Enzymatic Hydrolysis of Corn Gluten Meal

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Corn gluten meal was hydrolyzed with Alcalase 2.4L, an alkaline protease. The effects of enzyme concentration and gluten size reduction on the hydrolysis were studied. Extent of reaction was expressed in terms of both the degree of hydrolysis (using the pH-stat technique) and the concentration of soluble protein. Linear and product inhibition kinetic models were compared to the experimental results after parameter estimation by minimizing the residual sum of squares. The models describe the time-dependent behavior of three protein/peptide pools—insoluble protein, TCA-insoluble proteins, and TCA-soluble peptides. A simplified product inhibition model gave the best fit to the experimental data.

Corn gluten meal is a byproduct of the corn wet-milling process and contains approximately 60% protein. The major protein fractions in corn gluten meal are zein (68%), glutelin (28%), and globulins (1.2%) (Neumann and Wall, 1984; Buck et al., 1987). Currently, its main use is as an animal feed. The major drawback of using corn gluten meal in foods is that it is very insoluble in water (Ofelt and Evans, 1949; Russell and Tsao, 1982). There is, therefore, motivation to try to increase its solubility.

Enzymatic hydrolysis has been used to favorably modify the functional properties of proteins, such that the hydrolysates could be incorporated into foods for their flavor and functional and nutritional value (Constantinides and Adu-Amankwa, 1980). Corn gluten meal has been hydrolyzed by alkaline microbial proteases (Adler-Nissen, 1977). The effects of temperature, pH, and enzyme concentration were studied.

Kinetic studies have been conducted on soluble enzyme/insoluble substrate systems with soy protein (Constantinides and Adu-Amankwa, 1980; Adler-Nissen, 1977), fish protein (Archer et al., 1973), cellulose (Howell and Stuck, 1975; Mandels et al., 1971), gelatin (McLaren, 1963), and starch (McLaren, 1963). Three different approaches have been used to model kinetic mechanisms. Michaelis–Menten kinetics, derived for the enzymatic hydrolysis of soluble substrates (Constantinides and Adu-Amankwa, 1980; Howell and Stuck, 1975), have also been applied to insoluble substrate systems. Alternatively, a model based on adsorption of excess enzyme onto substrate surface sites, followed by subsequent hydrolysis, was used to model hydrolysis of fish protein (Archer et al., 1973), cellulose (Mandels et al., 1971; McLaren, 1963), and gelatin (McLaren, 1963). The hydrolysis of fish protein concentrate has also been modeled as two simultaneous first-order reactions (Archer et al., 1973).

The main objective here was to study the kinetics of the hydrolysis. After an initial screening for the effects of enzyme dosage and meal size, the time course of extent of hydrolysis and appearance of soluble protein fractions was followed. A useful feature of a kinetic model is the ability to describe the product size distribution, one of the determinants of functional properties.

MATERIALS AND METHODS

Materials. Alcalase 2.4L (Novo Laboratories, Inc., Wilton, CT) is a food-grade preparation of subtilisin Carlsberg in the liquid form. The activity was 2.4 AU/g. The enzyme was diluted by 1:10 to reduce its viscosity for ease of addition to the reactor. The corn gluten meal was supplied by Grain Processing, Muscatine, IA. It was used as an 8% by weight protein slurry made up with deionized water (Adler-Nissen, 1977).

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Analyses. Nitrogen content of the corn gluten meal was determined by micro-Kjeldahl (AOAC, 1970) and protein content as 6.25 × N (the conversion factor for corn gluten proteins) (Adler-Nissen, 1985). Soluble protein was determined by the biuret method (Doumas, 1975), with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard.

Trichloroacetic acid (TCA) was used to precipitate proteins and higher molecular weight peptides from the hydrolysates. A 5-mL portion of 2.4 N TCA was added to 10 mL of hydrolysate (Adler-Nissen, 1985) and the precipitate removed by centrifugation ($2.7 \times 10^3 g$ for 30 min).

The particle size distribution was determined for the nonreduced and size-reduced corn gluten samples by sieve analysis. A 30-g sample was placed on the coarsest (top) in a series of six 8-in. sieves and shaken in a sieve shaker for 30 min (Willis, 1981). The fractions were collected and weighed. Sieve sizes of the nonreduced samples were between 1400 μm (12 mesh) and 45 μm (350 mesh), whereas for a size-reduced sample the size range was between 1180 μm (14 mesh) and 38 μm (400 mesh). Surface areas were measured by BET analysis using krypton gas as the adsorbate (McClellan and Hamsberger, 1967). External surface areas were calculated by assuming that the corn gluten meal consisted of spheres of the average diameter of the sieving fraction.

Experimental Procedure. *Procedure I.* Hydrolysis was performed on nonreduced and size-reduced corn gluten. The dry corn gluten meal was pulverized on a rotating hammer mill (Micro switch, Freeport, IL). Enzyme concentrations used were between 0.375 and 0.75 AU/L.

The corn gluten slurry, containing 8% protein by weight, was agitated at 450 rpm for 1 h at pH 9 and 50 °C to allow for extraction of the soluble fractions. After an initial sample (15 mL) was withdrawn, the enzymatic hydrolysis began with addition of enzyme. The pH-stat meter was zeroed at this point. The hydrolysis time was 4 h, with samples taken at 2 and 4 h. Concentrated hydrochloric acid was added to the samples to deactivate the enzyme. The samples were then centrifuged and the supernatants assayed for soluble protein.

Volume of base addition was continuously recorded. Before calculation of degree of hydrolysis (DH), base consumption was corrected for the combined effects of CO_2 adsorption and residual nonenzymatic dissolution. The contribution of the former has been shown to be less than 1% for the sample sizes used here (Adler-Nissen, 1985).

Procedure II. To study the time course of the hydrolysis, the procedure was essentially the same except samples were taken more frequently. Samples of 10 mL were taken every 10 min for samples taken during the first hour, every 15 min during the second hour, and every 30 min during the remaining 2 h of the 4-h hydrolysis. The enzyme was deactivated by placing the sample in a water bath at 90 °C for 15 min. Temperature was used to deactivate the enzyme to avoid protein precipitation that occurred with concentrated hydrochloric acid. The samples were centrifuged, and supernatants were assayed for soluble protein before and after TCA precipitation.

Kinetic Model Development. The kinetic models considered were all based on three compartments (see Figure 1). I represents insoluble protein, while compartments S and P both represent soluble material. S is the group of high molecular weight proteins/peptides precipitated by trichloroacetic acid (TCA), and

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Table I. Kinetic Expressions Used To Model the Rates r_I and r_S

model	insol substr r_I	sol substr r_S
linear/linear	$k_I e_0([I] - [IE])$	$k_S e_0[S]$
Michaelis–Menten/ Michaelis–Menten	$k_I e_0([I] - [IE])/ [1 + ([I]/K_{mI}) + ([S]/K_{mS})]$	$k_S e_0[S]/ [1 + ([I]/K_{mI}) + ([S]/K_{mS})]$
Freundlich adsorption/linear	$k_I(e_0([I] - [IE]))^{2/3}$	$k_S e_0[S]$
simplified product inhibn/ product inhibn	$k_I e_0([I] - [IE])/ [1 + ([P]/K_{mP})]$	$k_S e_0[S]/ [1 + ([P]/K_{mP})]$

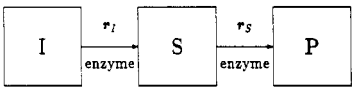

Figure 1. Illustration of the different compartments used in the kinetic modeling of the hydrolysis.

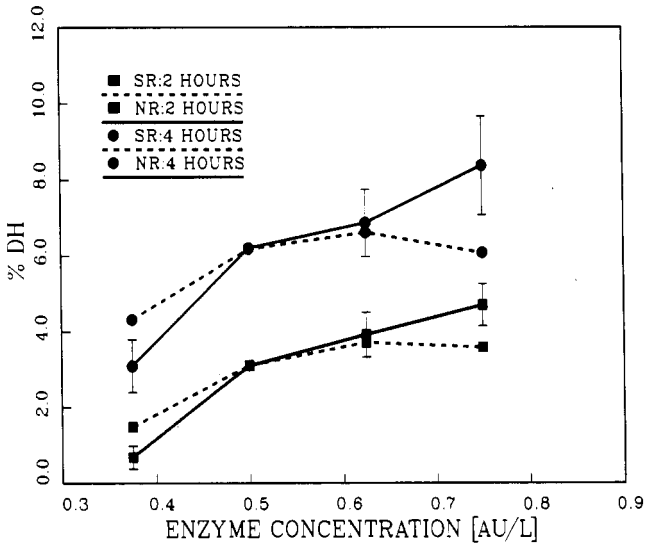

Figure 2. Variation with enzyme concentration (AU/L) of percent degree of hydrolysis for nonreduced (NR) and size-reduced (SR) corn gluten meal after 2 and 4 h of hydrolysis. Error bars represent low and high values for two NR observations. Reaction conditions: pH 9, 50 °C.

P is the low molecular weight (TCA soluble) peptide pool.

The rate expressions r_I and r_S may be written as

$$r_I = \frac{-d[I]}{dt} = \frac{d([S] + [P])}{dt} \quad (1)$$

$$r_S = \frac{d[P]}{dt} = -\frac{d([I] + [S])}{dt} \quad (2)$$

Various kinetic expressions for both r_I and r_S were tested in different combinations as shown in Table I. A linear rate expression is the simplest and is a limiting case of the Michaelis–Menten expression, classically used for soluble substrates. Product inhibition has been reported for enzymatic hydrolysis of soybean protein and lean meat proteins (Constantinides and Adu-Amankwa, 1980; O'Meara and Munro, 1985). The Freundlich adsorption model would be most applicable where enzyme adsorbs to a limited number of substrate surface sites.

Treatment of Hydrolysis Data. DH was calculated from the values of base consumption (corrected). This procedure has been linearly correlated with the trinitrobenzenesulfonic acid (TNBS) method, giving slightly lower values (Adler-Nissen, 1985). The rate of hydrolysis (dh/dt) was obtained by smoothing the base consumption versus time data and then calculating the rate with Lagrange's forward, central, and backward difference formulas for equally spaced points (Hoerl, 1973). Plots of logarithm of the rate versus time for typical simultaneous reaction systems (Ronca et al., 1975; Mihalyi and Godfrey, 1962; Mihalyi and Harrington, 1959) are initially curved (fast and slow reactions), followed by a linear region (slow reaction only).

RESULTS AND DISCUSSION

Effect of Enzyme Dosage. The rate of hydrolysis and the soluble protein concentration increased with increasing enzyme concentrations (Figures 2 and 3). All enzyme concentrations demonstrated an acceleration in the rate of hydrolysis (Figure 4), 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 slowest; consequently, the acceleration in the rate of the hydrolysis occurred at later times. Initially the most accessible bonds are cleaved. As a result of these cleavages further accessible bonds would be exposed, causing an acceleration in the rate of hydrolysis. The lower the enzyme concentration, the longer it takes to reach the same extent of cleavage as reached at higher enzyme concen-

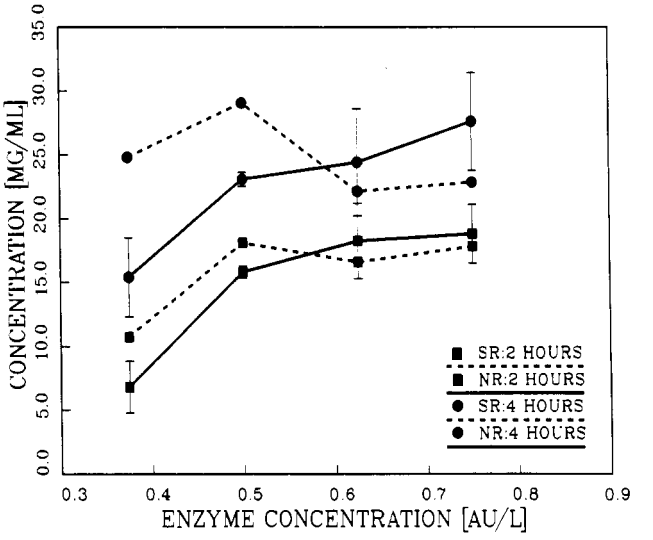

Figure 3. Variation with enzyme concentration (AU/L) of soluble protein (mg of protein/mL) for nonreduced (NR) and size-reduced (SR) corn gluten meal after 2 and 4 h of hydrolysis. Error bars represent high and low values for two NR observations. Reaction conditions: pH 9, 50 °C.

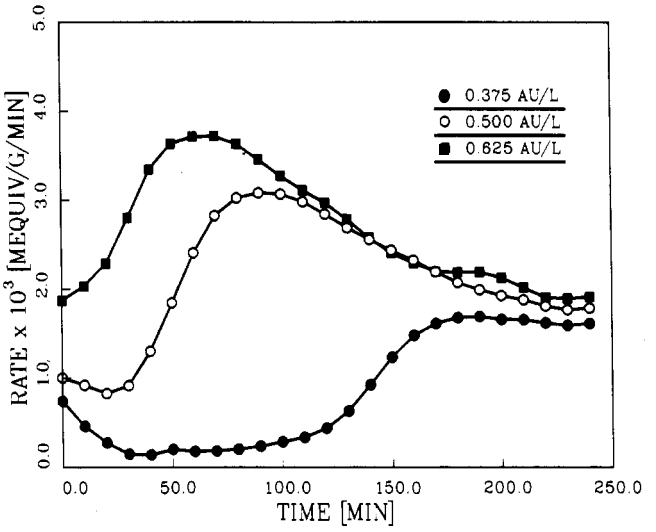

Figure 4. Variation in the rate of hydrolysis (mequiv/g per min) with time (min) at three levels of enzyme concentration (AU/L). Smoothed values of h were used to calculate the rate dh/dt. Reaction conditions: pH 9, 50 °C.

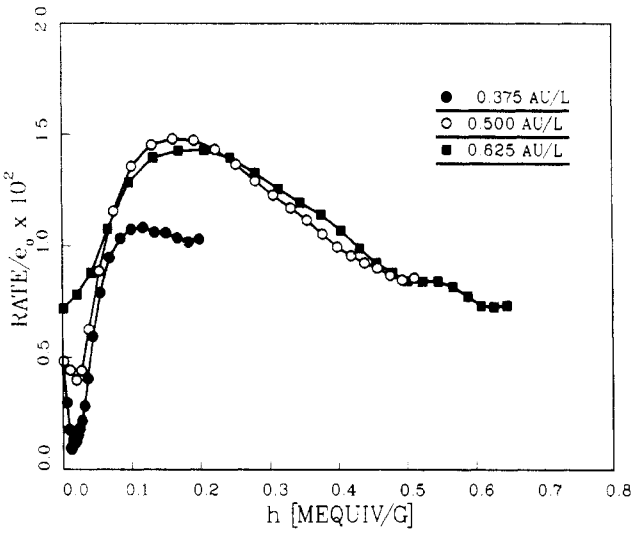

Figure 5. Variation in the rate of hydrolysis (mequiv L/g^2 per min) with the number of peptide bonds cleaved (mequiv/g) at three levels of enzyme concentration (AU/L). Smoothed values of h were used to calculate the rate dh/dt. Reaction conditions: pH 9, 50 °C.

Table II. Actual Surface Area of Ground and Unground Corn Gluten Meal (CGM) Compared to the External Surface Area Based on Perfect Spheres

size	ext surface area, m^2/g	actual (BET) surface area, m^2/g
unground CGM (355–180 μm)	0.063	0.1069
ground CGM (75–35 μm)	0.31	0.432

trations. Figure 5 shows that the increase in the rate for different enzyme concentrations occurs when a certain number of peptide bonds have been cleaved—when h reaches 0.02 mequiv/g.

Effect of Size Reduction. Nearly 75% of the unground corn gluten was between 355 μm (44 mesh) and 180 μm (85 mesh). Pulverizing the corn gluten reduced 85% of the sample to a size between 75 μm (200 mesh) and 38 μm (400 mesh). The gluten meal had very little internal surface area, and surface area was increased by size reduction (Table II). The internal surface area accessible to the enzyme will be even less than that measured with krypton gas as the adsorbent.

The degree of hydrolysis (DH) and the yield of soluble protein did not increase significantly (t-test) for the size-reduced corn (Figures 2 and 3). These figures show that, at the highest enzyme levels, the extent of reaction (in terms of both soluble protein and DH) at 2 and 4 h is no longer increasing with enzyme concentration, a possible indication of surface area limitation. But for both measures the extent is less (though not at the 90% significance level) for the material with the higher initial surface area. The reason for the result at 2 and 4 h may be that the rate increase observed early on (Figures 4 and 5) overcomes any initial advantage of increased surface area. Only at the lowest enzyme concentration is the rate increase late enough (ca. 2 h) that this effect would not obscure a surface area effect (Figure 4). The latter complication can be avoided by looking at the initial rate data. The initial rate of hydrolysis at 0.375 AU/L was higher for the size-reduced corn ($1.20 \times 10^{-3} \pm 0.53 \times 10^{-3}$ mequiv/g per min) compared with the unground corn ($0.68 \times 10^{-3} \pm 0.03 \times 10^{-3}$ mequiv/g per min). At the highest enzyme concentration (0.75 AU/L), the initial rates were statistically the same—$1.35 \times 10^{-3} \pm 0.12 \times 10^{-3}$ mequiv/g per min for the unground corn and $1.45 \times 10^{-3} \pm 0.12 \times 10^{-3}$ mequiv/g per min for the size-reduced corn.

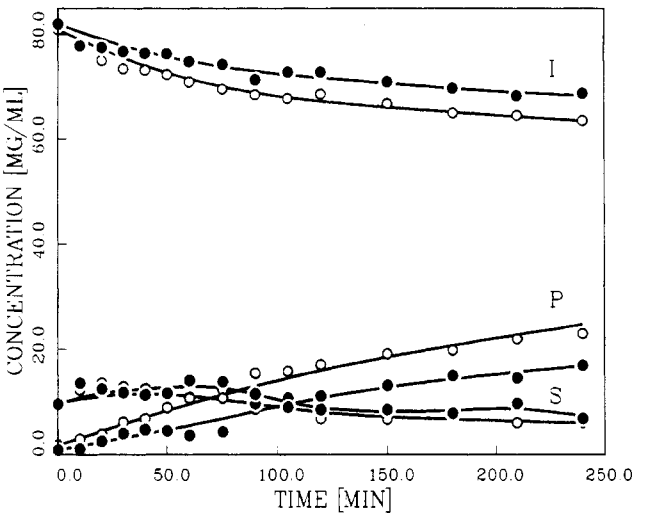

Figure 6. Experimental values of protein concentration (mg of protein/mL) with time (min): ●, low enzyme concentration (0.375 AU/L); ○, high enzyme concentration (0.625 AU/L).

Time Course of the Hydrolysis. Initially the level of soluble protein was relatively low so that most of the hydrolysis was in the insoluble phase and produced soluble proteins and high molecular weight peptides (S). At this point, the rate of hydrolysis of I (r_I) was greater than that of S (r_S), resulting in an overall increase in S. The concentrations of pools S and P for the course of the hydrolysis are shown in Figure 6 for low (0.375 AU/L) and high (0.625 AU/L) enzyme concentrations. The 10–20% greater reduction in I (or an increase in (S + P)) at the higher enzyme concentration shows up predominantly in pool P. This implies that the additional enzyme preferentially hydrolyzes soluble protein.

The low rate of conversion by 4 h (Figure 6) suggests that there is a hydrolysis-resistant core (Mihalyi and Harrington, 1959). An estimation of the content of this unreactive material (IE) was made by performing an extended hydrolysis of 48 h. At this point 46.8% (43.26 mg/mL) remained insoluble, and this was used as the value for IE in all the kinetic models studied.

Comparison of Kinetic Models. The expressions used to describe the kinetic models are shown in Table I. The constants in each compartmental model were evaluated by finding the values giving the least-squares fit to the experimental data of (S + P) and P versus time (Levenburg-Marquardt subroutine ZXSSQ) (IMSL, 1987).

The values of the linear model (solid lines) compared to the experimental values (symbols) are shown in Figures 7 and 8 at low (0.375 AU/L) and high (0.625 AU/L) enzyme concentrations. The linear model was able to predict the main trends of the experimental data, but it tended to overpredict the values of P toward the end of the hydrolysis. This effect was more pronounced for the higher enzyme concentrations. The linear model allows for reduction in substrate concentrations, but not for bonds of different susceptibility or product inhibition, two other possible causes of decreasing rates.

The experimental values in pool S increased initially and then gradually decreased. The linear model fitted this poorly during the first 50 min of the hydrolysis. It did, however, predict the later times well. 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 over all runs were found to be $1.29 \times 10^{-2} \pm 0.18 \times 10^{-2}$ and $4.60 \times 10^{-2} \pm 0.79 \times 10^{-2}$ mL/mg per min, respectively. The constants

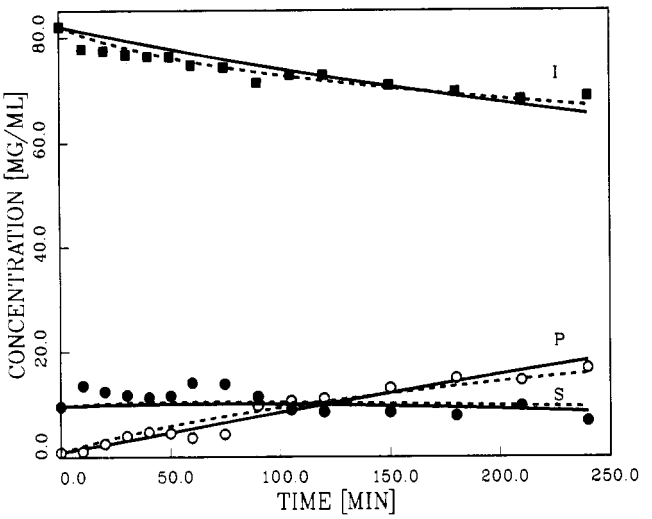

Figure 7. Comparison of the linear (—) and the simplified product inhibition model (---) with experimental data: ■, insoluble, I; ●, soluble proteins/peptides, S; ○, soluble peptides, P. Reaction conditions: enzyme concentration 0.375 AU/L, pH 9, 50 °C.

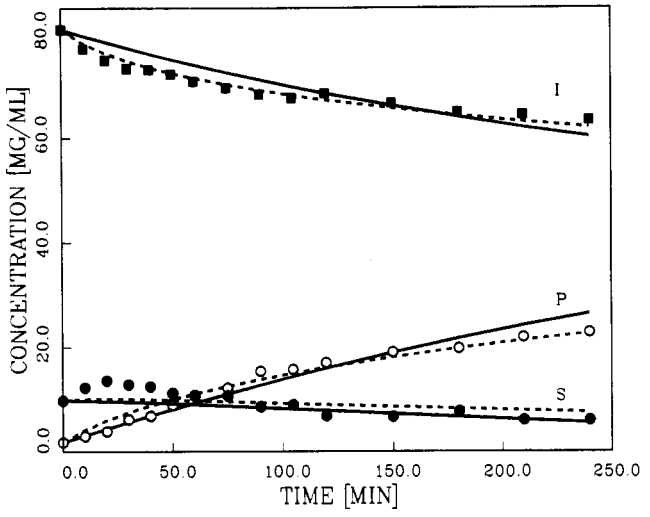

Figure 8. Comparison of the linear (—) and the simplified product inhibition model (---) with experimental data: ■, insoluble, I; ●, soluble proteins/peptides, S; ○, soluble peptides, P. Reaction conditions: enzyme concentration 0.625 AU/L, pH 9, 50 °C.

k_I and k_S were shown to be statistically "constant" for a small population (t-distribution).

The calculated values for the simplified product inhibition model (dashed lines) are also shown in Figures 7 and 8. The calculated P values fit the experimental values much closer than the linear model during the later stages of the hydrolysis. The main weakness of this model was an underestimation of S during the first 50 min of the hydrolysis. The improved fit is reflected in the lower sums of squares at all enzyme concentrations (Table III). The reduction in the residual sum of squares was more pronounced at higher enzyme levels where product levels, and consequently product inhibition, would be greater. The average values for k_I, k_S, and K_{mP} were found to be $5.27 \times 10^{-2} \pm 4.38 \times 10^{-2}$ mL/mg per min, $1.65 \times 10^{-1} \pm 1.43 \times 10^{-1}$ mL/mg per min and 3.79 ± 2.36 mg/mL, respectively. Variations in these parameters between individual runs were also shown to be within the bounds of statistical error.

With the product inhibition model, a second region of convergence appears in the least-squares procedure. The values reported were chosen on the bases of lower sums of squares and consistency with the values of k_I and k_S from the linear model.

Table III. Comparison of the Residual Sum of Squares at Different Enzyme Concentrations (AU/L) for Linear and Product Inhibition Models

enzyme concn, AU/L	residual sum of squares: linear model	residual sum of squares: product inhibn model
0.375	90.92	52.44
0.375	17.25	[a]
0.500	87.72	57.99
0.500	110.15	60.81
0.625	243.60	35.97
0.625	138.87	32.10
0.750	157.70	59.01
0.750	220.14	73.05

[a] Did not converge.

The two other models shown in Table I did not result in any improvement over the linear and simplified inhibition models. The Michaelis–Menten/Michaelis–Menten model resulted in the same values for the constants k_I and k_S as were found for the linear model, while the constants K_{mI} and K_{mS} were found to be so large (approximately 10^{17}) that their influence in the denominator was negligible. The Freundlich adsorption/linear model also failed to represent the mechanism any better than the linear model, another indication that the hydrolysis was not limited by available surface sites during these 4-h runs.

Finally, a scheme of simultaneous fast and slow cleavage sites is not appropriate to describe the rate of hydrolysis (Figure 4). Such a model would show a monotonic decrease in rate with time (Ronca et al., 1975; Mihalyi and Godfrey, 1962; Mihalyi and Harrington, 1959).

CONCLUSIONS

The rates of hydrolysis and formation of soluble protein increase with enzyme concentration. The increase in soluble protein was accounted for by an increase in low molecular weight peptides (P). The rate of hydrolysis increases during the early stages of the hydrolysis due to increased bond accessibility for cleavage. The eventual decline is attributed to disappearance of substrate and 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 was increased 4 times. Over an extended period, the hydrolysis was apparently not limited by the initial availability of surface sites at which the enzyme can bind.

Of the kinetic models studied, the linear and the simplified product inhibition models generate the best representation of the experimental data. The addition of the product inhibition term to the linear model results in improvement in the residual sum of squares of between 33 and 85%. The largest improvement is observed at the highest enzyme concentrations where product inhibition is most likely. The kinetic model helps us to understand the change in product distribution with continued hydrolysis. Clearly, there is a tradeoff between the yield of soluble protein and the product distribution. Further work is needed to devise a means to react the resistant fraction.

ACKNOWLEDGMENT

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ABBREVIATIONS

CGM, corn gluten meal; e_0, initial enzyme concentration (mg/mL); h, hydrolysis equivalents (mequiv of peptide

bonds/g); [I], insoluble substrate concentration (mg/mL); [IE], insoluble substrate concentration, resistant to hydrolysis (mg/mL); k_I, reaction rate constant for insoluble pool I (mL/mg per min); k_S, reaction rate constant for soluble pool S (mL/mg per min); K_{mi}, Michaelis–Menten constant for pool i (mg/mL); [P], concentration of soluble low molecular weight peptides, not precipitated by TCA (mg/mL); r_I, rate of hydrolysis of substrate I (mg/mL per min); r_S, rate of hydrolysis of substrate S (mg/mL per min); [S], soluble protein/high molecular weight peptides, precipitated by TCA (mg/mL); t, time (min).

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Monoclonal Antibody-Based Enzyme Immunoassay of the Bowman–Birk Protease Inhibitor of Soybeans

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Monoclonal antibodies that bind the Bowman–Birk protease inhibitor (BBI) of soybeans were derived from mice inoculated with an emulsion of native, un-cross-linked protein. The antibodies did not cross-react with the soybean Kunitz trypsin inhibitor or the homologous inhibitors from lima beans and chickpeas. One epitope of BBI, which is destroyed by heat and by disulfide-modifying reagents, was defined. Monoclonal antibodies that recognize this epitope can be used for specific recognition of native BBI in the presence of denatured forms, both in purified samples and in extracts of soybean cultivars and processed foods. An enzyme-linked immunosorbent assay (ELISA) for BBI was developed with these antibodies, and the results from the ELISA agreed with enzymatic assays of inhibitory activity in heat-treated soy meal. The monoclonal antibodies were also effective for affinity isolation of BBI from soy isolates. The demonstration that BBI retained its native conformation following conjugation with enzyme and that the antibody was stable during immunoaffinity procedures indicates that these immunochemical methods could readily be used for screening plant germplasm and in biomedical studies.

The protein of soybeans (*Glycine max*) is widely used in human foods in a variety of forms including infant formulas, soy protein isolates, soy flour, textured soy fibers, soy sauce, and tofu as well as in animal feeds. Protease inhibitors constitute about 6% of the proteins of soybeans (Rackis et al., 1986). The two major inhibitors are the Kunitz trypsin inhibitor (KTI) and the Bowman–Birk inhibitor (BBI), a low molecular weight (M_r 8000) double-headed inhibitor of chymotrypsin and trypsin (Birk, 1985). The presence of inhibitors of digestive enzymes in soybeans impairs their nutritional quality and promotes the development of pancreatic nodular hyperplasia and acinar cell adenomas in rats (Gumbmann et al., 1986). On the other hand, recent evidence suggests that BBI may

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