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Kinetic-conceptual model of the hydrolysis of bovine plasma proteins - ALCALASE® 2.9L: The role of inhibition by product

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ABSTRACT

In the present work, the inhibitory effect of the peptide fractions, obtained through enzymatic hydrolysis of bovine plasma was evaluated, on the enzyme used in the reaction (Alcalase 2.4 L). In this sense, Ultra-filtered peptide fractions of different molecular sizes (A: Fraction>10; B: Fraction 10-3 kDa; and C: Fraction <3 kDa), were used to verify the impact on the total hydrolysis rate. The Fractions between 3 and 10 kDa were refined to fit a conceptual kinetic model which considers inhibition by product and substrate. Additionally, the inactivation of the enzyme through the reaction time was evaluated and its effects incorporated into the model. It was shown that some peptides released in the successive stages of the reaction can in turn inhibit the activity of the hydrolyzing enzyme. The model evaluated suggests a time-varying expression of inhibition parameters as a function of the initial substrate concentration in the reaction. This is based on the kinetic changes of the product profiles for each reaction time in the evaluated operating conditions (S_0 variable). A greater inhibitory effect due to the products is evidenced when the reaction occurs with a higher load of the initial substrate (S0 = 20 g/L).

1. Introduction

Peptides from protein enzymatic hydrolysis have been proven to have important bioactive potentials [1]. *In vitro* antioxidant and antihypertensive power exerted by peptide fractions from bovine blood proteins hydrolysis with Alcalase (subtilibsin) has been discussed [2,3]. The main proteins in bovine plasma include albumins, immunoglobulins, α- and β-globulins, and fibrinogen, all of which have a significant bioactive potential, and whose hydrolysates are an important source of antioxidant and antihypertensive peptides, when Alcalase is used as enzyme [4,5]. This protease extract is able to recognize a wide variety of amino acids positioned at P1 and P2′ or P3', so it shows broad specificity and enzyme selectivity, being able to hydrolyse a large variety of protein substrates and yielding a hydrolysate with a high small peptides content (Tacias-Pascacio et al., 2020). From a chemical point of view, proteases act as catalysts of peptide bonds hydrolysis in portions of the protein sequence, attending their own specificity, which allows for the release of peptides with a different chemical nature, in time, through a dynamic process that depends on the reaction mechanism [6].

In the case of protein hydrolysis, this mechanism is particularly complex since, despite theoretically, the enzyme is capable of

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splitting all bonds by which it has an affinity, in practice, this breakage efficiency is conditioned to several aspects. Some of such aspects are external and controllable, such as pH, Temperature, and enzyme/substrate ratio (E/S) [7]. However, the probability of the layout of the 20 natural amino acids in the protein chains infers a high degree of chemical complexity in the splitting dynamics from the protease. Therefore, the hydrolysis result is a varied physicochemical, qualitative and quantitative composition [8], which becomes more complex in the case of substrates with several reactivity proteins and different balance nature, like the case of food matrixes or non-purified protein sub-products.

The production of enzymatic hydrolyzates in controlled reaction systems usually occurs at constant temperature and pH, according to the optimal values of the enzyme selected [9]. E/S/ratio and time are set according to the functionalities desired and protein recovery of the final hydrolyzates [10]. As defined by Linderstrøm-Lang Theory, the breakage mechanism may change, in varied substrate conditions, approximating its behavior to a Zipper or a one-by-one mechanism, according to the preference of the enzyme by the original protein chain or by the intermediate fragments derived from the first breakages [11]. About that, it has been proven that enzymatic affinities toward intact proteins were different in Alcalase hydrolysis when the reaction system operated at different concentrations of the initial substrate [12]. These changes in the hydrolysis mechanism are induced by the different concentrations of substrate, but not for the changes in E/S [11]. Due to these different mechanisms, the hydrolyzates produced by hydrolysis at different substrate concentrations have different peptide profiles, and some of these peptides can inhibit proteases [11], slowly causing a modification in the peptide formation dynamics in each substrate condition.

On their analysis of the kinetic mechanisms implied in enzymatic hydrolysis of salmon muscle, Valencia et al. (2014) concluded that the decrease in hydrolysis rate of the reaction (typical shape of the hydrolysis curve), occurs mainly due to the inhibition of the reaction by hydrolysis products and thermal deactivation of the enzyme. Their results indicate that larger peptides have a higher inhibiting effect than smaller fractions. However, the inhibiting effect is not necessarily stronger for low GH compared with the highest ones because this also depends on the concentration of these peptides [13]. The results are relevant when the proposal by Ref. [14] is considered, regarding the reasons why the typical form of the hydrolysis curve is given, which have been discussed by several authors in enzymatic reactions from several protein sources, where it has been suggested that this shape is basically due to: (a) Decrease in the concentration of peptide bonds susceptible to hydrolysis by proteases (depletion of substrate), (b) enzyme inhibition, caused by hydrolysis products; (c) thermal denaturation of the enzyme.

The kinetic models for the study of protein hydrolysis usually assume that the inhibitor joins the ES complex in the region where: $K_m \ll S_0$ and $[S] = S_0$, this allows integrating the phenomenon in the form of an exponential equation, there the reaction velocity is: $r = S_0 h_{tot} * d(GH)/dt = a * \exp(-b * GH)$. Expressions of this nature have been used in batch reactors to explain zero order enzymatic hydrolysis velocity and enzyme deactivation in substrates such as caseins [15], lacto-albumins [16], bovine hemoglobin [17], and bovine plasma [18] whereas enzymatic deactivation and inactivation by substrate, were corroborated by a BSA-trypsin system [19].

Despite, in some cases, inhibition by products is observed in kinetic models analysis, this analysis does not always come with experimental evidence of the phenomenon, and it is not explained, either, to what extent these inhibitors may affect the curve shape [20]. There is an inhibiting effect about it, proven from some peptides formed during protein enzymatic hydrolysis, Valencia et al. (2014), have shown that an adequate strategy for the development of a full kinetic model is given by the analysis of the contributions of these effects on the enzyme's response. These authors propose a strategy in which inhibition constants are determined after adding hydrolyzates to the substrate with a mild (GH = 5 %) and an advanced (GH = 20 %) hydrolysis degree, as an indirect measure of the presence of high and low molecular weight, respectively. It is proposed to analyze this behavior with ultrafiltrates of controlled sizes.

In this paper, it was proposed to validate a kinetic model based on inhibition mechanisms by effects of the substrate and product, and thermal deactivation of the enzyme in presence of the substrate as a strategy to analyze the possible effect of inhibition phenomena on the production of peptides with bioactive potential in variable conditions of initial substrate, preserving the E/S substrate ration fixed. Inhibition was experimentally determined by adding products of molecular weights of controlled size and the influence of the concentration of inhibiting peptides in the response of the enzyme capacity (Alcalase® 2.4 L) to split bovine plasma protein peptide bonds. The model suggests a variable expression of inhibition parameters, based on kinetic changes of the product profiles for each reaction time in each condition of operation tested during the study.

2. Materials and methods

The methodology developed in this work is presented below in three subsections as follows: materials, reagents, and methods used in the experimentation phase, the cases and procedures for the construction of the kinetic model, and the procedure that was carried out for its validation.

2.1. Enzymatic hydrolysis

Fresh bovine blood, provided by a city cold store (Oviedo, Spain), was centrifuged at 5000 RPM for 10 min at 2 °C. The plasma obtained was dialyzed using cellulose dialysis membranes (D9402-100FT, Sigma-Aldrich) in distilled water. Protein concentration of 8.4 ± 0.42 % was determined using modified Lowry's method [21] and then this was diluted with distilled water at the concentrations required in each experimental curve. The reactions were carried out by phases; in Erlenmeyer, of 0.02 L and 0.1 L connected to an automatic pH adjustment system (pH-Buret 24 2s, Crison, Spain) and an isothermal stirrer. Temperature and pH were preserved constant in the optimal values of 60±1 °C, and 9.0, respectively. For hydrolysis, food-grade Alcalase® 2.9 L (2,4 AU-A/g), (EC: 3.4.21.14, CALBIOCHEM, Canada) was used.

The use of plasma without concentration of the protein fraction has been proposed, due to the high presence of these compounds in

total solids (Up to 70 % on a dry basis). Some preliminary fat extraction assays (results not shown) were attempted, but when comparing hydrolysis with whole plasma, no major differences in bond-breaking counted by pH-stat were evident. The agility and economy that the use of plasma implies in the conditions in which it is obtained industrially, provides an opportunity to think about its rapid industrial processing and without the use of prior extraction and/or concentration technologies.

Monitoring of reaction products was carried out with the pH-stat method, using NaOH solutions of 0.1–1.0 M, according to experiment conditions. The <<mmo» of α-NH bonds, split in time, were calculated using equation (1) and the concentration (mM) was estimated according to each volume used.

$$\alpha - \mathrm{NH(mM)} = \frac{V \bullet N}{\alpha} \tag{1}$$

where B is the NaOH volume; N, Molar concentration; and α, a function of pK = f (T, pH) (Figueroa et al., 2016).

Enzymatic activity tests were carried out in non-reactive conditions (dissolving the enzyme in distilled water), and in reactive conditions (dissolving the enzyme in 1 g/L of substrate used in the reaction). The enzyme underwent the same temperature and pH conditions that would be used later in the reaction. Enzyme activity in time was monitored, taking an aliquot of 100 μL until 2 h of reaction. Enzymatic activity was registered separately, using the azocasein method, where 1 mL of azocasein dissolved (0,25 % m/V), is incubated with the enzyme for 5 min at a temperature of 37 °C. The reaction was stopped with the addition of TCA acid at 10 % and the supernatant was collected in NaOH 2 M, after centrifuging at 10,000 g for 5 min absorbance of the sample at 430 nm was registered, used to calculate the relative activity concerning the activity measured in zero time. The inactivation constant (k_d) is estimated with the adjustment of the relative activity in time data (t), at a first-order kinetics (equation (2)).

$$\frac{A}{A_0} = e^{-k_d * t} \tag{2}$$

in order to analyze the influence of the peptide fractions on the initial rate of the reaction at different concentrations of initial substrate (1–20 g/L), ultrafiltered enzymatic hydrolysates of three different molecular sizes were obtained: A (Fraction>10 kDa), B (Fraction 10-3 kDa) and C (Fraction <3 kDa). These hydrolysates were filtered using centrifuge tubes with a MWCO of 3.0 and 10.0 kDa (Macrosep® Advance, Pall Corporation, USA). The object of the experiment was to determine which fraction exerted the greatest inhibitory effect on the initial rate of the hydrolysis reaction. These fractions were added at a concentration of 3.7 mM at the start of the reaction, in a system operated with an initial substrate concentration of 10 g/L (which coincides with the estimated Km value). Fraction B, which inhibits at least 40 % of the reaction rate compared to hydrolysis without inhibitor, was selected for the inhibition tests. It was added at two different concentrations (I1 = 3.735 and I2 = 7.47 mM) for the determination of the kinetic parameters of the reaction with the double reciprocal methodology.

2.2. Hydrolysis kinetic model

Some considerations at the time of establishing the model were: 1) since there are several proteins with different peptide sequences and in different proportions in the system, and considering that the chemical attack of the enzyme is aimed at the peptide bond, considered as substrate, the initial concentration of peptide bonds, defined as its own moles in solution. Alcalase selectivity is related to its own specificity toward specific residues. This selectivity may change with T, pH, and substrate concentration in the operation. T and pH remain constant, and only the effects related to the substrate are analyzed. 2) In each step of the reaction, (an enzymatic attack) only one peptide bond is split, releasing an α-NH group that can be quantified using the pH-stat method. 3) The equation governing the reaction mechanisms includes the joint effects of inhibition by substrate, products, and enzyme deactivation (first order). 4) The product inactivation constant is a function of the breakage, since very breakage releases different peptides, which array a community of different compounds in each time.

The experimental data for the definition of the model were taken at different initial substrate (protein) concentrations from 1 to 20 g/L. This means, concentrations of 41.5–166 mM total peptide bonds. Assuming a value of 8.3 mmol (peptide bonds/g of protein, as the total number of bonds per unit of protein mass [22]. Alcalase concentration (E_0) was the same, with 300 (mg/L), for all the executions.

2.3. Estimation of kinetic parameters

In this analysis, the concentration of bonds produced in hydrolysis was used as the descriptor for the progress of the reaction, instead of the GH, since this provides a feasible alternative for the calculation of constants in consistent units. Kinetic parameters were obtained from the graphic analysis through the development of the classic saturation curve (S_0 Vs v_0) (Valencia et al., 2014). Initial velocities were determined by duplicate, with the pending of the product curve Vs time for the first 7 min of reaction, using the pH-stat method to calculate the concentration of broken bonds. Parameters k_m, k_s, and V_{max} were estimated by non-linear analysis of equation (3), using Matlab's Curve Fitting Tool, with the experimental data of curve S_0 Vs v_0.

$$v_0 = \frac{V_{max} \bullet S_0}{K_m + S_0 + (S_0^2/K_s)} \tag{3}$$

Table 1
Experimental planning to validate the mathematical model proposed.

$[S_0]$ (g/L)	$[S_0]$ (mM)	[S_0 Truly cleavable by Alcalase] (mM)	E_0 (g/L)
5	41.5	8.3	0.2
10	83	16.6	0.4
15	124.5	24.9	0.6
20	166	33.2	0.8

Table 2
Kinetic constants tested by non-linear adjustment of equation (1) with inhibition effects by substrate. Experimental data taken at pH = 9 and T = 60 °C.

Source	V_{max} (mM)	K_m (mM)	K_s (mM)	V_{max}/K_m
Bovine plasma	1.25	83	951	0.015
Valencia et al.; (2014)	2.12	9.54	233	0.058

2.4. Model validation

A set of experiments at different levels of initial substrate was used in the validation, where E:S ratio is kept constant (1:25). Preliminary results have shown that, under these conditions, different peptide profiles are obtained when bovine plasma is hydrolyzed with Alcalase despite values of GH_{final} are statistically non-differentiable (unpublished data). The experimental approach used to validate the model is shown in Table 1.

2.5. Quantification of molecular weights and peptide identification

Quantification of molecular weights in bovine plasma hydrolyzates with Alcalase (2 mg/mL) was carried out with HPLC-SE. a high-resolution liquid chromatographer (Agilent 1200, Agilent Technologies Inc., California, USA.) was used, equipped with a diode matrix UV detector operating at 214 nm, and at room temperature. A Yarra SEC- 2000 (300 × 7.8 mm) column was used, and as mobile phase, water and acetonitrile in a 90/10 (v/v) proportion with 0.03 % TFA (v/v) was used. Samples were eluted with a constant flow of 1 mL/min.

The column was previously calibrated using proteins with different sizes as standard: Ribonuclease A (13,7 kDa), Albumin (44,3 kDa), Thyroglobulin (670 kDa), and triglycine (0,225 kDa), as well as a low molecular weight marker, Glycine (0,075 kDa). The resulting calibration curve showed a determination coefficient $R^2 = 0.96$. to analyze the distribution of molecular weights of the hydrolyzates in the reaction times, the Schedule was divided into 3 characteristic regions of the sizes according to elution time and calibration curve: Fractions PM > 10, 10 > PM > 3, and PM < 3 kDa. The identification of the peptides present in ultra-filtrates lower than 3 kDa of each hydrolyzed fraction was carried out by high-pressure liquid chromatography coupled to mass spectrometry in tandem (RP-UPLC-MS/MS) with a Dionex Ultimate 3000 RS UHPLC (Thermo Fisher Scientific, USA) connected online to a Bruker Impact II Q-ToF mass spectrometer (Bruker, Billerica, MA, USA). Before the analysis, 200 μL of the initial simple were desalted in Millipore ZipTip C18 columns. 5 μL were recovered and taken to a final volume of 25 μL for the analysis. A Bruker Intensity Trio C18 (50 × 2.1 mm, 3 μm) column was used for the analysis. Solvent "A" was composed of MilliQ ultrapure water and formic acid 0.1 % (v/v). Solvent "B" was composed of acetonitrile and formic acid at 0.1 % (v/v), used in a 2 % gradient for 1 min, followed by an increase of 2 %–35 % for a period of 30 min, of 35 % during 1 min, of 35 % at 80 % during 4 min, and finally 80 % of B during 1 min. The injection volume was 2 μL with a flow rate of 150 μL/min. The separation was carried out at 30 °C and the mass spectrums were obtained in the mode of positive ions, *m/z*. Nitrogen was used as drying gas at a flow rate of 6,0 L/min and at 300 °C, and as nebulizing gas at a pressure of 0,31 MPa.

3. Results and discussion

The results of the different stages of the analysis process are described below, indicating the relevant results found in the experimental construction and validation of the model.

3.1. Adjustment of the kinetic model with rigid parameters

The parameters tested are shown in Table 2, compared to those obtained by Valencia et al.; (2014), despite the difference in pH values with which they were obtained. K_s values for the plasma-Alcalase system are substantially higher than those obtained when salmon muscle is used as substrate. This suggests a lower inhibition effect due to substrate, in the case of plasma. The specificity in change (V_{max}/K_m), is lower for the case of bovine plasma, due to the large differences found in K_m values. On similar research with Alcalase and sesame cake protein, a K_m higher than 41,17 g/L is evinced for pH = 8.5 and T = 55 °C [23], a bit lower than the levels used on this study. On the other hand, Sousa Jr et al. (2004), reported $K_m = 47.3$ mM, in an Alcalase system (immobilized) and whey serum proteins.

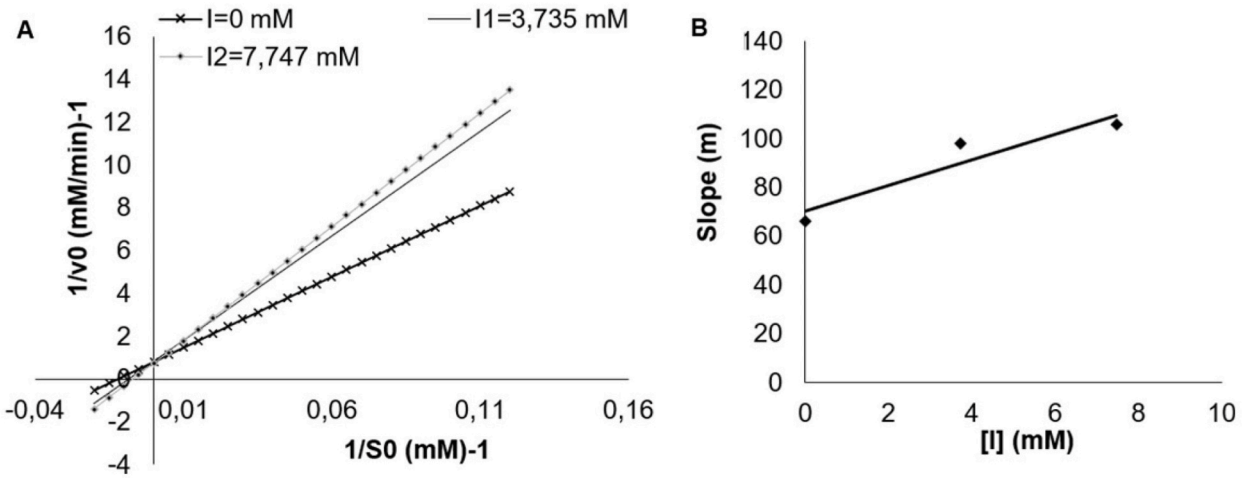

Fig. 1. A) Lineweaver–Burk's graphic for experimental data of bovine plasma hydrolyzate with Alcalase at T = 60 °C and pH = 9.0, without inhibitor, with inhibitor (I_1 = 3.735 e I_2 = 7.47 mM); B) Secondary graph that represents Lineweaver–Burk's curve slope (m) for Ki estimation.

Table 3
Effect of the addition of inhibitors to the Alcalase bovine plasma reaction system on apparent constants to calculate inhibition constant ($R^2 > 0.87$ for all cases).

[I] (mM)	Slope (m)	K_m^{app} (mM)	V_{max}^{app} (mM/min)	K_m^{app}/V_{max}^{app}	K_i (mM)
0.000	66.284	82.432	1.277 ± 0.05	61.964	13.330
3.735	97.884	121.995		97.840	
7.470	|105.65	133.467		106.440	

3.2. The effect of inhibition by product

Alcalase can be inhibited during the reaction similarly to those of several proteases due to the presence of resulting peptides in the hydrolysis. This phenomenon is similar to that, for example, with the Angiotensin converting enzyme (ECA), in the exposition of the hypertensive capacity of peptides resulting from the protein breakage of several sources [24]. For instance, the peptide molar proportion, needed to completely inhibit ECA changes from 26:1 to >1300:1, and it is also known that changes in pH, temperature, and E:S of protein hydrolysis alter the ECA inhibiting activity of hydrolyzates [11], due to the presence of different peptides at different concentrations, according to each operation condition and reaction time.

As proposed, there are three important elements to consider in these inhibition effects: 1) the inhibition source; 2) the inhibitor concentration; and 3) its kinetic mechanism. The typical strategy to study these effects is the addition of inhibitors at different concentrations in the reaction system to assess the effect on K_m and V_{max}. Following the logics of Valencia et al. (2014) and Demirhan et al. (2011), ultra-filtrates of three different molecular sizes were taken: A (Fraction>10 kDa); B (Fraction 10-3 kDa); and C (Fraction <3 kDa). These ultra-filtrates, which only contained peptides in a range of sizes described, were added to different concentrations until obtaining, at least, a 40 % inhibition in the initial velocity values. Larger (A) and smaller (C) fractions had little effect on speed, compared to that obtained with fraction B. Respectively, in the case of fractions A and C, with an addition of 3.7 mM they barely reduced initial velocity 5 and 17 %, measured with substrate concentrations around the value of K_m.

For this reason, fraction B was selected for this exploring phase of the inhibition analysis. Two concentrations of these fractions were used in the study (I_1 = 3.735 e I_2 = 7.47 mM) to analyze the effect on k_m^{app} and V_{max}^{app} in the model of equation (4), through the analysis of curve (v_0 Vs S_0).

$$\frac{\frac{V_{max}}{\left(1+\frac{[I]}{K_i}\right)}[S]}{\frac{K_m}{\left(1+\frac{[I]}{K_i}\right)}+[S]}=\frac{V_{max}^{app}[S]}{K_m^{app}+[S}} \tag{4}$$

Data $1/v_0$ and $1/S_0$ of the plasma curve with and without inhibitor at the two concentrations were used to build Lineweaver-Burk's graphic representation (Fig. 1A), which evinces a typical effect of competitive inhibition. The variations in km^{app}, $Vmax^{app}$ values, and slopes (m) are represented in Table 3, products of the regression analysis that were used in the secondary graph for estimating the inhibition constant (Ki) (Fig. 1B) of the model assumed (competitive inhibition) according to equations (5)–(7).

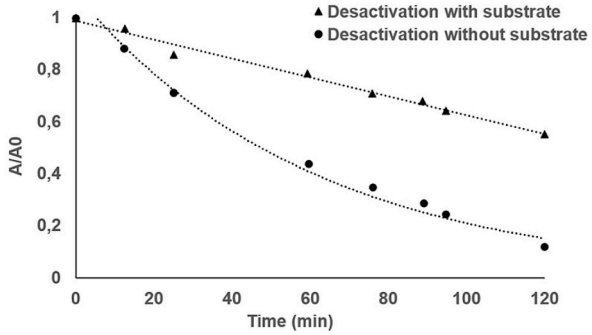

Fig. 2. Enzymatic deactivation of Alcalase 2.4 L with adjustment to first-order kinetics in reactive conditions and in presence of hydrolysis substrate at 1 g/L.

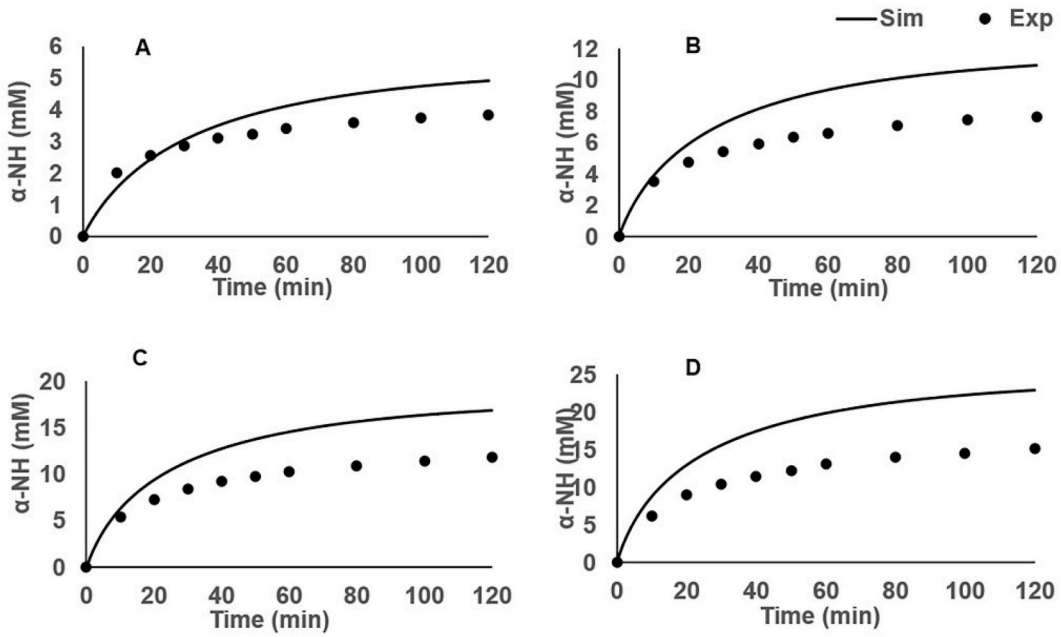

Fig. 3. Validation of the α-amino bonds concentration in time with the model proposed for the case in which the Ki inhibition parameter is constant. Prediction of the model proposed (continuous line), and the experimental data (markers) for different operation conditions A = 5; B = 10; C = 15; and D = 20 g/L of initial substrate.

$$v_0 = \frac{V_{max}[S]}{K_M\left(1 + \frac{[I]}{K_I}\right) + [S]} \tag{5}$$

$$\frac{1}{v_0} = \frac{K_M}{V_{max}}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \tag{6}$$

$$m = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max} \bullet K_I} \bullet I \tag{7}$$

3.3. Enzymatic deactivation and global definition model

The analysis of Alcalse residual activity in time suggests that, indeed, the enzyme undergoes an important thermal deactivation, positively modulated by the presence of substrate in a mild form. Fig. 2, shows the adjustment of the analysis of the enzyme relative activity to a first-order kinetics for both cases: when the enzyme is in an aqueous non-reactive medium, and in presence of the test substrate, bovine plasma at 1 g/L.

The kinetic parameters estimated were used in the definition of the global model shown in equation (8). The model considers the

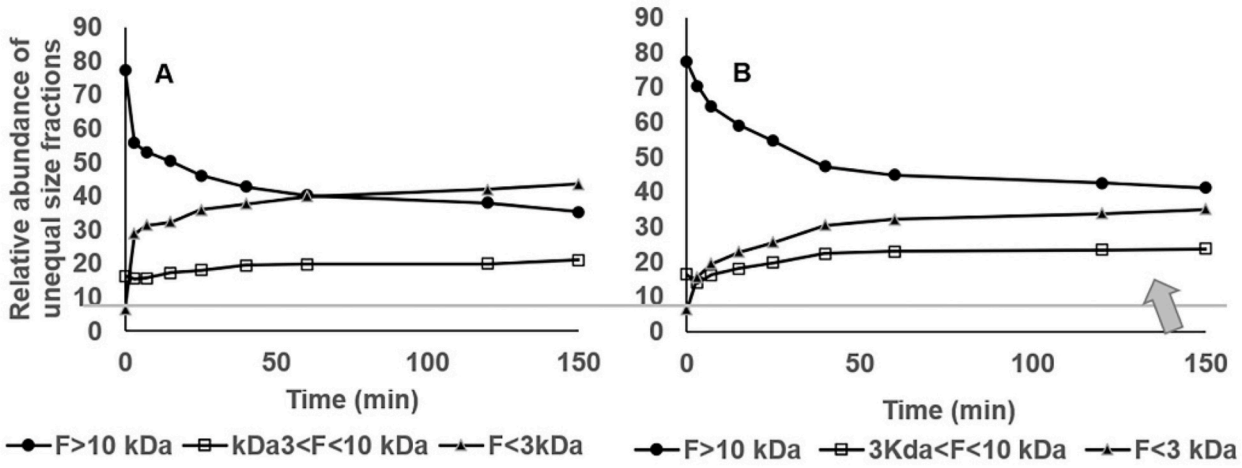

Fig. 4. Molecular weight distribution Vs time for different levels of initial substrate S_0. A) 5 g/L substrate, and B) 20 g/L substrate.

inhibition effects by substrate, competitive inhibition by hydrolysis products, and Alcalase thermal deactivation in time for constant pH and temperature conditions.

Even though K_{cat}, catalytic constants, K_m affinity, k_d enzyme deactivation, K_s and K_i inhibition by substrate and product are actually a function of temperature, these effects are not described in this analysis, since the purpose of this study is to relate the inhibition effects with kinetic parameters under different E/S. the numerical solution of this equation can be compared with experimental data for different E/S relations and analyze the goodness-of-fit.

$$\frac{dS}{dt} = -\frac{K_{cat} \bullet E_0 \bullet e^{-k_d \bullet t} \bullet S}{K_m\left[1+\frac{P}{K_i}\right] + S\left[1+\frac{S}{K_s}\right]} \tag{8}$$

Alcalase has great specificity, as it is capable of splitting around a wide number of amino acids. Especially when Glu, Met, Leu, Tyr, Lys, and Gln are located in the P1 position of the catalytic site [25]. External phenomena such as the role of pH, Temperature, substrate concentrations, and inhibition effects, among others, lead to think that, definitively, not all bonds present in the substrate are effectively and actually breakable by the enzyme. An extended hydrolysis experiment worked as the basis to establish that no more than 20 % of total bovine plasma bonds break in the pH, T (constant), and E/S (variable) conditions tested within the validation experiment set. This means, out of the 8.3 mmol/g, corresponding to the total amino bonds of the plasma, only 20 % is likely to be hydrolyzed by Alcalase in the conditions given.

This means that the initial bonds' concentration range (used for the construction of adjustment curves) actually hydrolysable is 1.66–33.2 mM. It is convenient to use these values of real concentrations and express the curves based on this analysis. On Fig. 3(A-D), the model validation curves are represented, using the numerical solution of equation (8) in a routine programmed on MATLAB, with the estimated values of the kinetic constants, Vs experimental data of hydrolysis carried out under the conditions described on Table 1. It is possible to analyze that there is a certain adjustment level in the first hydrolysis stage for all hydrolysis conditions. However, this capacity of the model to predict the response of the α-amino bonds released is better when less initial substrate is used in all the system. Graphically, it is possible to notice that, when the initial substrate is 5 g/L protein, the model predicts with a satisfactory adjustment the first 30 min of reaction. From that time, it starts to overestimate the data of products generated. In contrast, when the initial substrate concentration is 20 g/L, the model adjusts only for the first 5 min. This behavior is the same for intermediate concentrations tested (10 and 15 g/L) with approximate satisfactory adjustment times of 10 and 7 min respectively.

Each time the enzyme splits a peptide bond, different chemical nature peptides are generated in the process. When changes in pH and temperature are registered, it is normal to expect changes in the enzymatic action mechanism, for reasons widely explained in extended scientific literature [26]. However, only a few scientific papers have approached the changes of these mechanisms when initial substrate conditions are modified, preserving the E/S ratio invariable. An indication of this change in the mechanism was found in bovine lacto-whey hydrolysis by Alcalase, where a graphic of the percentage of intact proteins against GH was different for the different concentrations of initial substrate [11,27]. Assuming that one of the reasons of this change in product profile was due to enzymatic inhibition phenomena, the production route of these compounds at every level of operation substrate is expected to be different. Therefore, it is highly likely that heterogeneous peptides, present at different concentrations, have different inhibitory effects.

The results of the molecular weights' distribution of the enzymatic hydrolyzates for different concentrations of initial substrate in the reaction time are shown in Fig. 4. Data indicates that at a higher split velocity of the large-size fractions, compensated with a proportional appearance of small-size fractions, is evinced when the initial substrate in the reaction is lower (Fig. 4A with a substrate load of 5 g/L), when compared with the estimated proportion when the load of initial substrate is higher (Fig. 4B with a substrate load of 20 g/L). According to the effects of lack of adjustment of the model, the proportion of intermediate-size products (3–10 kDa), is each

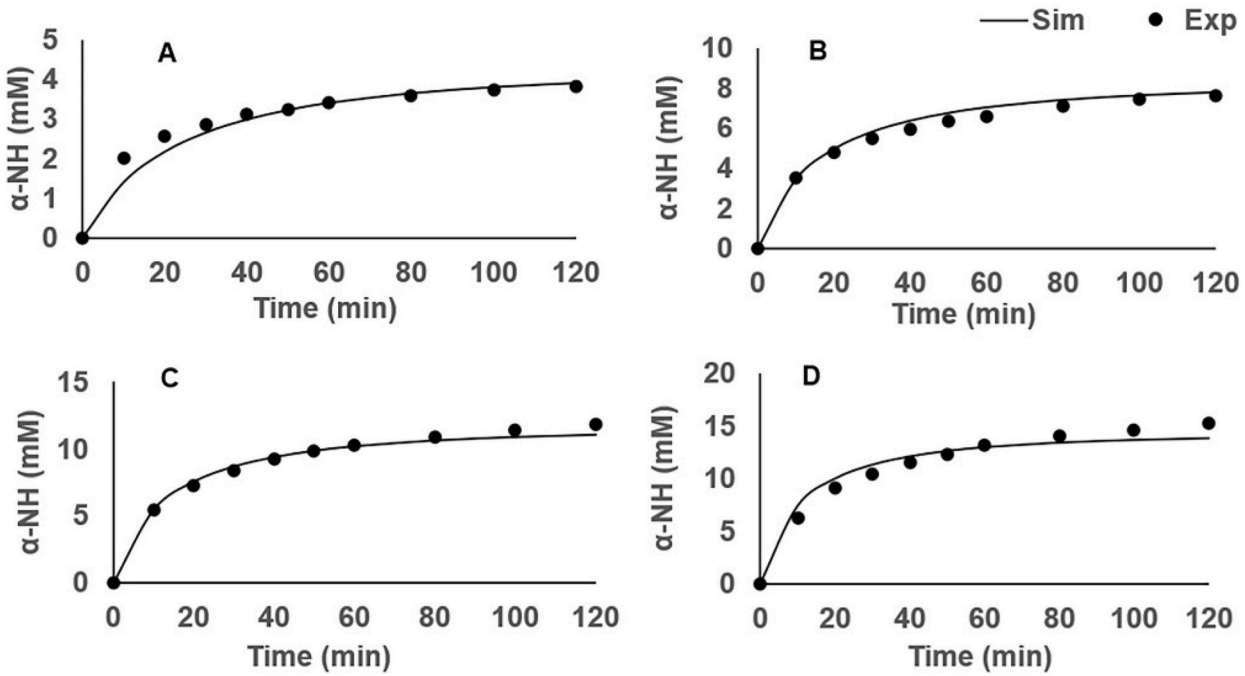

Fig. 5. Validation of the α-amino bonds concentration in time with the model proposed for the case in which the K_i inhibition parameter is a function of the bond breakage. Prediction of the model (continuous line). Experimental data (markers). For the different initial substrate conditions: A = 5; B = 10; C = 15; and D = 20 g/L of initial substrate.

Table 4
Some of the peptides identified in the hydrolyzed fractions (obtained with GHs_{max}) with different concentrations of initial substrate F1, F2, F3, and F4 (S = 5,10,15, and 20 g/L respectively). In silico ACE inhibitory activity (AHTpin; https://webs.iiitd.edu.in/raghava/ahtpin/help.php) and mean relative abundance of each sequence in the different fractions.

Sequences	ECA- Inhibition Capacity (SVM)	Origin Fraction	Relative abundance (%)
VSLPEVPGEY	(2.48)	F5	0.53
GEVLPLPEANFPSF	(2.02)	F5	0.04
LRPVPPPISGGGY	(1.79)	F10	0.30
		F15	0.30
		F20	1.09
VSFTLPRSPTSQE	(2.32)	F5	0.31
YNPDIIKVK	(2.77)	F10	0.03
		F15	0.03
LSGQLPSPSKPVPF	(1.85)	F5	0.15
		F10	0.81
		F15	0.81
		F20	1.04
SPDLEPVLRY	(1.79)	F5	0.02
		F10	0.03
		F15	0.03
		F20	1.04
EAPSLRPVPPPISGGGY	(1.71)	F20	2.8

time lower in the reaction system as the initial operation substrate increases. In practice, these intermediate-size products have a higher inhibitory effect on the plasma-Alcalase hydrolysis reaction. Therefore, it is highly likely that this concentration increase generates the inhibition effects that can be the reason of the lack of adjustment of the model in the consecutive phases of the reaction.

This implies that the inhibition constant, expressed as a rigid value, may not be enough to explain the phenomenon. Valencia et al. (2014), has warned that inhibition by product and enzymatic deactivation of the enzyme phenomena explain the typical form of the hydrolysis curve, given that the variety and quantity of products generated at each time are different, the inhibition constant is expected to be different as the reaction advances. A strategy for the definition of a variable expression of the K_i constant, recognizes that this has a change proportional to the fraction of relative α-amino bonds split: $K_i \propto (S_0–P/S_0)$.

Judging by the satisfactory adjustments of the model in the initial phase of the reaction, K_i must change from its initial value estimated, to a different <<L» value for each substrate level, due to the variability of inhibitors in the reaction, for each substrate

concentration tested, which means that this change is a function of the substrate concentration in the form of equation (9), with linear β adjusted with other experimental data (10).

$$K_i = K_{i0} - (\text{Lineal function } S_0 \ll \beta \gg) \left(\frac{S_0 - P}{S_0}\right) \tag{9}$$

$$\beta = 0.07 * S_0 + 3.25 \tag{10}$$

This auxiliary equation (9) is used in expression (8) to complete the model that assumes the changes in values of the constants as the reaction takes place. The results of the validation of the model with the adjustments made are shown in Fig. 5 (A-D). The new results indicate that the model, with the variations suggested, is capable of satisfactorily predicting the experimental results throughout the whole reaction time for the different initial substrate conditions tested with $R^2 \geq 0.95$ in all cases. Under these conditions, changes in inhibition constants for each operation condition tested, expressed as an interval, regarding the K_{i0} initial value, are: $S_0 = 5$ g/L $[K_{i0} - 0.3K_{i0}]$; $S_0 = 10$ g/L $[K_{i0} - 0.18K_{i0}]$; $S_0 = 15$ g/L $[K_{i0} - 0.11K_{i0}]$ $S_0 = 20$ g/L $[K_{i0} - 0.07K_{i0}]$.

The results indicate that the peptides produced after hydrolysis with Alcalase can have the potential to inhibit the reaction at certain stages. Whereas, understanding these effects in the mathematical model may help to understand the optimal substrate conditions to produce peptides with Biological properties. Table 4 shows some of the peptides identified in each fraction (<3 kDa) obtained after hydrolysis with different initial substrates: $S_0 = 5$, 10, 15, and 20 g/L (F5, F10, F15, and F20, respectively). Evidently, peptides with an important capacity to inhibit ACE is potentiated in the fractions obtained with 5 g/L of initial substrate, but a higher relative abundance has other less active peptides in fractions with a higher load, like those obtained from 20 g/L of initial substrate. Therefore, as mentioned above, peptides with the potential to inhibit ACE may potentially be involved in inhibiting effects on the hydrolyzing enzyme. These results provide evidence which shows that, indeed, peptides of varied nature and reactivity persist in the reaction according to the operating conditions analyzed (different initial substrate concentrations).

4. Conclusions

The results indicate that the model explains enzymatic hydrolysis of bovine plasma proteins with Alcalase, includes the joint effects of inhibition by substrate, competitive by product, and thermal deactivation of the enzyme, positively modulated by the presence of the substrate. The effect of hydrolysis products inhibition is essential in the experimental behavior adjustment. Intermediate molecular weight fractions have a major inhibition effect on the enzyme. This effect is different in every condition of initial substrate (S_0) tested, as long as the (E/S) ratio is preserved in system. A higher inhibiting effect by products occurs when the reaction takes place with a higher load of initial substrate ($S_0 = 20$ g/L). For a fixed (E/S) ratio, inhibition constants are related to substrate conversion, which is explained because varied physicochemical nature peptides appear and disappear in time as the reaction advances until reaching a set of different peptides and in relative concentrations also varied for every substrate level used. This peptide community exerts a different inhibitory action before the enzyme in each operation condition.

CRediT authorship contribution statement

Omar A. Figueroa: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Jader D. Alean:** Formal analysis, Writing – original draft. **Ismael Marcet:** Formal analysis, Writing – original draft. **Manuel Rendueles:** Conceptualization, Formal analysis, Resources. **José E. Zapata:** Conceptualization, Formal analysis, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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