

KINETIC MODEL FOR WHEY PROTEIN HYDROLYSIS BY ALCALASE MULTIPPOINT- IMMOBILIZED ON AGAROSE GEL PARTICLES

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Abstract - Partial hydrolysis of whey proteins by enzymes immobilized on an inert support can either change or evidence functional properties of the produced peptides, thereby increasing their applications. The hydrolysis of sweet cheese whey proteins by alcalase, which is multipoint-immobilized on agarose gel, is studied here. A Michaelis-Menten model that takes into account competitive inhibition by the product was fitted to experimental data. The influence of pH on the kinetic parameters in the range 6.0 to 11.0 was assessed, at 50°C. Initial reaction-rate assays in a pHstat at different concentrations of substrate were used to estimate kinetic and Michaelis-Menten parameters, k and K_M . Experimental data from long-term batch assays were used to quantify the inhibition parameter, K_I . The fitting of the model to the experimental data was accurate in the entire pH range.

Keywords: Enzymatic proteolysis, Michaelis-Menten kinetic model, competitive inhibition, whey hydrolysis.

INTRODUCTION

Cheese whey is a by-product of the dairy industry that for a long time has been considered a residue. However, its high BOD [35000 mg/l – compare, for example, with the 300 mg/l BOD of domestic drains (Viotto, 1993)] and the high cost of its treatment have stimulated the search for commercial uses for it or its derivatives (Demetrakakes, 1997).

Partial hydrolysis of whey proteins can either change or evidence the functional properties of the peptides in this industrial residue, thereby increasing their applications. Hydrolyzed cheese whey can be a suitable protein source for infants and for individuals with a reduced digestive capacity. A tailor-made hydrolysis with specific enzymes, followed by convenient downstream processes, can also reduce phenylalanine contents in the product, which would thus be fitted to the diet of phenylketonuria patients.

Products containing bioactive peptides that are free of any bitterness can be obtained by partial hydrolysis of cheese whey proteins, making use of different proteases (Mann, 2000).

In general, the reaction rates of enzymatic proteolyses are assumed to follow either a Michaelis-Menten or a first-order kinetics. First-order kinetics is the consequence of a mechanism where the rate-controlling step is the first attack on the tertiary structure of the protein (Vorob'ev et al., 1996). The substrate and product concentrations in these rate equations are generally expressed either in terms of the number of hydrolyzable peptide bonds or of the degree of hydrolysis. It is also important to point out that competition for the active site between the original substrate and the peptides that are continuously produced during enzymatic proteolysis could bring about a substantial reduction in the reaction rates of hydrolysis.

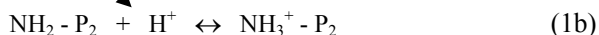
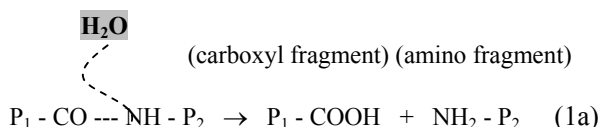
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Making use of a pHstat, it is possible to observe the progress of the reaction by monitoring the consumption of a titrant, necessary to control the system pH during batch hydrolysis assays carried out in a laboratory-scale reactor.

This work focuses on the case of whey protein hydrolysis by alcalase, multipoint-immobilized on agarose gel particles. Initial reaction-rate assays in a pHstat, with different substrate concentrations and different pH's made it possible to estimate the Michaelis-Menten kinetic parameters as a function of pH. Long-term assays showed that a third additional parameter should be considered, that would take into account the inhibitory effect of the products. Data from long-term batch assays also permitted quantification of this additional parameter by the Marquardt algorithm (1963). Assays at pH 6, 7, 8, 9, 10 and 11 were carried out. All assays were run at 50°C, a temperature where high productivity is achieved without any significant loss of enzymatic activity (Lopes, 2001).

ENZYMATIC PROTEOLYSIS

In general, proteolytic reactions can be summarized as follows:



and



According to Svendsen (1976) and Adler-Nissen (1986), it is generally accepted that serine proteases like alcalase, when acting on peptide bonds, follow Michaelis-Menten kinetics (see Equation 2, from Segel, 1975). Postolache and Oncescu (1989) also characterized the hydrolysis of casein and hemoglobin by alcalase according to Michaelis-Menten kinetic models.

$$V = -\frac{k \cdot E \cdot N}{K_M + N} \quad (2)$$

On the other hand, it could be expected that the original substrate and some of the peptides that are

continuously released during the hydrolytic process would compete for the active site in the enzyme. Adler-Nissen (1986) and González-Tello et al. (1994) had already pondered the possibility of product inhibition. Equation 3 shows a Michaelis-Menten model with competitive inhibition (Segel, 1975).

$$V = -\frac{k \cdot E \cdot N}{K_M \left(1 + \frac{I}{K_I}\right) + N} \quad (3)$$

where N is the molar concentration of peptide bonds in the substrate that can be hydrolyzed by alcalase, I is the molar concentration of the inhibitor and k, K_M and K_I are the kinetic model parameters. Alcalase can hydrolyze about 25% of the total number of peptide bonds in the protein substrate.

When the pHstat technique is used, the consumption of base necessary for controlling system pH during the batch assay provides the degree of hydrolysis (DH) in a straightforward manner (Equation 4). The initial reaction rates can be determined from the base consumption during the initial minutes of each assay (Adler-Nissen, 1986).

$$\text{DH} = \frac{h}{h_{\text{tot}}} \times 100\% = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{\text{MP}} \times \frac{1}{h_{\text{tot}}} \times 100\% \quad (4)$$

where h_{tot} is the total number of peptide bonds in the protein substrate, in meqv/g_{protein} (or simply mmol/g_{protein}); h is the number of hydrolyzed peptide bonds; B is the base consumption in ml; N_b is base normality; α is the average degree of dissociation of the α -NH groups and MP is the mass of protein in g.

Note that for the range of pH considered here, the carboxyl radical will be completely dissociated (reaction 1c will be displaced to the right). For this reason, the correction factor, α , only considers the equilibrium of reaction 1b.

MATERIALS AND METHODS

Cooperativa de Lactínios São Carlos donated *in natura* cheese whey, which was micro- (membrane A/G Technology Corporation®, 0.45 μm) and ultra-filtered (membrane Amicon®, 10000 Da) up to a concentration of 60 g/l.

The alcalase enzyme (Subtilisin) was multipoint-immobilized on agarose gel particles (donated by Hispanagar, Spain). Based on Tardioli (2003), activated agarose gel (glixyl agarose, 75 $\mu\text{mol/ml}$)

was suspended in bicarbonate buffer 50 mM, pH 10 at a 1/10 ratio ($V_{\text{gel}}/V_{\text{total}}$). Then 100 $\mu\text{l}_{\text{alcalase}}/\text{ml}_{\text{gel}}$ were added to the suspension and maintained under gentle shaking at 20 °C for 24 hours. Then 1 $\text{mg}_{\text{sodiumborohidrete}}/\text{ml}_{\text{suspension}}$ was added, maintaining the same stirring at 20 °C for 30 minutes. Finally, the reduced derivative was washed using distilled water in abundance.

In the jacketed reactor, coupled to the pHstat, the temperature of the concentrated cheese whey was raised to 50 °C and its pH adjusted by the manual addition of HCl and/or NaOH. The immobilized enzymes were then quickly added to the reactor and the automatic pH control of the equipment switched on.

Initial-rate assays for different substrate concentrations and pH's made it possible to estimate the parameters k and K_M of the Michaelis-Menten kinetic model. Eighteen experiments lasting 15 minutes were carried out at $T = 50$ °C. In each assay, 15 ml of cheese whey (20, 40 and 60 g/l) and 0.04 g of gel containing 15.7 $U_{\text{BAEE}}/\text{g}_{\text{gel}}$ were utilized. One U_{BAEE} (of alcalase) hydrolyzes 1 μmol of benzoil

arginine ethylic ester per minute at pH 8 and 25°C. For each concentration, six pH values (6, 7, 8, 9, 10 and 11) were taken into account.

Four long-term hydrolysis experiments (lasting 5 hours) were also carried out, which made it possible to evaluate the adequacy of the pure Michaelis-Menten model, and subsequently quantify the parameter K_I . In each assay, 30 ml of cheese whey at 60 g/l and 0.30 g of gel containing 13.4 $U_{\text{BAEE}}/\text{g}_{\text{gel}}$ were utilized. The assays were carried out at different pH: 7, 8, 9 and 10 ($T = 50$ °C).

Despite alcalase was immobilized on agarose gel particles, the vigorous stirring and high porosity of the gel substantially reduced convective and diffusive mass transport resistances. Therefore, we assumed that we were observing inherent reaction rates in our experimental conditions, i.e. free from mass transfer delays.

For pH control, NaOH 0.2 N or 0.5 N was utilized. For parameter α , the values presented by Adler-Nissen were adopted (1986) (Table 1).

For cheese whey, h_{tot} is 8.8 $\text{meqv}/\text{g}_{\text{protein}} = 8.8$ $\text{mmol}/\text{g}_{\text{protein}}$ (Adler-Nissen, 1986).

Table 1: Calibration values for the pHstat (Adler-Nissen, 1986).

pH	$1/\alpha$
11	1.00
10	1.00
9	1.01
8	1.13
7	2.27
6	13.6 (extrapolation)

RESULTS AND DISCUSSION

From the number of hydrolyzed peptide bonds per gram of gel calculated for the first minutes of reaction of the initial-rate assays, $h_{g0} \times \text{time}$ curves, like the one shown in Figure 1, were built. Initial reaction rates (V_0) were obtained by linear regression.

Making use of the Lineweaver-Burk equation [Equation 5 (Segel, 1975)], k and K_M were estimated, as shown in Figure 2.

$$-\frac{1}{V_0} = \frac{K_M}{k \cdot E} \cdot \frac{1}{N} + \frac{1}{k \cdot E} \quad (5)$$

The results are presented in Table 2. Figures 3 and 4 show them graphically. The point

corresponding to pH 7 (an outlier) was discarded. It is important to note that assays with finely crushed gel particles indicated the absence of diffusion resistance effects. The experimental effectiveness factor η was always essentially equal to unity, for the gel enzymatic load used here.

The curves in Figures 3 and 4 are typical results (activity and K_M vs. pH).

The model was validated against long-term batch assays. Without the inhibition term, the predicted reaction rates were noticeably higher than the experimental. Figure 5 shows the model predictions and the experimental values for the number of peptide bonds hydrolyzed by the enzyme ($N_0 - N$ in $\text{mmol}/l_{\text{solution}}$ with $N_0 = 8.8 \times 60 \times 0.25 = 132$ $\text{mmol}/l_{\text{solution}}$), for one of the five-hour assays in the pHstat.

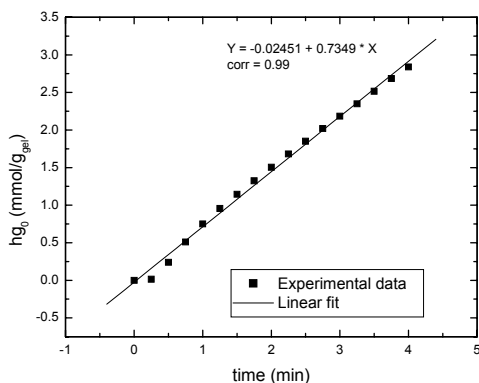


Figure 1: Determination of initial reaction rate of whey protein (60 g/l) hydrolysis by alcalase immobilized on agarose gel particles at pH 10 and $T=50^{\circ}\text{C}$.

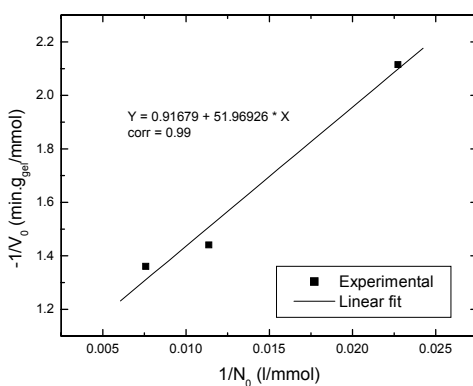


Figure 2: Lineweaver-Burk plot for whey protein hydrolysis by alcalase immobilized on agarose gel particles at pH 10 and $T = 50^{\circ}\text{C}$.

Table 2: Michaelis-Menten kinetic parameters k and K_M ; whey protein hydrolysis by alcalase immobilized on agarose gel particles; $T=50^{\circ}\text{C}$.

pH	$k.E$ (mmol/g _{g_{gel}} min)	k (mmol/U _{BAAE} min)	K_M (mmol/l)
11	1.07	0.068	54.3
10	1.09	0.069	56.7
9	0.86	0.055	47.3
8	0.49	0.031	36.2
7	0.70	0.044	21.1
6	0.34	0.022	28.8

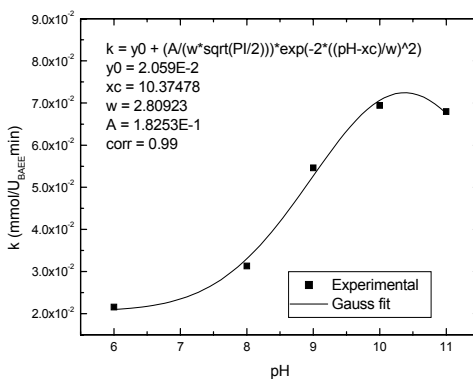


Figure 3: Influence of pH on the kinetic rate constant (k) for whey protein hydrolysis by alcalase immobilized on agarose gel particles at $T=50^{\circ}\text{C}$.

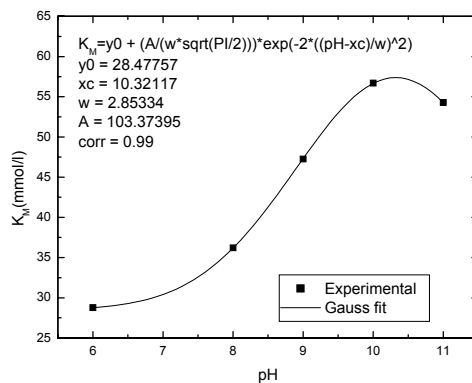


Figure 4: Influence of pH on the kinetic rate constant (K_M) for whey protein hydrolysis by alcalase immobilized on agarose gel particles at $T=50^\circ\text{C}$.

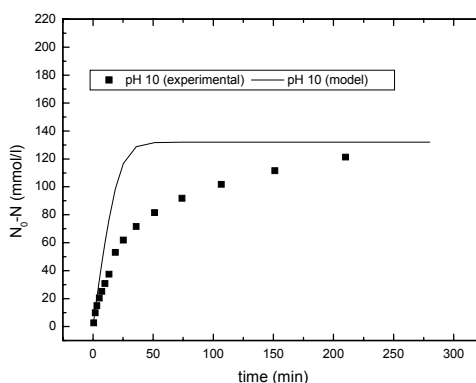


Figure 5: Michaelis-Menten model predictions (without inhibition) and experimental values for the number of hydrolyzed peptide bonds for whey protein hydrolysis by alcalase immobilized on agarose gel particles at pH 10 and $T=50^\circ\text{C}$.

These data were used for adjusting K_I . During the fitting procedure, it was also possible to confirm the consistency of the values previously obtained for K_M and k . Table 3 shows the values of K_I as a function

of pH. Each new acyl-terminal, produced after the hydrolysis of a peptide bond, may link to the enzyme active site (a serine), making a non-productive acyl-enzyme complex. Therefore, $I = (N_0 - N)$.

Table 3: Inhibition parameter K_I ; whey protein hydrolysis by alcalase immobilized on agarose gel particles; $T=50^\circ\text{C}$.

pH	K_I (mmol/l)
10	7.0
9	4.0
8	1.2
7	0.8

Graphically we have

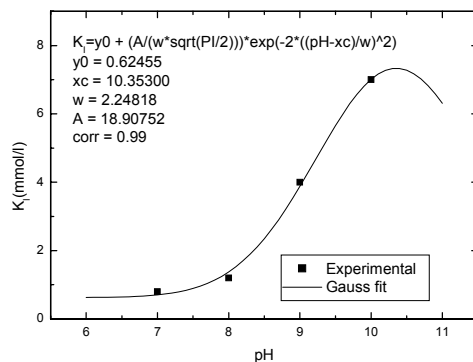


Figure 6: Influence of pH on the inhibition kinetic parameter (K_i) for whey protein hydrolysis by alcalase immobilized on agarose gel particles at $T=50^\circ\text{C}$.

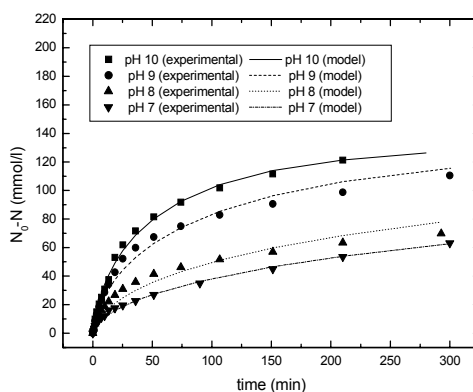


Figure 7: Michaelis-Menten model predictions (with inhibition) and experimental values for the number of hydrolyzed peptide bonds for whey protein hydrolysis by alcalase immobilized on agarose gel particles at pH 10 and $T=50^\circ\text{C}$.

Figure 7 shows the model predictions and the experimental values for the number of hydrolyzed peptide bonds for the long-term assays carried out in the pHstat.

It is possible to observe the very good agreement between model predictions and experimental data.

CONCLUSIONS

By making use of the pHstat technique and the Marquardt algorithm, it was possible to estimate kinetic parameters for the enzymatic proteolysis of cheese whey, using alcalase, multipoint-immobilized on agarose gel particles. A process of competition between the original substrate and the peptides that are continuously produced during the reaction brings about a substantial reduction in the reaction rates of proteolysis. Therefore, neither pure Michaelis-

Menten nor first-order kinetics could represent our data, although these are the two usual forms of rate equation proposed in the literature for enzymatic proteolysis. A kinetic model with product competitive inhibition, however, provided an accurate fit for our experimental data.

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NOMENCLATURE

B base consumption (ml)

DH	degree of hydrolysis
E	enzyme contents in the agarose gel (U_{BAEE}/g_{gel})
h_{tot}	the total number of peptide bonds in the protein substrate (mmol/ $g_{protein}$)
h	the number of hydrolyzed peptide bonds (mmol/ $g_{protein}$)
hg_0	number of hydrolyzed peptide bonds per gram of gel calculated for the first minutes of reaction in the initial rate assays (mmol/ g_{gel})
I	molar concentration of inhibitor (mmol/l)
k, K_M	kinetic model parameter and K_i
MP	mass of protein (g)
N	molar concentration of peptide bonds in the substrate hydrolyzable by alcalase (mmol/l)
N_b	base normality
V	proteolysis reaction rate (mmol/ $g_{gel} \cdot \text{min}$)
α	the average degree of dissociation of α -NH groups
η	measured reaction rate/reaction rate in the absence of mass transfer effects

REFERENCES

- Adler-Nissen, J., *Enzymic Hydrolysis of Food Proteins*. Elsevier Applied Science Publishers (1986).
- Demetrakakes, P., Waste Not, Want Not, *Food Processing*, July, 75-79 (1997).
- González-Tello, P., Camacho, F., Jurado, E., Páez, M.P. and Guadix, E.M., *Enzymatic Hydrolysis of Whey Proteins*, *Biotechnology and Bioengineering*, 44, 523-528 (1994).
- Lopes, G.P., *Estudo do Controle do pH da Hidrólise Enzimática de Soro de Queijo*. Master's thesis, Universidade Federal de São Carlos-São Paulo (2001).
- Mann, E., *Whey Products and Their Uses*, Dairy Industries International, December, 13-14 (2000).
- Marquardt, D.W., An Algorithm for Least-Squares Estimation of Nonlinear Parameters, *J. Soc. Indust. Appl. Math.*, 11, 431-441 (1963).
- Postolache, C. and Oncescu, T., Kinetics of Hemoglobin and Casein Hydrolysis Catalyzed by Alcalase, *Revue Roumaine de Chimie*, 34, 581-584 (1989).
- Segel, I.H., *Enzyme Kinetics*. A Wiley-Interscience Publication (1975).
- Svendsen, I., Chemical Modifications of the Subtilisins with Special Reference to the Binding of Large Substrates. A Review, *Carlsberg Res. Commun.*, 41, 237-291 (1976).
- Tardioli, P.W., *Hidrólise Controlada de Proteínas do Soro Láctico Usando Carboxipeptidase A e Agarose Imobilizadas Multipontualmente em Agarose*. Ph.D Thesis, Universidade Federal de São Carlos-São Paulo (2003).
- Viotto, W.H., *Ultrafiltração de Soro Doce de Queijo Minas Frescal. Efeito de Pré-tratamento do Soro no Desempenho da Membrana de Ultrafiltração e na Composição e Solubilidade do Concentrado Protéico de Soro*. Ph.D. Thesis, Universidade Estadual de Campinas-São Paulo (1993).
- Vorob'ev, M.M., Levicheva, I.Y. and Belikov, V.M., Kinetics of the Initial Stage of Milk Protein Hydrolysis by Chymotrypsin, *Applied Biochemistry and Microbiology*, 32, 219-222 (1996).