Enzymatic Hydrolysis of Whey Proteins: I. Kinetic Models

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We have studied the enzymatic hydrolysis of whey proteins at pH 8 and 50°C with two proteases of bacterial origin, MKC Protease 660 L and Alcalasa 0.6 L, and one of animal origin, PEM 2500 S. Our results show that a greater degree of hydrolysis is achieved under the same experimental conditions with the bacterial proteases than with the animal one. In our interpretation of the results we propose a mechanism in which the hydrolytic reaction is a zero-order one for the substrate, and the enzyme denaturalizes simultaneously via a second-order kinetic process due to free enzyme attacking enzyme bound to the substrate. Our results also indicate that there is an irreversible serine-protease inhibitor in whey proteins.
Key words: whey proteins • proteases • enzymatic hydrolysis • peptides

INTRODUCTION

Enzymatic hydrolysis of biopolymers in foodstuffs, i.e., polysaccharides, proteins, pectins, etc., is a process of considerable importance that can be used to improve the physical, chemical, and organoleptic properties of the original food without prejudicing its nutritive value, and also improve its intestinal absorption characteristics.[5,6] It is a mild process, occurring over a series of stages, and does not produce parallel degradation reactions in the separate components, as does both acid and alkaline hydrolysis. Protein hydrolysates are used in the preparation of special diets for the enteric feeding of babies and sick adults. Thus, they should be osmotically balanced, hypoallergenic, and have an acceptable flavor, all of which are achieved when the hydrolysates are composed of peptides with two to six amino acids.

A kinetic study of the process is quite complicated due to the various types of peptide bonds involved and their differing vulnerability to attack by enzymes during the hydrolytic process. These effects make it difficult to interpret the experimental results via simple kinetic equations and reduce the significance of the kinetic parameters obtained.[9] Furthermore, give the economic importance of such processes, most of them are protected by patent; those that are published tend to study the characteristics of the hydrolysates in terms of the degree of hydrolysis achieved.

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MATERIALS AND METHODS

The substrate used was a commercial preparation of whey proteins, Lactoalbumin 75 L (bought from MILEI, Germany). This prime material is used in industry to prepare enzyme hydrolysates from proteins. Its total nitrogen content was determined, using Kjeldahl's method, to be 73% by protein weight, and its water was 6% by weight, as calculated by dehydration in a vacuum oven at 60°C for 24 h.

The proteases used were:

- *MKC Protease 660 L*, obtained from *Bacillus subtilis* and provided by Miles Laboratories. This enzyme complex is stable between 50° and 70°C and at pH values between 7 and 10. Its activity is 6.60 AU/mL, as measured by Anson's modified method.[1] We have previously shown that the enzyme is stable at experimental conditions of pH 8 and 50°C, and that it is deactivated by heating to 100°C for 5 min.[3]
- *Alcalase 0.6 L*, a proteolytic enzyme prepared by controlled fermentation of a selected strain of *Bacillus licheniformis* and commercially available from Novo Industries. Its principal enzyme component is Subtilisin A, which is a serine-type endoproteinase. Its optimum conditions are 50° to 70°C and pH 6 to 10. Its activity is 1.01 AU/g.[1] The enzyme is stable at 50°C, and can be deactivated by heating to 85°C for 2 min.[11]
- *PEM 2500 S*, a proteolytic compound made up of a mixture of purified pancreatic enzymes, including bovine trypsin, porcine trypsin, and bovine chymotrypsin, commercially available from Novo Industries. Its maximum activity occurs between 38° and 50°C at pH 8. Its overall activity is 20.84 AU/g,[1] and is deactivated by heating to 100°C for 5 min.[7]

The experimental apparatus was a well-stirred batch reactor fitted with both temperature and pH controls. The kinetics of the process was monitored by the pH-stat method as the quantity of base needed to maintain pH constant is proportional to the degree of hydrolysis, x, defined as the fraction of peptide bonds hydrolyzed. The relationship between x and the consumption of base is arrived at via the equation[11]

$$x = \frac{E}{M_p \cdot \alpha \cdot h_t} \qquad (1)$$

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where E represents the equivalents of base (NaOH 2N) needed to keep pH constant; α is the mean degree of dissociation, which is a function of the temperature and pH (under our experimental conditions $\alpha = 0.885$); h_t is the total number of peptide-bond equivalents per unit of protein mass (8.8 for whey proteins); and M_p is the protein mass in kilograms. The values for α and h_t have been taken from tables published by de Novo Industries.[11]

In some experiments we also calculated the degree of hydrolyzation following the method proposed by Snyder and Sobocinsky,[12] based on the reaction of 2,4,6-trinitrobenzenesulfonic acid with groups of free amines from amino acids, peptides, and proteins (TNBS method[12]); the values for x determined by either method coincide very closely, above all when a di- or tripeptide is used as reference.[3]

All experiments were made at 50°C and pH 8, which correspond to maximum activity and stability conditions for the proteases in question, except for bovine chymotrypsin, which is a minor component of PEM 2500 S.

DISCUSSION OF RESULTS

The degrees of hydrolysis achieved with different initial enzyme concentrations are shown in Figures 1, 2, and 3. In these graphs, and in the rest of the experiments, it can be seen that the rate of hydrolysis decreases appreciably with time and that the conversion, x, seems to tend toward a limit value, which increases with the initial enzyme concentration.

The decrease observed in the rate of hydrolysis may be due to three factors: (a) a decrease in the concentration of peptide bonds susceptible to hydrolysis by the proteases; (b) a possible inhibition of the enzymes caused by the products of hydrolysis; (c) the denaturation of the enzyme. The first of these effects would make the curves tend toward the same value for x and is thus an improbable explanation as the x–t curves tend toward different values. Furthermore, on studying the influence of the initial substrate concentration, S_0, it is clear that it has a negative influence on the value of x (Figs. 4, 5, and 6). This effect suggests that the substrate may play a part in deactivating the enzyme and lends weight to the idea that the concentration of hydrolyzed bonds is not the controlling factor in the rate of hydrolysis.

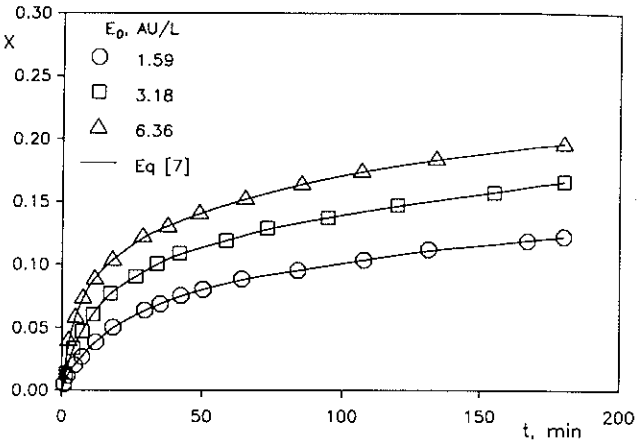

Figure 2. Variation in the degree of hydrolysis versus initial enzyme concentration: Alcalasa 0.6 L; S_0 = 38.45 g/L.

As an example, the variation of x versus the product of the initial enzyme concentration, E_0, multiplied by the time of hydrolysis elapsed, t, is shown in Figure 7. It can be seen that all the experiments made with the same initial substrate concentration fall along one single line, whatever the initial enzyme concentration.

The overall rate of hydrolysis at constant pH and temperature is

$$r = S_0 \frac{dx}{dt} = F(S_0, x) \cdot E \tag{2}$$

by separating variables and integrating (2) if there is no denaturation, then

$$\int_0^x \frac{dx}{F(S_0, x)} = E_0 \cdot t \tag{3}$$

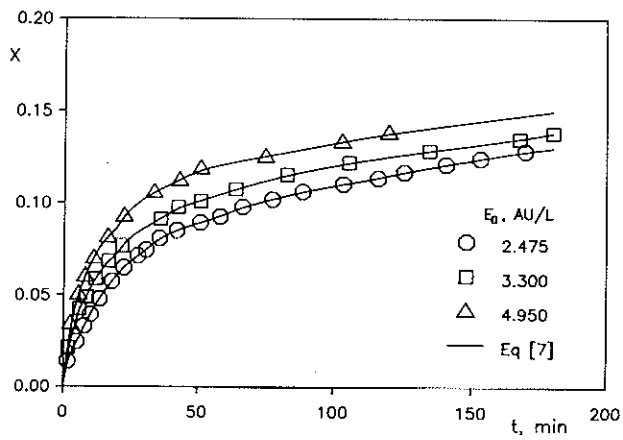

Figure 1. Variation in the degree of hydrolysis versus initial enzyme concentration: Protease 660 L; S_0 = 38.45 g/L.

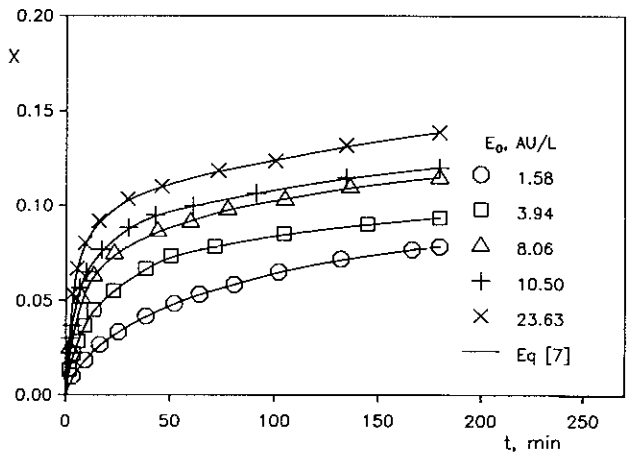

Figure 3. Variation in the degree of hydrolysis versus initial enzyme concentration: PEM 2500 S; S_0 = 38.45 g/L.

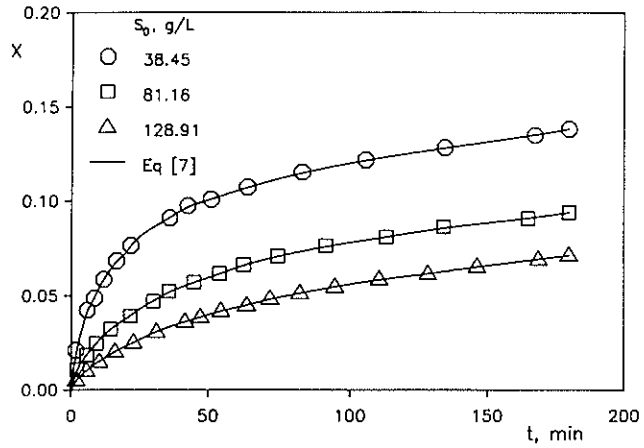

Figure 4. Influence of S_0 upon the degree of hydrolysis. Protease 660 L; E_0 = 3.30 AU/L.

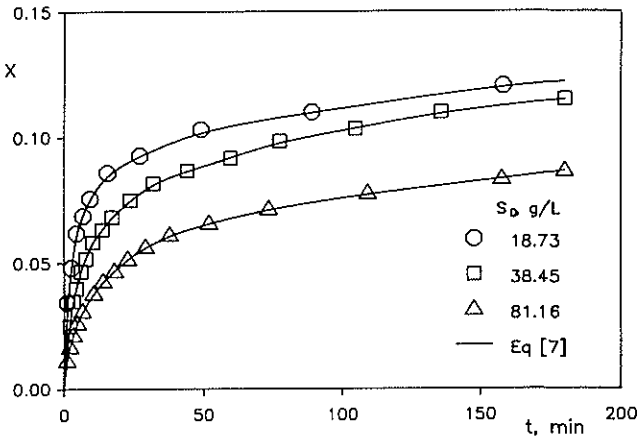

Figure 6. Influence of S_0 upon the degree of hydrolysis. PEM 2500 S; E_0 = 8.06 AU/L.

If denaturation is a second-order one for the protease, then

$$-\frac{dE}{dt} = k_d \cdot E^2 \tag{4}$$

and the integration of (2) leads to

$$\int_0^x \frac{dx}{F(S_0, x)} = \frac{1}{k_d} \ln [1 + k_d \cdot E_0 \cdot t] \tag{5}$$

Equations (3) and (5) show a univocal relationship between the degree of hydrolysis achieved and the product $E_0 \cdot t$, which indicates that in our experiments either there is no enzyme denaturation or it is a second-order one for the protease.

To fit the x–t data we used cubic spline functions,[8,13] choosing the knots so that they corresponded to equal intervals in the dependent variable and their number so as to minimize the summation of the residual squares. From the spline functions we could determine dx/dt for different degrees of hydrolysis. As can be seen in Figure 8, the dx/dt values decrease exponentially with x, so that

$$\frac{dx}{dt} = a \cdot \exp(-b \cdot x) \tag{6}$$

Integrating Eq. (6) we get

$$x = \frac{1}{b} \cdot \ln(1 + a \cdot b \cdot t) \tag{7}$$

This expression allows us to determine a and b by nonlinear regression, thus avoiding the errors inherent in the differential method. The values of these parameters are set out in Tables I, II, and III. The value of b always remains approximately constant, around an average of 33.0 for the Lactoalbumin–Protease 660 L system, 26.3 for the Lactoalbumin–Alcalasa 0.6 L system, and 48.9 for the Lactoalbumin–PEM system. On the other hand, a increases with E_0 and falls with S_0. With the mean values of b we were able to recalculate the values for a_m, which appear in Tables I, II, and III. With the values of a_m and b we could reproduce the experimental results shown in Figures 1 to 6, with errors of less than ±5%.

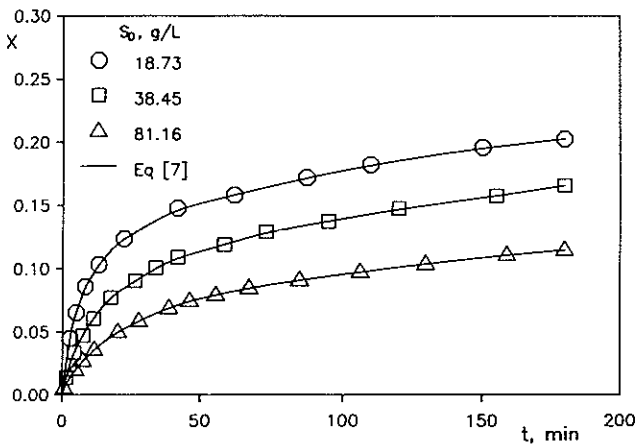

Figure 5. Influence of S_0 upon the degree of hydrolysis. Alcalasa 0.6 L; E_0 = 3.18 AU/L.

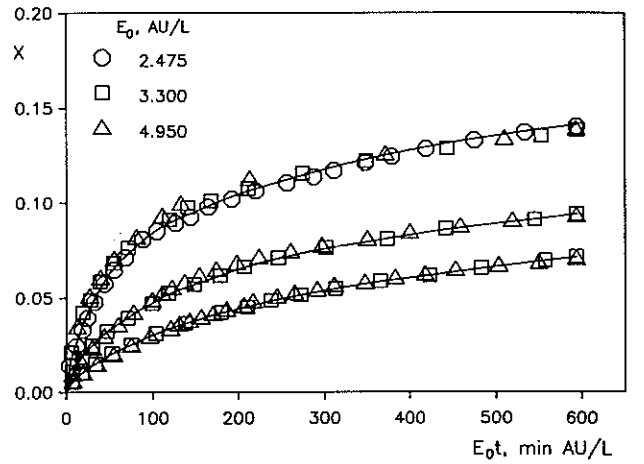

Figure 7. Variation in the degree of hydrolysis with $E_0 \cdot t$. Lactoalbumin 75 L/Protease 660 L; S_0 = .38.45 g/L to 81.16 g/L to 128.91 g/L.

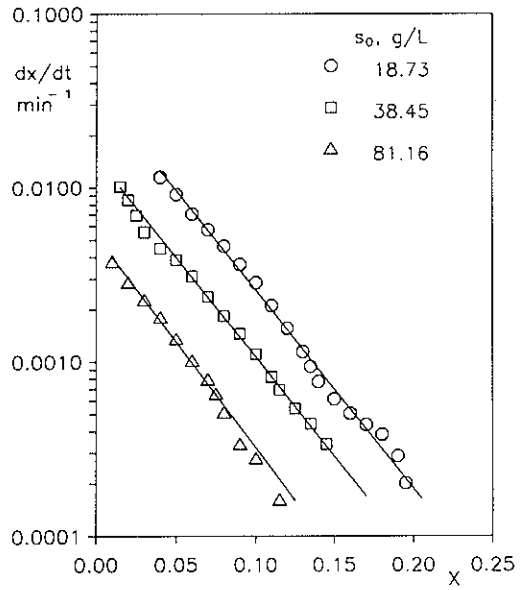

Figure 8. Decrease in dx/dt with the degree of hydrolysis. Protease 660 L; S_0 = 38.45 g/L.

When the values of a_m are represented versus E_0/S_0 they fit a straight line with its ordinate in the negative origin.

bacterial proteases: $$a_m = 0.226 \frac{E_0}{S_0} - 0.0049 \quad (8)$$

PEM: $$a_m = 0.171 \frac{E_0}{S_0} - 0.0049 \quad (9)$$

The overall rate of hydrolysis for the three systems assayed, Eqs. (6), (7), and (8), is

$$r_h = S_0 \frac{dx}{dt} = \beta \left[E_0 - \frac{\delta \cdot S_0}{\beta} \right] \exp(- b \cdot x) \quad (10)$$

This equation indicates that, below a certain critical enzyme concentration,

$$E_{0c} = \frac{\delta \cdot S_0}{\beta} \quad (11)$$

hydrolysis does not take place, as it would make no sense to consider the rate of hydrolysis to be negative. This result may be due to the fact that Eq. (10) does not work for very small E_0/S_0 values, or else to the presence of an irreversible inhibitor in the substrate or its instantaneous generation by hydrolysis of some component of the substrate.

In fact, Weber and Nielsen[14] have demonstrated the existence in whey proteins from cows' milk of an inhibitor of serine proteinases, which they identify as being similar to the α-AT inhibitor present in human and bovine blood. According to Beatty et al.,[2] these inhibitors combine with the enzyme very rapidly and irreversibly, thus making it impossible for a certain amount of the protease to take part in the hydrolytic reaction:

$$E + I \xrightarrow{k_i} EI \quad (12)$$

Thus, accepting the presence of the inhibitor is practically equivalent to saying that, at the initial moment of the reaction, the active enzyme concentration is

$$E_{0a} = E_0 - \epsilon \cdot S_0 \quad (13)$$

where $\epsilon \cdot S_0$ is the fraction of substrate constituting the inhibitor. If we substitute Eq. (13) into (10) we get

$$r_h = S_0 \frac{dx}{dt} = \beta \cdot E_{0a} \exp(- b \cdot x) \quad (14)$$

KINETIC MODEL

To explain Eq. (14) we propose the following mechanism:

$$E + S \underset{}{\overset{k_s}{\rightleftarrows}} ES \xrightarrow{k_h} E + 2P \quad (15)$$

If we assume that equilibrium has been reached in the equation above, and carry out an active-enzyme balance we get

Table I. Lactoalbumin 75 L/Protease 660 L. Kinetic parameter values.

S_0 (g/L)	E_0 (AU/L)	a (min^{-1})	b	a_m (min^{-1})
38.45	2.48	0.0080	28.79	0.0102
38.45	3.30	0.0152	32.53	0.0157
38.45	4.95	0.0238	32.03	0.0253
81.16	3.30	0.0043	36.10	0.0040
81.16	4.95	0.0054	29.59	0.0059
128.91	3.30	0.0019	37.05	0.0018
128.91	4.95	0.0026	35.03	0.0025

Table II. Lactoalbumin 75 L/Alcalasa 0.6 L. Kinetic parameter values.

S_0 (g/L)	E_0 (AU/L)	a (min^{-1})	b	a_m (min^{-1})
38.45	1.59	0.0058	27.71	0.0055
38.45	3.18	0.0123	24.36	0.0138
38.45	6.36	0.0278	24.48	0.0325
81.16	3.18	0.0057	29.90	0.0050
18.73	3.18	0.0286	24.57	0.0331

Table III. Lactoalbumin 75 L/PEM 2500 S. Kinetic parameter values.

S_0 (g/L)	E_0 (AU/L)	a (min^{-1})	b	a_m (min^{-1})
38.45	1.58	0.0028	38.42	0.0040
38.45	3.94	0.0107	46.38	0.0115
38.45	8.06	0.0285	47.39	0.0308
38.45	10.50	0.0393	47.19	0.0431
38.45	23.63	0.0994	48.96	0.0992
18.73	8.06	0.1229	57.62	0.0703
81.16	8.06	0.0129	56.08	0.0100

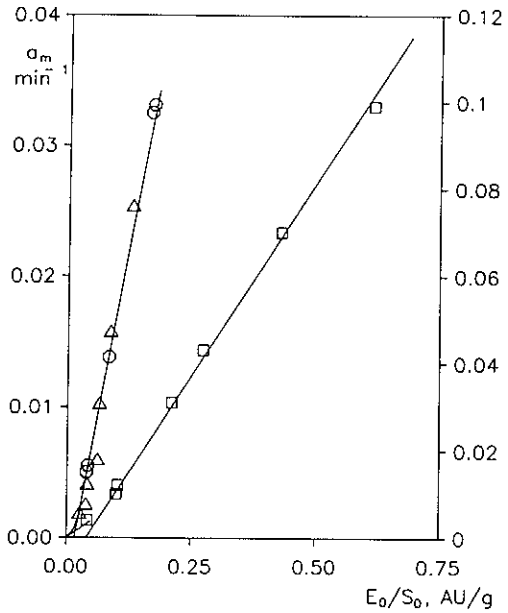

Figure 9. Influence of E_0/S_0 upon a_m, Eq. (8) and Eq. (9).

$$[ES] = \frac{K_s[S]E}{1 + K_s[S]} \tag{16}$$

$$[E] = \frac{E}{1 + K_s[S]} \tag{17}$$

If the enzyme has a high affinity for the substrate $k_s \cdot [S] \gg 1$, then Eqs. (16) and (17) can be reduced to

$$[E] \approx \frac{E}{K_s[S]} \tag{18}$$

$$[ES] \approx E \tag{19}$$

And thus the rate of hydrolysis will be

$$r_h = S_0 \frac{dx}{dt} = k_h \cdot [ES] = k_h \cdot E \tag{20}$$

It is accepted that the denaturalization of the enzyme is of second order and that it is caused by free enzyme attacking the enzyme bound to the substrate. Thus,

$$E + ES \xrightarrow{k_d} E + E_d + S \tag{21}$$

Equation (21) explains the variation in x versus $E_0 \cdot t$ and that the denaturation does not occur when no substrate is present, as has been shown experimentally with Protease 660 L.[3] Thus, the kinetic equation for the denaturation process is

$$-\frac{dE}{dt} = k_d \cdot \frac{E}{S_0} \cdot E \tag{22}$$

in which S can be put in place of S_0 because the peptides deriving from the hydrolytic process revert to being substrate for hydrolysis. Furthermore, the decrease in the concentration of peptide bonds available for hydrolysis is made up for by their greater susceptibility to enzyme attack. If we divide (22) by (20), separating variables and integrating, we get

$$E = E_{0a} \exp\left(-\frac{k_d}{k_h} \cdot x\right) \tag{23}$$

By substituting (23) into (20), and taking into account Eq. (13), we are left with

$$r_h = S_0 \frac{dx}{dt} = k_h (E_0 - \epsilon \cdot S_0) \exp\left(-\frac{k_d}{k_h} x\right) \tag{24}$$

When we compare Eqs. (24) and (10) it can be seen that they are analogous and it can be deduced that

$$k_h = \beta \qquad e = \frac{\delta}{\beta} \qquad \frac{k_d}{k_h} = b \tag{25}$$

The values for these kinetic parameters are set out in Table IV.

The integrated equation for rate is obtained by integrating Eq. (24)

$$x = \frac{k_h}{k_d} \ln [1 + k_d(E_0 - \epsilon \cdot S_0) \cdot t] \tag{26}$$

which allows us to optimize the design and working of the enzyme reactor to $x \leq 0.20$.

CONCLUSIONS

Enzymatic hydrolysis of whey proteins can be explained in terms of a practically instantaneous and irreversible binding of the enzyme with an inhibitor present in the substrate or generated by the instantaneous hydrolysis of some minor component of the Lactoalbumin 75 L, followed by a zero-order hydrolytic reaction with regard to the substrate, which occurs simultaneously with a second-order denaturation of the protease, caused by free enzyme attacking the enzyme bound to the substrate.

The most notable experimental observation during the enzymatic hydrolysis of Lactoalbumin 75 L is that the rate of reaction diminishes exponentially versus conversion, Eq. (24). It would seem that this fact is not peculiar to whey proteins, as we have also seen in our laboratory that the overall rate of hydrolysis diminishes versus x during the

Table IV. Kinetic constant values.

Enzyme	k_h [g/(AU min)]	ϵ (AU/g)	k_d [g/(AU min)]
Protease 660 L	0.241	0.025	7.95
Alcalasa 0.6 L	0.217	0.017	5.71
PEM 2500 S	0.171	0.029	8.36

enzyme hydrolysis of casein,[4] and even the hydrolysis of starch with α-amylase.[10]

NOMENCLATURE

a	kinetic parameter Eq. (6) (min^{-1})
a_m	value of a calculated with mean b
b	kinetic parameter Eq. (6)
DH	degree of hydrolysis (%)
E	concentration of active enzyme (AU/L)
E_0	initial enzyme concentration (AU/L)
E_{0a}	initial active-enzyme concentration (AU/L)
E_{0c}	critical enzyme concentration Eq. (11) (AU/L)
E_d	deactivated enzyme
EI	enzyme-inhibitor compound
ES	enzyme–substrate compound
h_t	number of peptide bonds in the substrate (eq g/kg)
k_d	kinetic denaturation constant (g protein/AU min)
k_h	kinetic hydrolysis constant (g protein/AU min)
k_i	kinetic constant Eq. (12) (M^{-1} s^{-1})
M_p	protein mass (kg)
r_h	rate of hydrolysis (g protein/L min)
S	substrate concentration (g protein/L)
S_0	initial substrate concentration (g protein/L)
TNBS	2,4,6-trinitrobenzenesulfonic acid
UA	Anson unit

x	degree of hydrolysis fraction
α	degree of dissociation Eq. (1)
β	kinetic parameter Eq. (10) (g protein/AU min)
δ	kinetic parameter Eq. (10) (min^{-1})
ϵ	kinetic parameter Eq. (13) (AU/g protein)

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