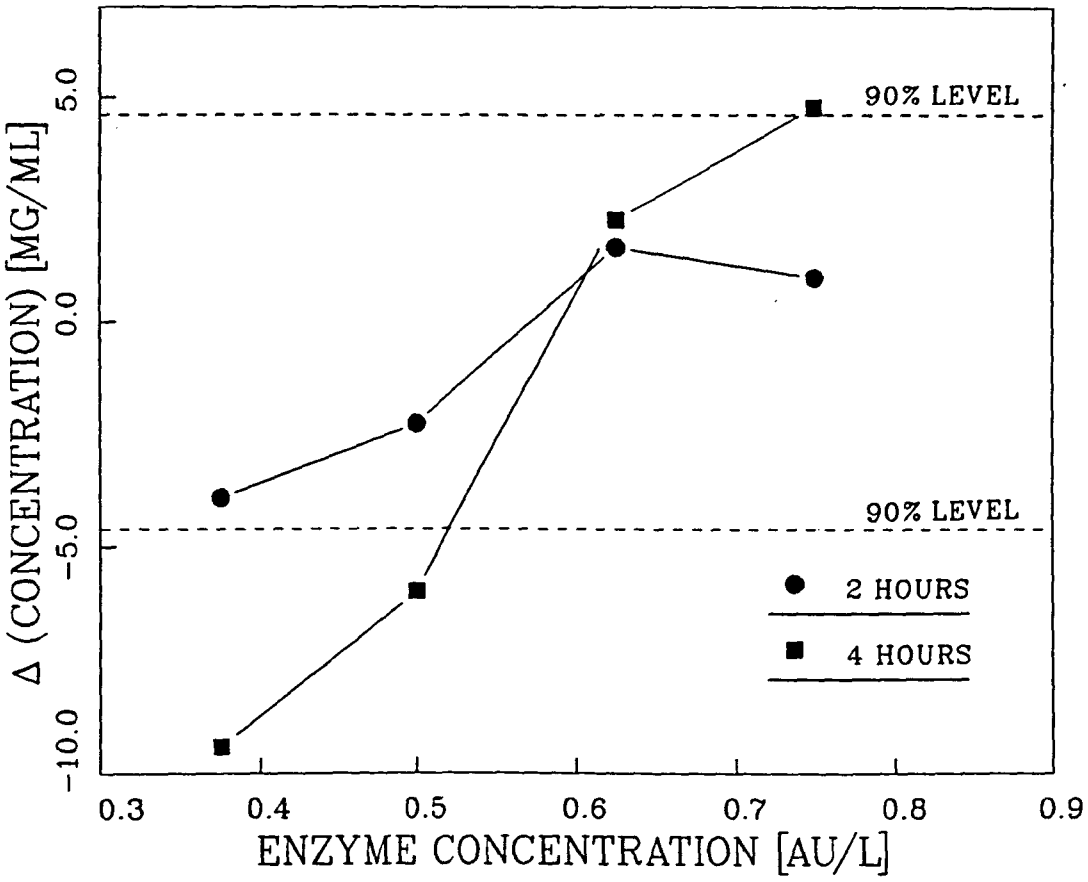

2 HOURS
4 HOURS
90% LEVEL
90% LEVEL
Δ (% DH)
2.5
1.5
0.5
-0.5
-1.5
0.3
0.4
0.5
0.6
0.7
0.8
0.9
ENZYME CONCENTRATION [AU/L]

Figure 11. Difference between the amount of soluble protein for non-reduced (NR) and size reduced (SR) corn gluten meal (NR-SR) versus enzyme concentration (AU/L). 90% confidence interval is shown. Reaction conditions: pH 9, 50°C


90% LEVEL
90% LEVEL
2 HOURS
4 HOURS
Δ (CONCENTRATION) [MG/ML]
5.0
0.0
-5.0
-10.0
0.3
0.4
0.5
0.6
0.7
0.8
0.9
ENZYME CONCENTRATION [AU/L]

indicated that the corn gluten meal has very little internal surface area (shown in Table 10). The internal surface area for unground and ground corn gluten was calculated as the difference between the actual and theoretical surface areas in Table 10. The internal surface area for the ground sample was found to be 0.078 m^2/g greater than the unground sample.

The measurement of total surface area depended on the diffusivity of krypton gas into the pores of the corn gluten. The rate of hydrolysis depends on the diffusion of the enzyme to the surface of the corn gluten. Since Alcalase has a large molecular weight (approximately 27300), the internal surface area available to the enzyme will likely be less than that measured.

A substantial amount of heat was generated during size reduction. The possibility of high temperatures modifying the physical properties of the corn gluten should not be ruled out.

The viscosity of the corn gluten slurry during the course of the hydrolysis did not increase to the extent reported in the literature (Adler-Nissen, 1978a). The viscosity of the hydrolysates was found to be around 13 cP. The original viscosity of the slurry could not be measured easily because the corn gluten meal quickly settled out. The viscosity of the slurry using Einstein's equation (Rosen, 1981) was 1.9 cP. Adler-Nissen (1978a) reported hydrolysate viscosities using corn gluten meal of 50-150 cP. These results were obtained, however, using a different source of corn gluten meal; they were also observed using corn gluten from

Table 10. Actual surface area of ground and unground corn gluten meal (CGM) compared to the theoretical surface area based on perfect spheres

	Theoretical surface area (m^2/g)	Actual surface area (m^2/g)	Ratio $\frac{\text{actual S.A.}}{\text{theoretical S.A.}}$
unground CGM (355-180 μm)	0.063	0.1069	1.70
ground CGM (75-38 μm)	0.31	0.432	1.39

only one source. It is likely that different observations will be made with corn gluten meal from different sources.

Time Course of Hydrolysis

Initially only insoluble protein was present so most of the initial hydrolysis would be in the insoluble phase, producing soluble proteins and high molecular weight peptides (S). At this point, the rate of hydrolysis of I (r_I) is greater than that of S (r_S) which results in an overall increase in S. The concentrations of pools S and P for the course of the hydrolysis are shown in Figure 12 for low (0.375 AU/L) and high (0.625 AU/L) enzyme concentrations. The increased enzyme concentration results in a decrease in I or an increase in the total soluble protein concentration (S + P) of between 10 and 20%. Throughout most of the decrease shows up in pool P. This implies that the additional enzyme preferentially hydrolyses soluble protein.

After four hours' hydrolysis, the enzyme concentration was doubled by adding a second aliquot of enzyme, but the reaction rate did not double. Alcalase 2.4L is stable at the reaction conditions used (Novo Technical Service Representative, 1987) and the insoluble substrate concentration after four hours' hydrolysis was at least 63 mg protein/mL. The observed rates (Table 11) must have been lower than expected due to either product inhibition or depletion of the susceptible bonds. The lower the enzyme concentration, the lower the extent of hydrolysis at four hours (Figure 9). Product concentration and reduction in susceptible bonds are less important at low enzyme concentrations and consequently rate does increase with addition of more enzyme.

Figure 12. Experimental values of protein concentration (mg protein/ml) with time (min). ■ denotes low enzyme concentration (0.375 AU/L) and ● denotes high enzyme concentration (0.625 AU/L)

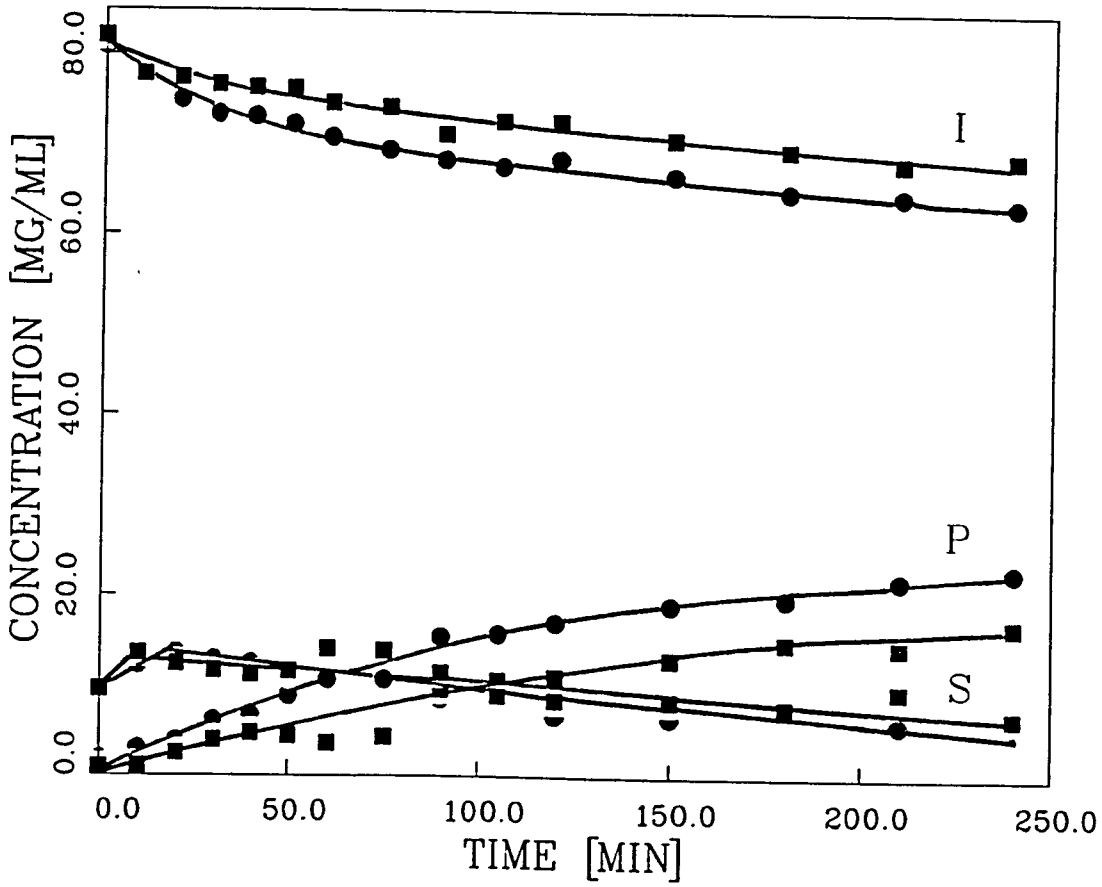

CONCENTRATION [MG/ML]
80.0
60.0
40.0
20.0
0.0
I
P
S
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

Table 11. Comparison of the initial rate of hydrolysis (%/hr)[a] with the rate of hydrolysis after four hours with the addition of a second equal aliquot of enzyme

Enzyme concentration (AU/L)	Initial rate of hydrolysis (first aliquot) (%/hr)	Rate of hydrolysis after addition of second equal aliquot (%/hr)	Ratio $\frac{\text{rate 2nd aliquot}}{\text{rate 1st aliquot}}$
0.375	0.74	2.00	2.70
0.50	1.45	1.79	1.23
0.625	2.57	2.50	0.97
0.750	2.54	2.48	0.98

[a]Rates of hydrolysis are average rates measured over a 60 minute period.

Experimental results (Figure 12) show that towards the end of the hydrolysis, the rate r_I was approaching zero. There was, however, a great deal of insoluble substrate remaining (at least 63 mg protein/mL), which suggests that there is a limit to the extent of hydrolysis and that the remainder tends to be a hydrolysis resistant core. An estimation of this solubility limit was made for corn gluten meal by performing an extended run over a period of forty-eight hours. This resulted in 53.2% (49.16 mg/mL) soluble protein. This value was represented by IE in all the kinetic models studied. Similar conclusions concerning an insoluble "core" have been made for other enzymatic hydrolyses, such as the tryptic digestion of myosin (Mihalyi and Harrington, 1959).

Kinetic Models

Linear/linear model

The expressions used to describe the linear model were shown in Table 5. The data values for pools (S + P) and P were calculated directly from the biuret analyses (Doumas, 1975). These values were accurate to ±0.36 mg protein/mL supernatant. The experimental values for the S pool were dependent on the values from two biuret analyses, before and after TCA precipitation (Adler-Nissen, 1985). The experimental error for the S values was therefore accurate to ±0.51 mg protein/mL supernatant. The experimental values of I were calculated as the difference between the amount of protein charged to the reactor and the experimental values of (S + P). The residual sum of squares was minimized using the experimental values of (S + P) and P.

Figures 13 and 14 show the values obtained for the linear model (solid lines) compared to the experimental values (symbols) for low (0.375 AU/L) and high (0.625 AU/L) enzyme concentrations. Samples were taken more frequently during the first two hours of the hydrolysis because the rate of hydrolysis was higher, and changes relevant to the hydrolysis mechanism occurred mostly during this time. Consequently, the frequency of sampling introduced a bias towards the first half of the hydrolysis during the minimization.

Figures 13 and 14 show that the linear model was able to predict the main trends of the experimental data. The values of the P pool fitted well for the first half of the hydrolysis. The model tended to over-predict the values of P towards the end of the hydrolysis. This deviation from the experimental values was also more pronounced for the higher enzyme concentrations. The linear model lacked the ability to distinguish if there exists bonds of different susceptibility or whether product inhibition was taking place, both of which would reduce the rate at the later stages of the hydrolysis. Pool S increased initially, then gradually decreased. The model fitted this poorly during the first fifty minutes of the hydrolysis. It did, however, predict the later times well. Increased weighting of the first six residuals leads to a closer fit for the first fifty minutes of the hydrolysis (Figure 15), but requires weighting factors of 5-10. However, the fit to the later experimental points became poor as a result of the improved fit at the early times.

Values for the constants k_I and k_S were estimated for each set of experimental data. The average values of k_I and k_S were found to be

Figure 13. Comparison between experimental data and the linear/linear model. Experimental data are denoted by symbols: ■ insoluble, I, ● soluble proteins/peptides, S, and O soluble peptides, P. Linear model denoted by lines. Reaction conditions: enzyme concentration 0.375 AU/L, pH 9, and 50°C

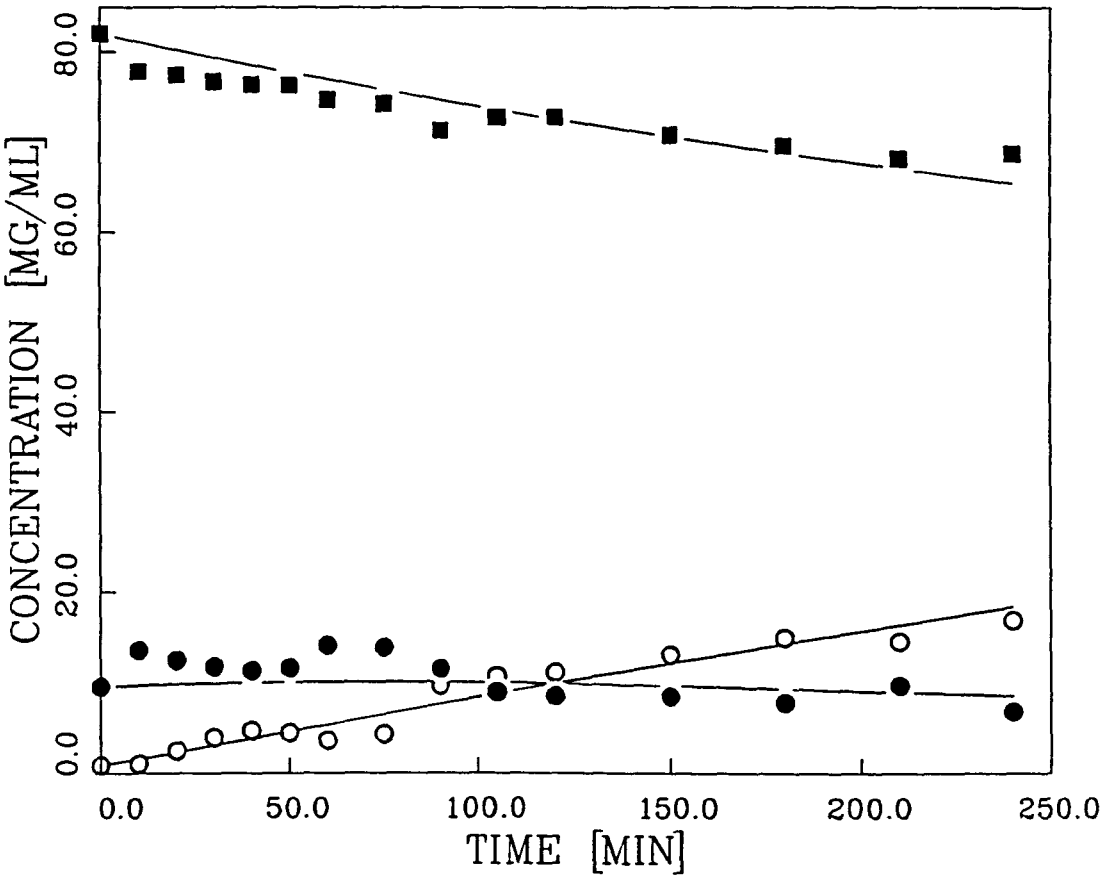

CONCENTRATION [MG/ML]
80.0
60.0
40.0
20.0
0.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

Figure 14. Comparison between experimental data and the linear/linear model. Experimental data are denoted by symbols: ■ insoluble, I, ● soluble proteins/peptides, S, and O soluble peptides, P. Linear model denoted by lines. Reaction conditions: enzyme concentration 0.625 AU/L, pH 9, and 50°C

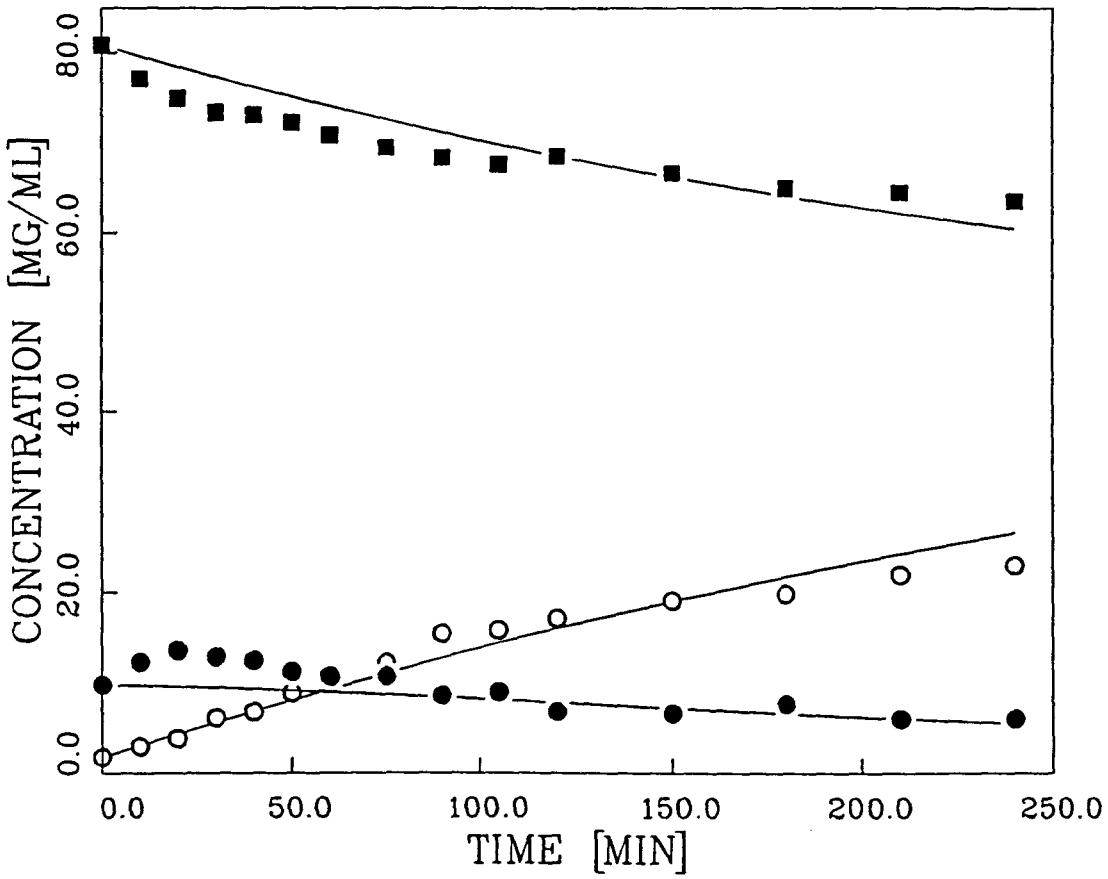
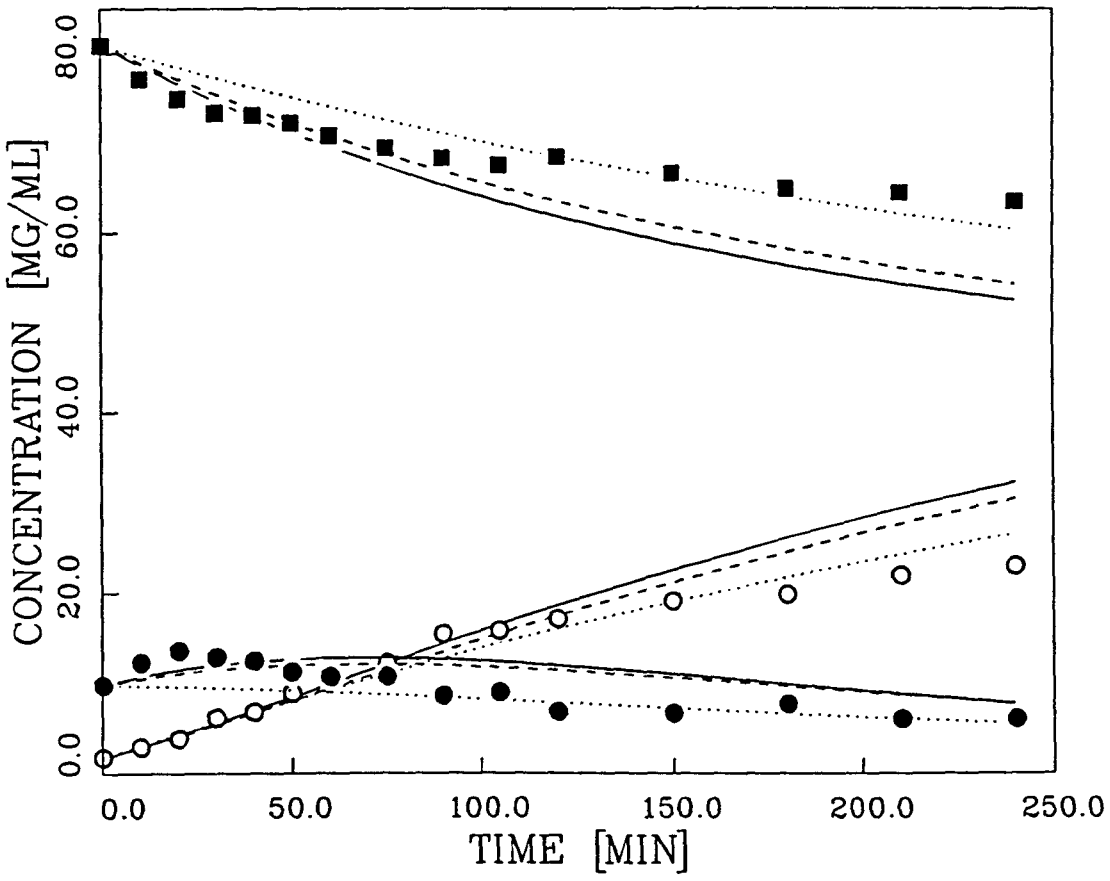

CONCENTRATION [MG/ML]
0.0
20.0
40.0
60.0
80.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

Figure 15. Comparison of the weighted linear model with experimental data. Weighting is applied over the first fifty minutes of hydrolysis for I(■), S(●), and P(○). ··· denotes weighting factor is 1, --- weighting factor is 5, and —— weighting factor is 10. Reaction conditions: enzyme concentration 0.625 AU/L, pH 9, and 50°C


CONCENTRATION [MG/ML]
0.0
20.0
40.0
60.0
80.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

1.29×10^{-2} ($\pm 0.18 \times 10^{-2}$) L/g min and 4.60×10^{-2} ($\pm 0.79 \times 10^{-2}$) L/g min, respectively. Figure 16 shows the normalized values $k_i - \bar{k}_i)/\sigma_i$ of the constants k_I and k_S, together with the 60%, 80%, and 95% confidence limits for a small population (t distribution). The scatter of the normalized constants (Figure 16) between the different confidence levels is typical of experimental data following a normal distribution. There is, therefore, no reason to disprove the hypothesis that the values are constant.

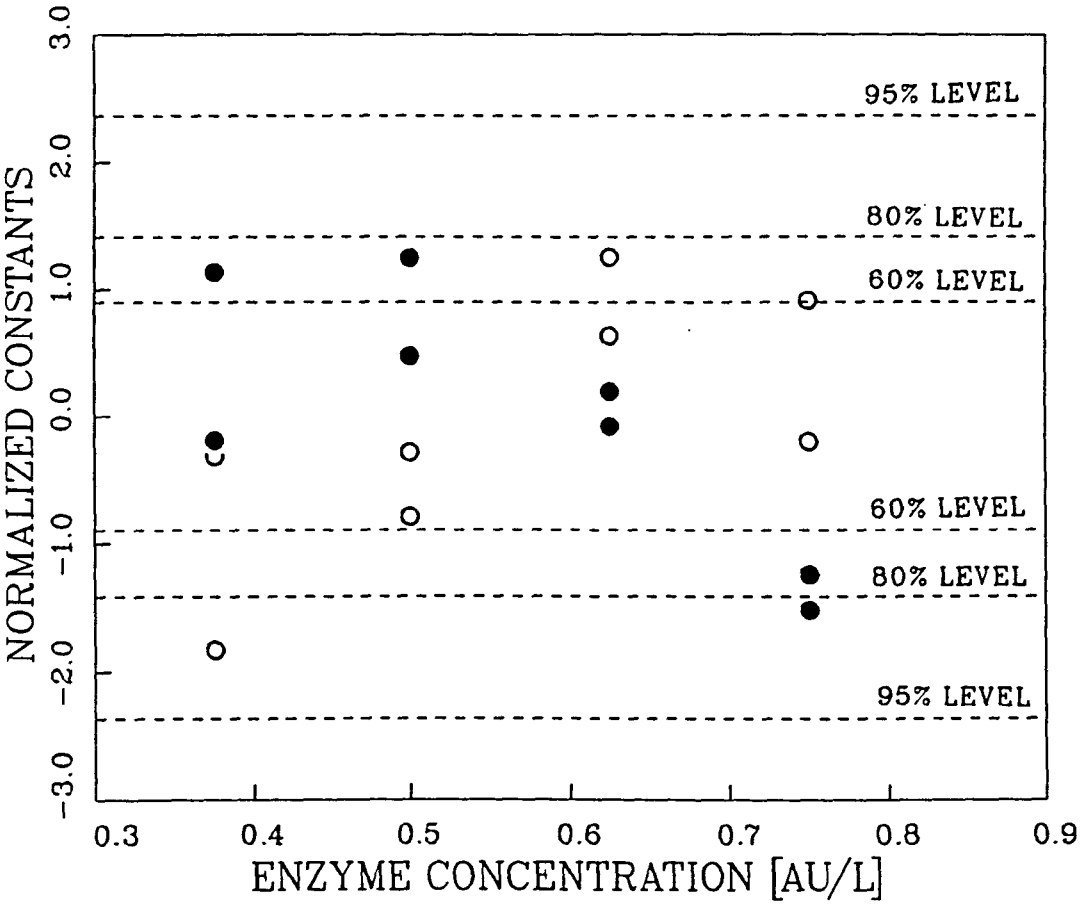
Michaelis-Menten/Michaelis-Menten model

The Michaelis-Menten model (shown in Table 5) didn't result in any improvement over the linear model. The minimization resulted in the same values for the constants k_S and k_I as were found for the linear model. The constants K_{mI} and K_{mS} were found to be so large (approximately 10^{17}), that their influence in the denominator became trivial. The effective fit of the Michaelis-Menten model was the same as the linear model. Consequently, the Michaelis-Menten model posed the same weaknesses as discussed for the linear model.

Freundlich adsorption/linear model

This model accounted for available surface sites at which the enzyme could bind. Experimental work has shown that the rate of hydrolysis was not dependent on the available surface sites. The Freundlich adsorption/ linear model didn't seem to represent the mechanism any better than the linear model. There was an increase between 10% and 20% in the residual sum of squares over the linear model.

Figure 16. Normalized values of the constants k_I(●) and k_S(○) versus enzyme concentration in AU/L. Constants are normalized according to $(k_i-\bar{k}_i)/\sigma_i$. 60%, 80%, and 95% confidence levels for a t distribution are shown


95% LEVEL
80% LEVEL
60% LEVEL
60% LEVEL
80% LEVEL
95% LEVEL
NORMALIZED CONSTANTS
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
0.3
0.4
0.5
0.6
0.7
0.8
0.9
ENZYME CONCENTRATION [AU/L]

Figures 17 and 18 show the experimental values (symbols) compared to the calculated values (lines). The weaknesses in the model were essentially the same as those discussed for the linear model. The linear model is therefore a better basis from which a more complex model may be developed.

Simplified product inhibition/product inhibition model

This model was a simple progression from the linear model allowing for the effects of product inhibition. The Michaelis-Menten model (Table 5) showed a tendency to approach the linear model. Consequently, the simplified product inhibition model enables product inhibition effects to be studied without introducing too many constants. Even the addition of a third constant increased the computation time required to find a solution. In some cases, convergence problems were encountered even when good initial guesses of the parameter constants were available.

The calculated P values fit the experimental values much closer than the other models during the later stages of the hydrolysis (shown in Figures 19 and 20). The main weakness of this model were the calculated values of S during the first fifty minutes of the hydrolysis. When the residual sum of squares was weighted during the early stages of the hydrolysis, convergence could not be achieved because the problem became stiff.

With equal weighting, different solutions could be found for the constants k_I, k_S, and K_{mP} depending on the initial guesses. When the different solutions were compared to the parameter values k_I and k_S for the linear model, it was easy to decide which solutions were reasonable.

Figure 17. Comparison between experimental data and the Freundlich adsorption/linear model. Experimental data are denoted by symbols: ■ insoluble, I, ● soluble proteins/peptides, S, and O soluble peptides, P. Model denoted by lines. Reaction conditions: enzyme concentration 0.375 AU/L, pH 9, and 50°C

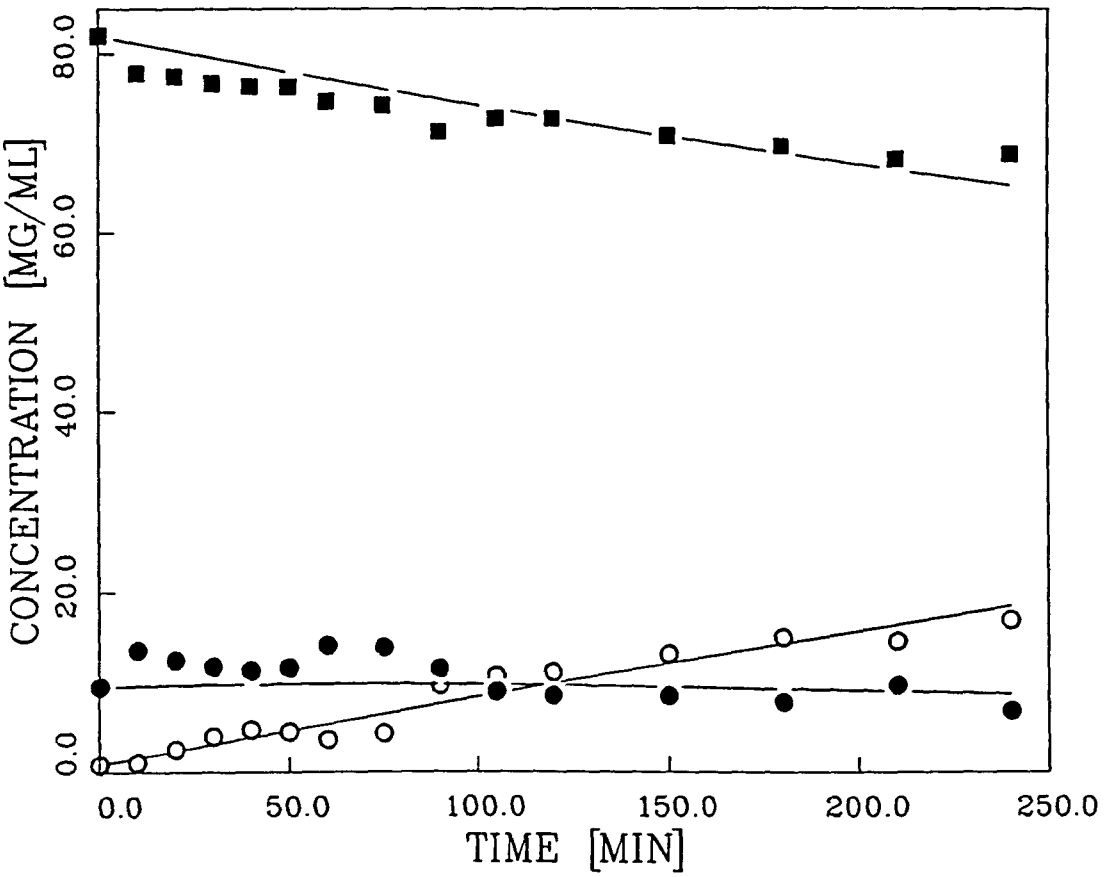

CONCENTRATION [MG/ML]
80.0
60.0
40.0
20.0
0.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

Figure 18. Comparison between experimental data and the Freundlich adsorption/linear model. Experimental data are denoted by symbols: ■ insoluble, I, ● soluble proteins/peptides, S, and O soluble peptides, P. Model denoted by lines. Reaction conditions: enzyme concentration 0.625 AU/L, pH 9, and 50°C

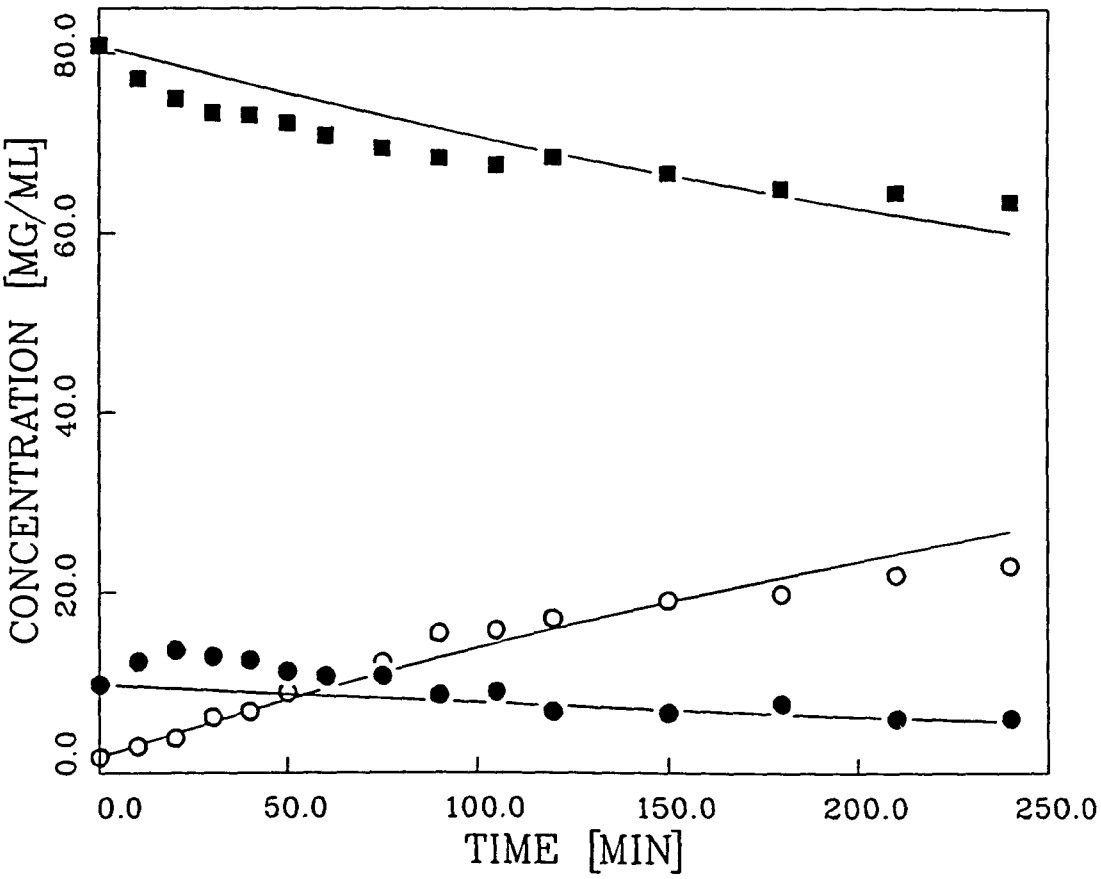

CONCENTRATION [MG/ML]
80.0
60.0
40.0
20.0
0.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

Figure 19. Comparison between experimental data and the simplified product inhibition/product inhibition model. Experimental data are denoted by ■ insoluble, I, ● soluble proteins/peptides, S, and ○ soluble peptides, P. Model denoted by lines. Reaction conditions: enzyme concentration 0.375 AU/L, pH 9, and 50°C

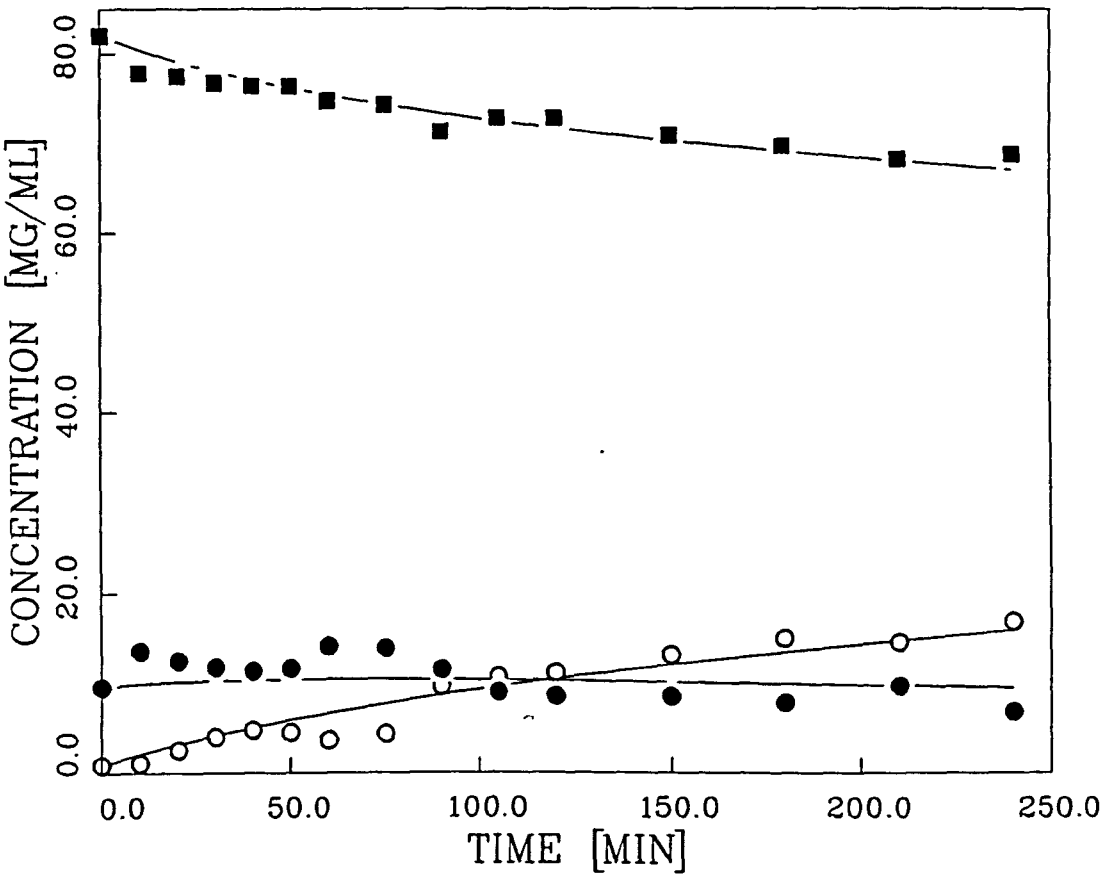
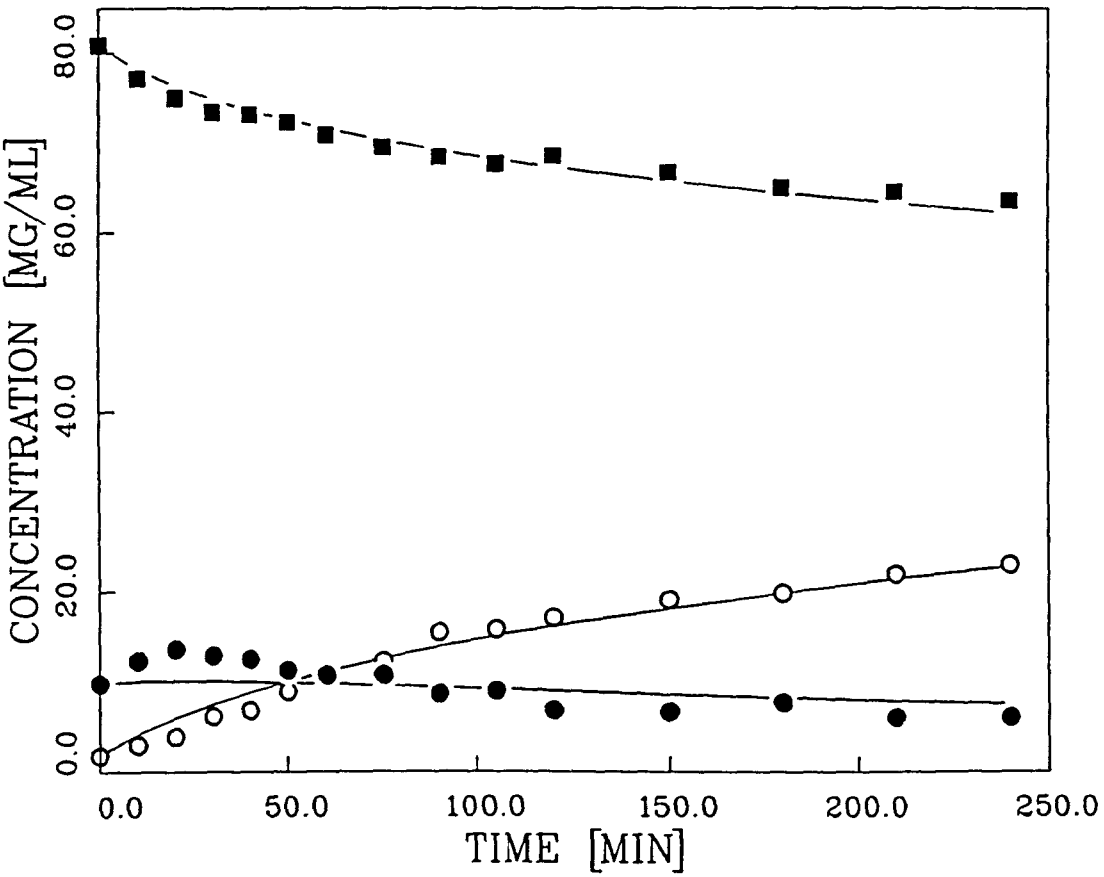

CONCENTRATION [MG/ML]
80.0
60.0
40.0
20.0
0.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

Figure 20. Comparison between experimental data and the simplified product inhibition/product inhibition model. Experimental data are denoted by ■ insoluble, I, ● soluble proteins/ peptides, S, and O soluble peptides, P. Model denoted by lines. Reaction conditions: enzyme concentration 0.625 AU/L, pH 9, and 50°C


CONCENTRATION [MG/ML]
80.0
60.0
40.0
20.0
0.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

The values of k_I and k_S for the product inhibition should increase a little due to the addition of the constant K_mP, but not by orders of magnitude. In some cases it was not possible to find reasonable values for the constants k_I, k_S, and K_{mP}, even with good initial guesses. The residual sum of squares for the "alternative solutions" was always the same or higher than the "correct" solution. The average values for k_I, k_S, and K_{mP} from five of eight data sets were found to be 5.27×10^{-2} ($\pm 4.38 \times 10^{-2}$) L/g min, 1.65×10^{-1} ($\pm 1.43 \times 10^{-1}$) L/g min, and 3.79 ($\pm$2.36) mg/mL, respectively. The constants were normalized in the same manner as the linear model, and are shown in Figure 21. They show no systematic variation, so the scatter must be due to experimental error. The normalized values are therefore within the bounds of statistical error and the hypothesis that they are constant is not contradicted.

Comparison of the linear and simplified product inhibition models

The incorporation of the product inhibition term into the linear model resulted in a lower residual sum of squares for both low and high enzyme concentrations (shown in Table 12). The reduction in the residual sum of squares was more pronounced where product levels, and consequently product inhibition, were higher.

There were two main areas where the linear model was not able to provide a good fit to the experimental data. Firstly, the calculated values of S were too low during the first hour of the hydrolysis. Secondly, the values of P were over-predicted during the later (product inhibition) stages of the hydrolysis.

Figure 21. Normalized values of the constants k_I (X), k_S (O), and K_{mP} (●) versus enzyme concentration in AU/L. Constants are normalized according to $(k_i-\bar{k}_i)/\sigma_i$. 60%, 80%, and 95% confidence levels are shown

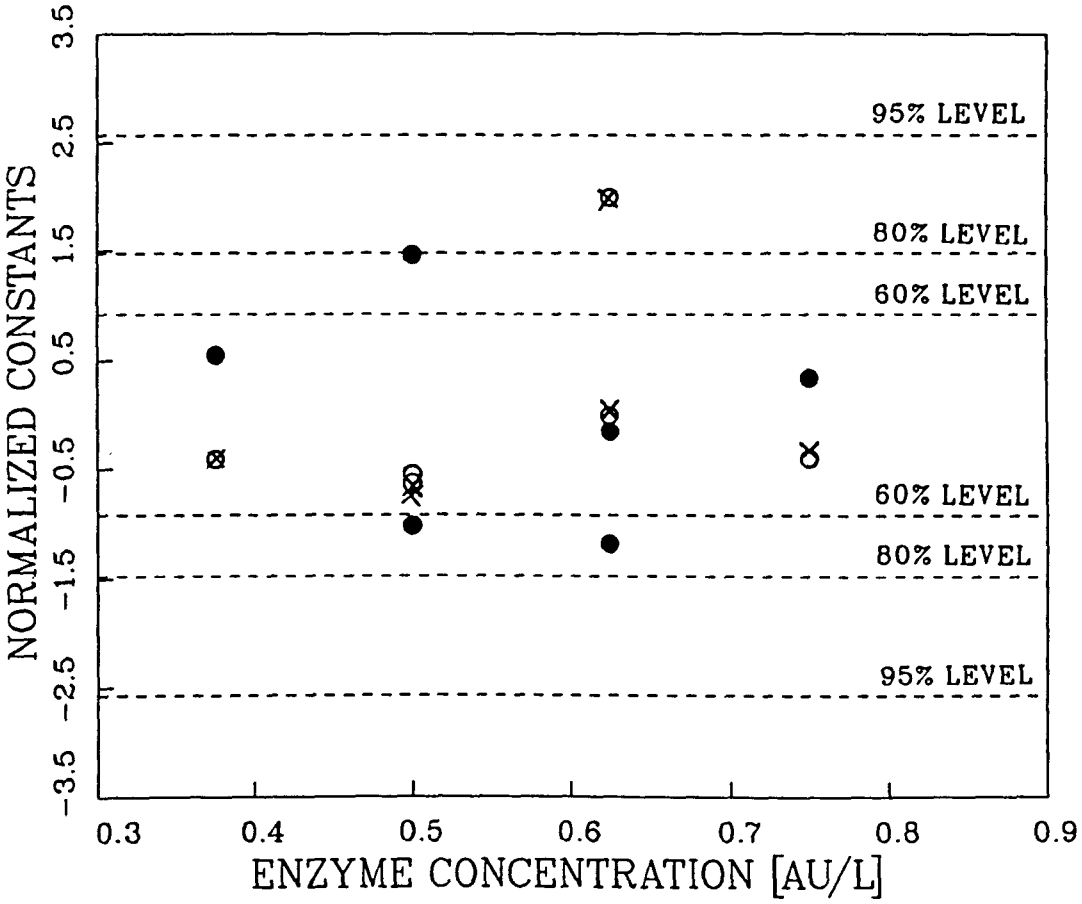

NORMALIZED CONSTANTS
3.5
2.5
1.5
0.5
-0.5
-1.5
-2.5
-3.5
95% LEVEL
80% LEVEL
60% LEVEL
60% LEVEL
80% LEVEL
95% LEVEL
0.3
0.4
0.5
0.6
0.7
0.8
0.9
ENZYME CONCENTRATION [AU/L]

Table 12. A comparison of the residual sum of squares at different enzyme concentrations (Anson/L) for the linear and product inhibition models

Run	Enzyme concentration (AU/L)	Residual sum of squares	
		Linear model	Product inhibition model
6M	0.375	90.92	52.44
12M	0.375	17.25	CP[a]
8M	0.5	87.72	57.99
7M	0.5	110.15	60.81
4M	0.625	243.60	35.97
9M	0.625	138.87	32.10
5M	0.75	157.70	59.01
11M	0.75	220.14	73.05

[a]Convergence problems.

The product inhibition model, on the other hand, proved to have problems only in calculating the values of S during the first hour of the hydrolysis. These observations can be seen more clearly when both models are plotted together with the experimental data (shown in Figures 22 and 23).

Any variations in the constants evaluated in both models did not appear to be related to the enzyme concentration. Any scatter in their values was probably as a result of experimental error. Statistically they are "constant".

Simultaneous fast and slow reactions

Figure 24 and the earlier Figure 9 do not show the form expected (Figure 2) for the simple case of fast and slow bond cleavages occurring simultaneously. This is not unexpected since each type of bond exists in both soluble and insoluble pools, and the relative amounts in the protein pools vary throughout.

Figure 24 also illustrates the effect of enzyme concentration on the rate of hydrolysis. The rate increases and drops off at a faster rate at the higher enzyme concentration. The initial increase in the rate (during the first fifty minutes) was because of the increased accessibility of bonds for cleavage. The rate dropped later in the hydrolysis as described previously, likely as a result of the depletion of susceptible bonds or product inhibition.

Figure 22. Comparison of the linear/linear model (—) and the simplified product inhibition model (---) with experimental data. Experimental data are denoted by: ■ insoluble, I, ● soluble proteins/peptides, S, and O soluble peptides, P. Reaction conditions: enzyme concentration 0.375 AU/L, pH 9, and 50°C

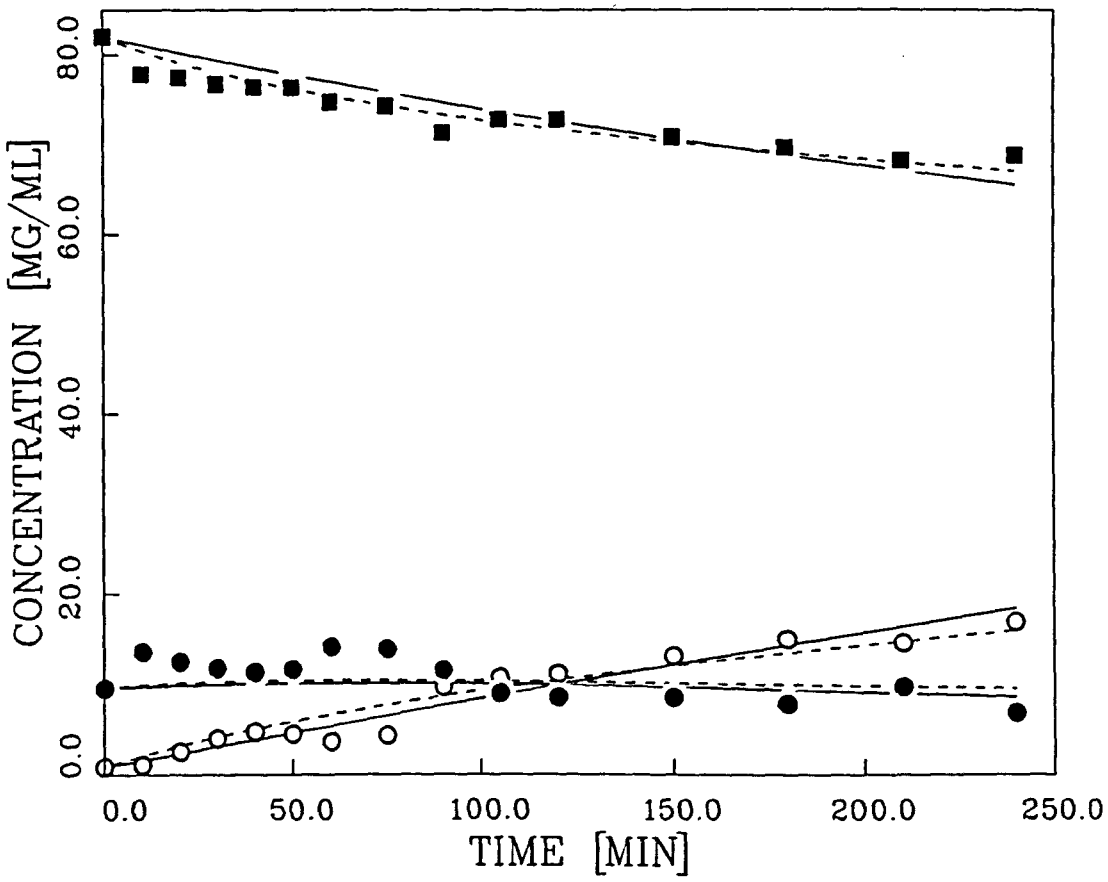

CONCENTRATION [MG/ML]
80.0
60.0
40.0
20.0
0.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

Figure 23. Comparison of the linear/linear model (—) and the simplified product inhibition model (---) with experimental data. Experimental data are denoted by: ■ insoluble, I, ● soluble proteins/peptides, S, and O soluble peptides, P. Reaction conditions: enzyme concentration 0.625 AU/L, pH 9, and 50°C

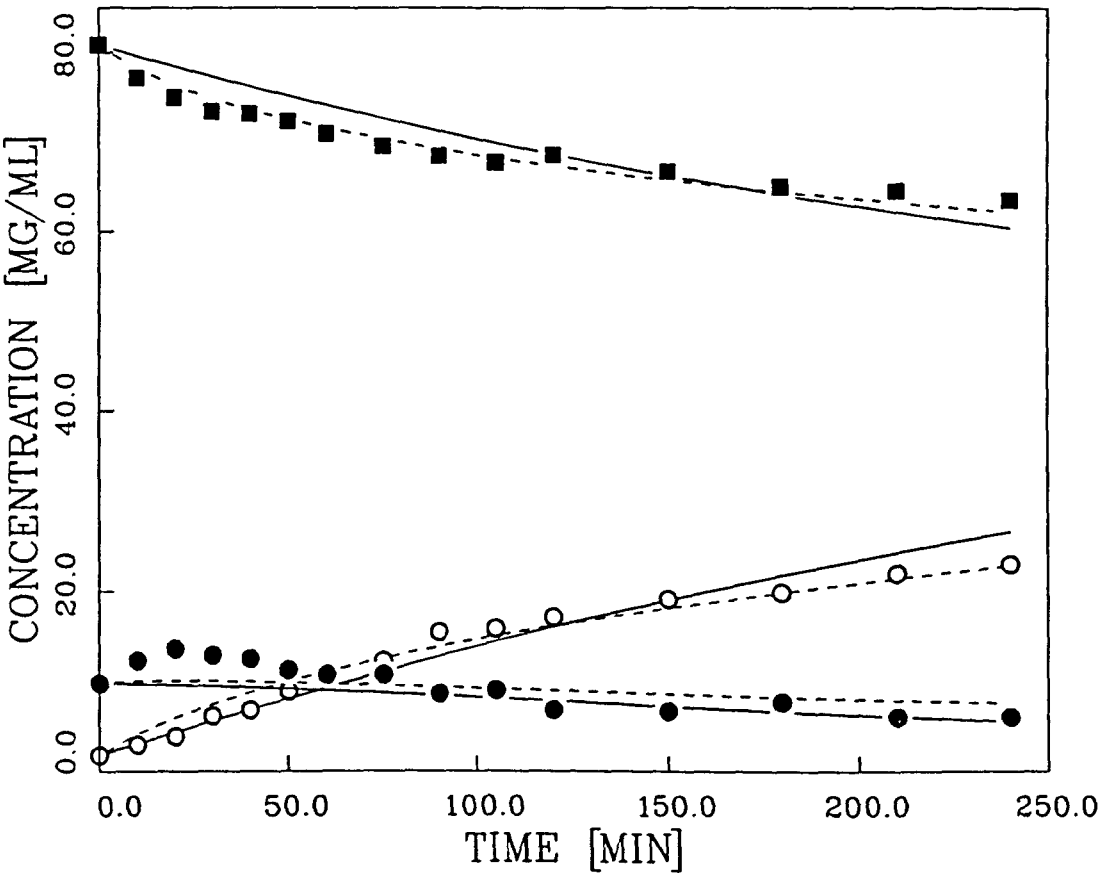
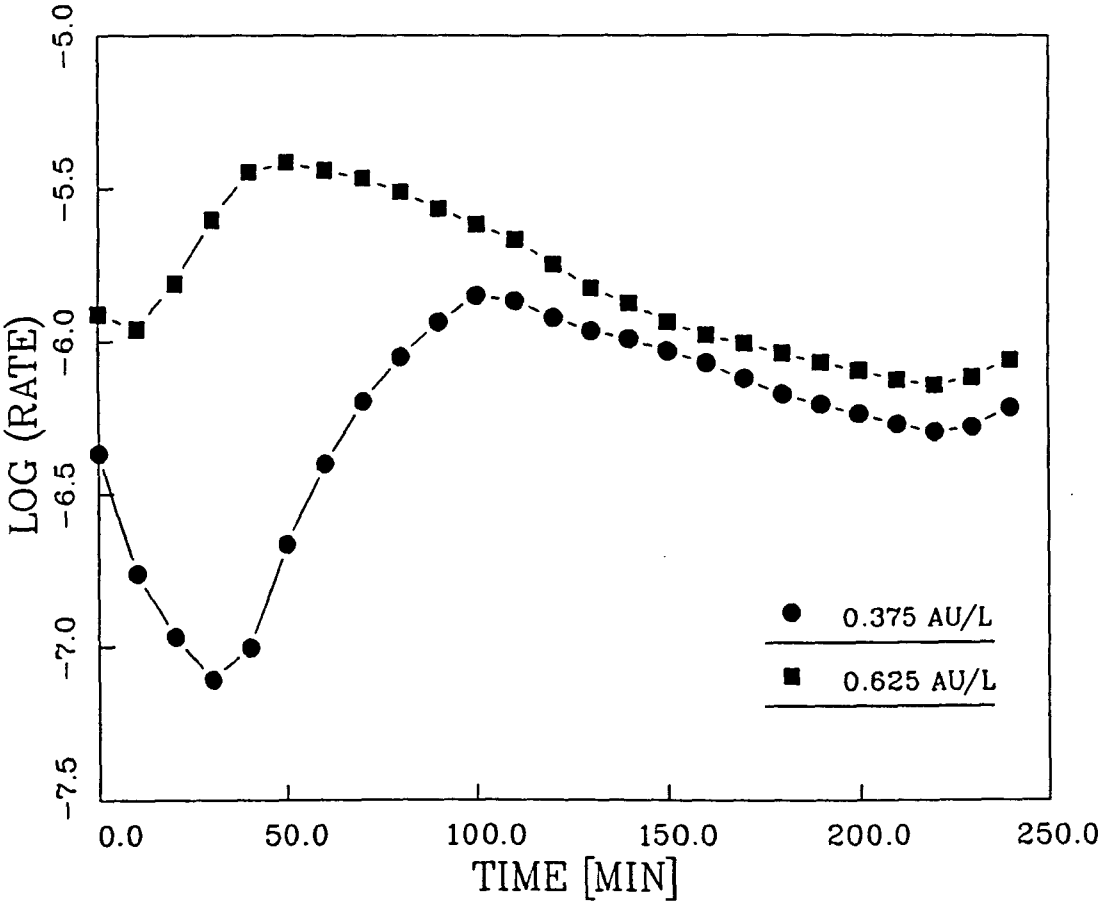

CONCENTRATION [MG/ML]
0.0
20.0
40.0
60.0
80.0
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]

Figure 24. Variation in the log(rate) with time for low (0.375 AU/L) and high (0.625 AU/L) enzyme concentrations. Smoothed values of h were used to calculate the rate - dh/dt. Reaction conditions: pH 9, 50°C


LOG (RATE)
-5.0
-5.5
-6.0
-6.5
-7.0
-7.5
0.0
50.0
100.0
150.0
200.0
250.0
TIME [MIN]
0.375 AU/L
0.625 AU/L

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The most suitable pH and temperature for the hydrolysis of corn gluten meal, using Alcalase 2.4L, were found to be 9 and 50°C, respectively. The range of enzyme concentrations used was 0.375 Anson units/L to 0.75 Anson units/L.

The rate of hydrolysis and the percent soluble protein, using Alcalase 2.4L, were shown to increase with enzyme dosage. The rate of hydrolysis accelerated during the first hour of hydrolysis, followed by an eventual decline. The acceleration was explained by an increase in the bond accessibility for cleavage. The decline, on the other hand, was attributed to either product inhibition or a reduction in the susceptible bonds.

Corn gluten meal does not exhibit a high internal surface area. Nevertheless, an increase in the rate of hydrolysis was not observed even when the total surface area was increased by four times. The hydrolysis was apparently not limited by the availability of surface sites at which the enzyme can bind.

An increase in soluble protein with an increase in enzyme concentration was accounted for by an increase in low molecular weight peptides (P). The high molecular weight proteins/peptides (S) in solution hardly varied with enzyme concentration. For longer term hydrolysis (48 hours), 53.2% of the available protein became soluble.

Of all the kinetic models studied, the linear and simplified product inhibition models generated the best representation of the experimental

data. These models predicted most of the trends of the experimental data. Product inhibition did, however, seem to be apparent. The addition of the product inhibition term to the linear model resulted in an improvement in the residual sum of squares of between 33% and 85%. The largest improvement was observed at the highest enzyme concentrations. In these cases, the highest concentration of low molecular weight peptides was produced.

Recommendations

Certain aspects of the enzymatic hydrolysis of corn gluten meal are still not thoroughly understood. Further experimentation should be carried out to try to improve the hydrolysis yields. In addition, the kinetic model should be tested further. Future work should involve in particular:

1) Specific sizes can be collected after sieving. Hydrolysis should then be carried out on the different sizes to try to determine if an optimum particle size exists for the hydrolysis.

2) Corn gluten meal should be steamed and thereafter hydrolyzed. Any improvement in the rate of hydrolysis would be due to swelling of the corn gluten meal.

3) Further extended hydrolysis runs should be performed with a view to obtaining as much soluble protein as possible.

4) Experiments can be carried out to test for product inhibition. The insoluble fraction should be separated from the soluble phase, resuspended in water, and the hydrolysis continued. The rate should increase due to the removal of the low molecular weight peptides responsible for the product inhibition, provided the enzyme is not removed in the soluble phase. Alternatively, low molecular weight peptides

could be added during the early stages of the hydrolysis, and the resulting product inhibition if present would cause the rate to decrease.

5) Steps should be taken to continually remove the low molecular weight peptides responsible for product inhibition. This could be achieved by using ultrafiltration in combination with the reactor.

6) A more complex model should be developed. This model should be able to account for the initial increase in high molecular weight proteins/peptides (S), and the eventual decline in the rate of hydrolysis due to product inhibition. The time course of the degree of hydrolysis could be coupled with the insoluble and soluble protein data.

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This would not have been possible without my parents' dedication to my education. Unfortunately, they cannot share these moments with me.

APPENDIX

Experimental conditions, experimental values of I, (S + P), and P used for modelling, and the estimated parameter constants calculated for each data set.

Table A-1. Run 4M, enzyme concentration: 0.625 AU/L, pH: 9, temperature: 50°C.
Linear model: k_I 1.32 x 10^{-2} L/g min, k_S 5.59 x 10^{-2} L/g min.
Product inhibition model: k_I 1.40 x 10^{-1} L/g min, k_S 4.46 x 10^{-1} L/g min, K_{mP} 1.01 mg/mL

Time (min)	% Degree hydrolysis	Insoluble protein (I) (mg/mL)	Total soluble protein (S + P) (mg/mL)	Low M.Wt. peptides (P) (mg/mL)
0	0	82.06	10.38	0.54
10	0.33	76.16	16.28	2.60
20	0.56	74.27	18.17	5.52
30	0.98	73.50	18.94	5.09
40	1.50	73.15	19.29	7.59
50	2.03	72.18	20.26	8.45
60	2.57	70.89	21.55	11.02
75	3.32	69.69	22.75	13.17
90	3.60	69.26	23.18	14.20
105	4.66	66.77	25.67	15.84
120	5.27	67.51	24.93	15.75
150	6.28	66.57	25.87	18.76
180	7.16	65.22	27.22	19.79
210	7.98	65.08	27.36	21.94
240	8.75	64.82	27.82	21.67

Table A-2. Run 5M, enzyme concentration: 0.75 AU/L, pH: 9, temperature: 50°C.
Linear model: k_I 1.06 x 10^{-2} L/g min, k_S 4.44 x 10^{-2} L/g min.
Product inhibition model: k_I 3.52 x 10^{-2} L/g min, k_S 1.18 x 10^{-1} L/g min, K_{mP} 4.59 mg/mL

Time (min)	% Degree hydrolysis	Insoluble protein (I) (mg/mL)	Total soluble protein (S + P) (mg/mL)	Low M.Wt. peptides (P) (mg/mL)
0	0	80.92	11.52	1.06
10	0.31	77.77	14.67	3.46
20	0.55	72.92	19.52	3.81
30	0.95	73.10	19.34	5.18
40	1.46	72.92	19.52	5.87
50	2.00	71.98	20.46	7.07
60	2.54	70.32	22.12	12.23
75	3.29	69.46	22.98	12.91
90	4.01	69.52	22.92	13.43
105	4.65	68.89	23.55	15.15
120	5.27	68.34	24.10	17.21
150	6.32	66.71	25.73	19.96
180	7.26	65.53	26.91	22.37
210	8.15	64.10	28.34	22.02
240	8.93	62.93	29.51	22.71

Table A-3. Run 6M, enzyme concentration: 0.375 AU/L, pH: 9, temperature: 50°C.
Linear model: k_I 1.49 x 10^{-2} L/g min, k_S 4.85 x 10^{-2} L/g min.
Product inhibition model: k_I 3.48 x 10^{-2} L/g min, k_S 1.06 x 10^{-1} L/g min, K_{mP} 5.08 mg/mL

Time (min)	% Degree hydrolysis	Insoluble protein (I) (mg/mL)	Total soluble protein (S + P) (mg/mL)	Low M.Wt. peptides (P) (mg/mL)
0	0	82.01	10.43	0.88
10	0.19	77.82	14.62	1.06
20	0.30	77.48	14.96	2.52
30	0.38	76.73	15.71	3.98
40	0.49	76.39	16.05	4.75
50	0.60	76.33	16.11	4.49
60	0.74	74.73	17.71	3.63
75	1.14	74.27	18.17	4.32
90	1.57	71.32	21.12	9.65
105	2.10	72.81	19.63	10.68
120	2.61	72.81	19.63	11.11
150	3.53	70.86	21.58	13.09
180	4.34	69.66	22.78	14.98
210	5.06	68.20	24.24	14.55
240	5.71	68.77	23.67	16.87

Table A-4. Run 7M, enzyme concentration: 0.5 AU/L, pH: 9, temperature: 50°C.
Linear model: k_I 1.37 x 10^{-2} L/g min, k_S 3.97 x 10^{-2} L/g min.
Product inhibition model: k_I 2.68 x 10^{-2} L/g min, k_S 7.69 x 10^{-2} L/g min, K_{mP} 7.23 mg/mL

Time (min)	% Degree hydrolysis	Insoluble protein (I) (mg/mL)	Total soluble protein (S + P) (mg/mL)	Low M.Wt. peptides (P) (mg/mL)
0	0	81.07	11.37	0.85
10	0.22	76.89	15.55	1.86
20	0.35	76.49	15.95	2.41
30	0.48	75.76	16.68	2.23
40	0.69	75.24	17.20	3.69
50	1.00	73.38	19.06	5.89
60	1.45	73.28	19.16	6.44
75	2.10	68.98	23.46	5.52
90	2.72	70.93	21.51	11.20
105	3.33	69.80	22.64	13.22
120	3.92	70.08	22.36	13.22
150	4.85	68.80	23.64	14.68
180	5.67	67.67	24.77	17.07
210	6.42	66.72	25.72	17.98
240	7.10	64.22	28.22	18.35

Table A-5. Run 8M, enzyme concentration: 0.5 AU/L, pH: 9, temperature: 50°C.
Linear model: k_I 1.51 x 10^{-2} L/g min, k_S 4.37 x 10^{-2} L/g min.
Product inhibition model: k_I 2.51 x 10^{-2} L/g min, k_S 6.83 x 10^{-2} L/g min, K_{mP} 1.39 mg/mL

Time (min)	% Degree hydrolysis	Insoluble protein (I) (mg/mL)	Total soluble protein (S + P) (mg/mL)	Low M.Wt. peptides (P) (mg/mL)
0	0	80.58	11.86	1.32
10	0.24	77.01	15.43	2.13
20	0.38	76.55	15.89	2.40
30	0.51	73.99	18.45	2.88
40	0.72	74.11	18.33	3.15
50	1.05	72.98	19.46	7.08
60	1.46	71.09	21.35	6.71
75	2.11	70.69	21.75	8.64
90	2.75	69.83	22.61	10.10
105	3.35	68.19	24.25	13.68
120	3.92	68.15	24.29	14.04
150	4.89	66.96	25.48	17.43
180	5.75	66.47	25.97	18.26
210	6.55	65.80	26.64	19.36
240	7.29	63.33	29.11	23.30

Table A-6. Run 9M, enzyme concentration: 0.625 AU/L, pH: 9, temperature: 50°C.
Linear model: k_I 1.27 x 10^{-2} L/g min, k_S 5.10 x 10^{-2} L/g min.
Product inhibition model: k_I 5.23 x 10^{-2} L/g min, k_S 1.72 x 10^{-1} L/g min, K_{mP} 3.43 mg/mL

Time (min)	% Degree hydrolysis	Insoluble protein (I) (mg/mL)	Total soluble protein (S + P) (mg/mL)	Low M.Wt. peptides (P) (mg/mL)
0	0	80.89	11.55	1.77
10	0.34	77.19	15.25	2.96
20	0.59	74.99	17.45	3.87
30	0.98	73.41	19.03	6.16
40	1.51	73.16	19.28	6.81
50	2.06	72.31	20.13	8.91
60	2.60	70.90	21.54	10.84
75	3.40	69.53	22.91	12.21
90	4.10	68.43	24.01	15.42
105	4.75	67.70	24.74	15.78
120	5.37	68.58	23.86	17.07
150	6.41	66.72	25.72	19.08
180	7.31	64.95	27.49	19.81
210	8.13	64.49	27.95	21.92
240	8.91	63.48	28.96	22.93

Table A-7. Run 11M, enzyme concentration: 0.75 AU/L, pH: 9, temperature: 50°C.
Linear model: k_I 1.01 x 10^{-2} L/g min, k_S 5.32 x 10^{-2} L/g min.
Product inhibition model: k_I 1.44 x 10^{3} L/g min, k_S 5.46 x 10^{3} L/g min, K_{mP} 8.35 x 10^{-5} mg/mL

Time (min)	% Degree hydrolysis	Insoluble protein (I) (mg/mL)	Total soluble protein (S + P) (mg/mL)	Low M.Wt. peptides (P) (mg/mL)
0	0	80.74	11.70	2.68
10	0.30	74.96	17.48	4.70
20	0.51	73.80	18.64	5.80
30	0.94	73.53	18.91	7.26
40	1.47	72.46	19.98	8.55
50	2.06	69.99	22.45	10.38
60	2.59	71.94	20.50	12.39
75	3.33	68.86	23.58	13.49
90	4.03	68.19	24.25	15.14
105	4.67	68.00	24.44	18.17
120	5.26	67.60	24.84	18.02
150	6.22	65.77	26.67	19.72
180	7.08	66.69	25.75	22.84
210	7.80	68.58	23.86	21.83
240	8.47	61.99	30.45	23.66

Table A-8. Run 12M, enzyme concentration: 0.375 AU/L, pH: 9, temperature: 50°C.
Linear model: k_I 1.25 x 10^{-2} L/g min, k_S 3.15 x 10^{-2} Lg/min.
Product inhibition model: convergence problems

Time (min)	% Degree hydrolysis	Insoluble protein (I) (mg/mL)	Total soluble protein (S + P) (mg/mL)	Low M.Wt. peptides (P) (mg/mL)
0	0	79.09	13.35	1.44
10	0.18	79.51	12.93	1.95
20	0.28	77.68	14.76	2.15
30	0.35	78.11	14.33	2.49
40	0.42	77.35	15.09	2.61
50	0.51	75.94	16.50	3.06
60	0.63	76.00	16.44	3.63
75	0.86	74.66	17.78	4.64
90	0.97	73.59	18.85	5.73
105	1.72	71.70	20.74	7.56
120	2.23	71.82	20.62	8.81
150	3.19	69.25	23.19	11.19
180	4.03	68.15	24.29	13.24
210	4.81	67.33	25.11	14.76
240	5.49	65.96	26.48	15.91