Current Enzyme Inhibition, 2019, *15*, 144-153

RESEARCH ARTICLE

Kinetic Models to Produce an Antioxidant by Enzymatic Hydrolysis of Bovine Plasma Protein Using a High Substrate Concentration

Nathalia A. Gómez[1,*], Leidy J. Gómez[1] and José E. Zapata[1]

[1]*Nutrition and Food Technology Group, Food Engineering Department, Faculty of Pharmaceuticals and Food Sciences, University of Antioquia, Medellín, Colombia*

ARTICLE HISTORY

Received: July 27, 2019
Revised: October 05, 2019
Accepted: October 07, 2019

DOI:
10.2174/1573408015666191009090742

Abstract: ***Background*:** The animal blood that is produced in a slaughterhouse is a potential source of inexpensive proteins used in the food industry around the world. However, 60% of it is surplus, and it ends with a negative environmental impact.

***Introduction*:** The enzymatic hydrolysis of proteins represents a good way to produce peptides with different biological activities.

***Methods*:** Enzymatic hydrolysis of bovine plasma with subtilisin at an alkaline pH and 61.5°C was performed using the pH-stat method. Experiments were conducted considering the effects of a high initial substrate concentration (So) and the enzyme/substrate ratio (E/S) minimizing the processing time necessary to obtain a specific degree of hydrolysis (DH).

***Results*:** The best conditions obtained were 42 g/L of So and 0.89 AU/g substrate of E/S until a DH of 20% in 11,1 ± 1,1 min was achieved to the tested conditions, which result in a fitted empirical polynomial equation of degree 3.

***Conclusion*:** A kinetic equation is established to relate the DH and the reaction time to a relative error of less than 5% in the fit, to obtain a good antioxidant product in an industrially interesting time. Additionally, the results suggest a good adjustment of the data with a determination coefficient (R2) of 0.9745 in validation.

Keywords: Antioxidants, degree of hydrolysis, enzymatic hydrolysis, kinetic model, pH-Stat method, protein hydrolysates.

1. INTRODUCTION

The animal blood that is produced in a slaughterhouse is a potential source of inexpensive proteins used in the food industry around the world [1]. One of the alternatives for the implementation of bovine blood derivatives is represented by the enzymatic hydrolysis of proteins, which may generate peptides with different biological activities [2]. Bovine plasma proteins are recognized as a valuable source of many biologically functional substances [3], that can display the inhibitory activity of Angiotensin-converting enzyme (ACE) [4], antigenotoxic activity [5], antioxidant activity [6], antimicrobial activity [7], and another potential use [8]. Different proteases, such as pepsin, trypsin and chymotrypsin, have been used to hydrolyze proteins in order to produce peptides that possess special bioactivities [9].

The process of enzymatic hydrolysis of a native protein with high molecular weight is complicated; therefore empirical kinetic models are applied to describe their hydrolytic behavior.

Some empirical kinetic models have been established in the different enzyme-protein systems such as milk whey protein-trypsin [10], bovine hemoglobin protein-subtilisin [11], casein-trypsin [12], and bovine plasma-subtilisin [13].

On the other hand, some phenomenological models based on semi-physical equations have been determined due to the great interest to understand in a quantitative way, the behavior of a process. Those models have been defined in the different enzyme-protein systems such as the chickpea protein - subtilisin [14], bovine hemoglobin protein-subtilisin [8], whey protein-subtilisin [15], camel and bovine milk proteins - pancreatic enzyme [16], egg white protein - pepsin [17], and fish protein-subtilisin [18].

The kinetic model of the hydrolysis for protein in the bovine plasma-subtilisin system with a substrate concentration of 4 to 8 g/L has been reported as zero-order kinetics with inhibition per substrate. In previous work, Figueroa and colleagues (2012) identified the presence of peptide fractions from bovine plasma hydrolysates with good antioxidant ac-

*Address correspondence to this author at the Nutrition and Food Technology Group, Food Engineering Department, Faculty of Pharmaceuticals and food sciences, University of Antioquia, Medellín, Colombia; Tel/Fax: +57-4-2195476, +57-316-865-9748; E-mail: nathalia.gomez@udea.edu.co

1875-6662/19 $58.00+.00

tivity in a DH of 20%, which is important to focus the hydrolysis reaction in order to obtain this DH [19].

The purpose of this work was to adjust a kinetical and an empirical predictive model where the input variables were the highest substrate concentration possible and the enzyme/substrate ratio required to obtain the smaller hydrolysis time in order to achieve a DH of 20% ($t_{DH20\%}$) in the experimental region. Additionally, the antioxidant capacity through 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was evaluated. Likewise, the antioxidant capacity of hydrolysate, Ascorbic acid and Trolox was compared, the last two of which are commercial antioxidants, through a kinetic reaction to compare the binding capacity of bovine plasma hydrolysates (HPB) with ABTS* radicals.

2. MATERIALS AND METHODS

2.1. Materials

Bovine plasma was purchased from a commercial supplier in Colombia. Plasma is defined as a liquid with a protein content of 7.0 ± 0.2%. Subtilisin (E.C. 3.4.21.62 with a specific activity of 2.45 ± 0.07 AU/g enzyme from *Bacillus Licheniformis*) (Novo Nordisk Co., Denmark). Sodium hydroxide (NaOH) analytical grade was used. Albumin from bovine serum, reference A7030, EC No. 232-936-2, was from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Testing chemicals, like Ascorbic acid, were purchased from Panreac Chemical S.A. (Barcelona, Spain); Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, and 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). All the other chemicals and reagents used were analytical grade. Ascorbic acid was prepared and used within one hour.

2.2. Determination of Protein Content

The protein concentration of the bovine plasma and the hydrolysate product was determined by the Biuret method [20]. The standard curve was constructed using bovine albumin. The spectrophotometric measurements were done using a UV-Vis spectrophotometer Genesys 10S. The absorbance was measured at 37°C at a wavelength of 540 nm.

2.3. DH Determination

DH is defined as the ratio of the number of peptide bonds cleaved and is expressed as hydrolysis equivalents (h), in relation to the total number of peptide bonds before hydrolysis (h_{tot}).

$$DH(\%) = \frac{h}{h_{tot}} \times 100 \quad (1)$$

The DH during enzymatic reactions of bovine plasma with subtilisin was determined through the pH-stat method. The base consumption may be related to the percentage of DH according to the Equations (1) and (2) [20]:

$$DH(\%) = \frac{V_B N_B}{M_p \alpha h_{tot}} \times 10 \quad (2)$$

where:

V_B- Base volume consumed (L); N_B- Concentration of the base (eqv/L); M_P- Mass of the protein (kg); α- The degree of dissociation of the group's α-NH (amino groups released in the reaction). This value is given as a function of the pH and the reaction temperature of the process. The value used is 0.9918 and it is calculated with the Equations (3) and (4) [21].

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

$$pK = 7{,}8 + \frac{298 - T}{298.T} 2400 \quad (4)$$

h_{tot}- Calculated from the amino acid analysis by summing the moles of each individual amino acid per gram of bovine plasma, in this case, is used as the value of 8.3 eqv/kg that has been reported in the literature for blood proteins [21].

2.4. Enzymatic Hydrolysis of Bovine Plasma Protein

Bovine plasma with a protein concentration of 22 g/L, 32 g/L and 42 g/L in distilled water was dissolved. The hydrolysates were prepared according to the method described by Liu *et al*. (2009) [22]. The hydrolysis reaction in a bioreactor with a working volume of 1 L, adding an enzyme/substrate ratio (E/S) between 0.25-0.98 AU of subtilisin per g of bovine plasma protein at 61.5°C, 230 rpm was performed through a factorial design 3^2 (explained below). A pH of 9 was kept stable by adding a 2 M NaOH solution using an automatic potentiometric titrator Titrando 842 (Metrohm, Switzerland) controlled by a computer (software Tiamo 1.2.1). During the reaction time, the antioxidant capacity was measured. Six samples with different hydrolysis degrees (0; 3.8; 6.7; 10.8, 15.3 and 20%) were taken. The enzymatic reaction when the hydrolysis degree was 20% was stopped [23], inactivating and thermally denaturing the enzyme at 85°C in a water bath for 10 min after the hydrolysis [21]. Experiments were carried out in triplicates.

2.5. ABTS* Capacity

This method was used as described by Re *et al*. (1999) based on the capacity of a sample to inhibit the ABTS radical (ABTS*) compared to a standard antioxidant reference (Trolox®) [24]. The ABTS* radical was generated by a chemical reaction between potassium persulfate (2.45 mM) and ABTS (7 mM). The working solution was prepared by taking a volume of the previous solution and diluting it in a phosphate buffer solution (PBS) 5mM at pH 7.4 until the absorbance at λ=732 nm was 0.70 ± 0.02.

One milliliter of the ABTS* radical was mixed with 100 mL of the sample or standard and it was left in a dark place at 30°C for 30 min. Afterwards, the absorbance (732 nm) was measured. The Trolox calibration curve was prepared in

Table 1. Variables and levels used in the design 3^2.

Factors	Levels		
So (g/L)	22	32	42
Eo/So (AU/g substrate)	0.25	0.61	0.98

a concentration range of 0-250μM, and the absorbance value obtained for the samples was interpolated to calculate the concentration in Trolox equivalents (μM TE); the results were reported as micromoles of Trolox equivalents per gram of protein (μmol TE/g of protein).

In addition, a calibration curve with a solution of ABTS* was developed with a concentration between 0.00-2.45 mM at λ=732 nm, with the aim to obtain a comparison of the antioxidant capacity of Ascorbic acid 0,02 mg/mL, Trolox 0,10 mg/mL and the sample of HPB (0,16 mg/mL) with the best antioxidant capacity. Here, it is possible to determine the binding capacity of HPB with ABTS* radical. Finally, the solutions of HPB (0.0-0.2 mg/mL), Ascorbic acid (0.0-0.05 mg/mL) and Trolox (0.0-0.1 mg/mL) were prepared to calculate the half-maximum inhibitory capacity concentrations (IC50%) for each antioxidant analyzed.

2.6. Experimental Design to Evaluate the Effect of So and Eo/So on Hydrolysis Time to Achieve a DH of 20% ($t_{DH20\%}$)

Thirteen experimental runs were performed according to the factorial experimental design, central composite with response surface of two factors and three levels, and five replicates in the center point, which are shown in Table **1**. The effects of the factors (So and Eo/So) on the response ($t_{DH20\%}$: hydrolysis time to achieve a DH of 20 %) were evaluated through the analysis of variance (ANOVA).

From the results, a model quadratic was obtained to determine the best conditions of the So and the E/S in order to minimize the $t_{DH20\%}$ and maximize the initial substrate concentration (So), in the enzymatic hydrolysis process of bovine plasma.

2.7. The Kinetics of the Enzymatic Hydrolysis of Proteins

Phenomenological modeling to analyze the kinetics mechanism of the hydrolysis reaction was used. The reaction mechanism of protein enzymatic hydrolysis for the substrate inhibition and the enzyme inactivation can be modeled [17] to determine whether these effects occur under the analyzed working conditions:

$$E + S \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_2} E + P \tag{5}$$

$$ES + S \underset{k_{-3}}{\overset{k_3}{\Leftrightarrow}} SES \tag{6}$$

where:

E,S- Free enzyme and substrate; ES- Intermediate enzyme-substrate complexes; P- Final product of the enzymatic reaction; k_1, k_{-1}, k_2, k_3, k_{-3}- Reaction rate constants. The corresponding reaction rate depends on the irreversible step:

$$v = So\frac{d(DH)}{dt} = k_2[ES] \tag{7}$$

It is assumed that the balanced reaction is in a steady state when the variables in the kinetic of hydrolysis are constants, therefore from the mass balances for the ES and SES complex, simultaneously with the combinations of Eqs. 5 and 7, and the Eqs. 6 and 7, leading to the kinetics (Equations 8-11) for the inhibition process are given by:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] \rightarrow$$

$$\frac{d[ES]}{dt} = k_1[E][S] - [ES](k_{-1} - k_2) \rightarrow \tag{8}$$

$$[ES] = \frac{k_1[E][S]}{k_{-1} - k_2}$$

$$K_M = \frac{k_{-1} + k_2}{k_1} \rightarrow [ES] = \frac{[E][S]}{K_M} \tag{9}$$

$$\frac{d[SES]}{dt} = k_3[S][ES] - k_{-3}[SES] = 0 \rightarrow \tag{10}$$

$$[SES] = \frac{k_3}{k_{-3}}[S][ES]$$

$$K_{is} = k_{-3}/k_3 \rightarrow [SES] = \frac{[S][ES]}{k_{is}} \tag{11}$$

If [S]=So and the total enzyme concentration (e) at a given moment is expressed as $e = E + ES + SES$ e = E + ES + SES, the e concentration is reduced to:

$$e = [E] + \frac{[E][S]}{K_m} + \frac{[S]^2[E]}{K_m K_{is}} \rightarrow$$

$$[E] = \frac{eK_m}{K_m + S + S^2/K_{is}} \tag{12}$$

where

K_m- Michaelis-Menten coefficient; K_{is}- Inhibition constant kinetic $(K_{is} = k_{-3}/k_3)$.

If includes all the complexes formed with e_o during the reaction and the kinetic equation for the enzymatic inhibition process is related to the Eq.12, integrating the resulting equation 13, provides the Eq. 14 [25]: (DH: 0 to DH, e: e_0 to e).

$$-d(DH) = \frac{K_m + S + S^2/K_{is}}{k_1 . S^2 . K_m} . \frac{1}{e} de \quad (13)$$

$$e = e_0 . \exp\left(\frac{-k_1 . S^2 . K_m}{K_m + S + S^2/K_{is}} . DH \right) \quad (14)$$

From here, the relationship between Eqs.7, 12 and 14 makes it possible to obtain the following equation for the reaction rate:

$$v = \frac{k_2 e_0}{K_m + S + S^2/K_{is}} . \exp\left(\frac{-k_1 . S^2 . K_m}{K_m + S + S^2/K_{is}} . DH \right) \quad (15)$$

Eq. 15 is a mathematical equation based on the enzymatic action mechanism [26], which provides an exponential relationship between the reaction rate, the substrate concentration (S) and the initial concentration of the enzyme (e_0). The best concentration profile using MATLAB software was simulated. The fit was developed using the function ode45 by the Runge-Kutta method.

2.8. Statistical Analysis

All the tests of the DH determination were conducted per triplicate. The data results of ABTS* capacity are presented as mean ± standard deviation. The Design-Expert® 8.0.5 (Stat-Ease, USA) was used for the generation and analysis of the design's data. The statistical significance of the regression coefficients and the interaction between the different independent variables was tested through the analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

3.1. Effects of Different Hydrolysis Degrees Over the Antioxidant Capacity (ABTS)

As expected, the DH of bovine plasma protein increased with time during the hydrolysis. In Table **2**, it has been observed that the antioxidant capacity increases as a function of DH.

These results agree with previous studies [19, 22]. Seo *et al.* (2015) evaluated the radical scavenging activity of bovine plasma proteins through the DPPH method [13] which is another way to evaluate the antioxidant capacity of hydrolysate. They concluded that the antioxidant capacity improved gradually with the increase of the hydrolysis time in mackerel protein hydrolysates. According to their results, the largest antioxidant capacity was obtained with 20,46% of DH.

Table 2. Antioxidant activity (ABTS) to different DH.

DH (%)	CA (µmol ET/g)
0.0	12.53 ± 0.09[a]
3.8	53,63 ± 1,90[b]
6.7	62.28 ± 0,26[c]
10.8	62,96 ± 0,25[c]
15.3	65.43 ± 0.58[d]
20.0	65.71 ± 1,12[d]

[a-d] Each super index has a different significance ($p<0.05$).

The antioxidant capacity of HPB (0.16 mg/mL) with a DH of 20 %, Ascorbic acid (AA: 0.02 mg/mL) and Trolox (0.1 mg/mL) versus the time it takes to decrease of the amount of ABTS* was determined and compared (Fig. **1A**). The results in the kinetic shown in Fig. (**1B**), suggest that the antioxidant capacity of HPB in the first 5 minutes of the reaction with the ABTS* is lower than the reaction rate with AA and Trolox, a moment in which it rapidly starts its action as an antioxidant. The antioxidant capacity of AA and Trolox was stable until values lower than 50%, in which both finished their antioxidant function. However, the result also suggests that the antioxidant capacity of HPB with 20% of DH continues their capacity of binding the ABTS* radicals until it reaches a 65.83% value.

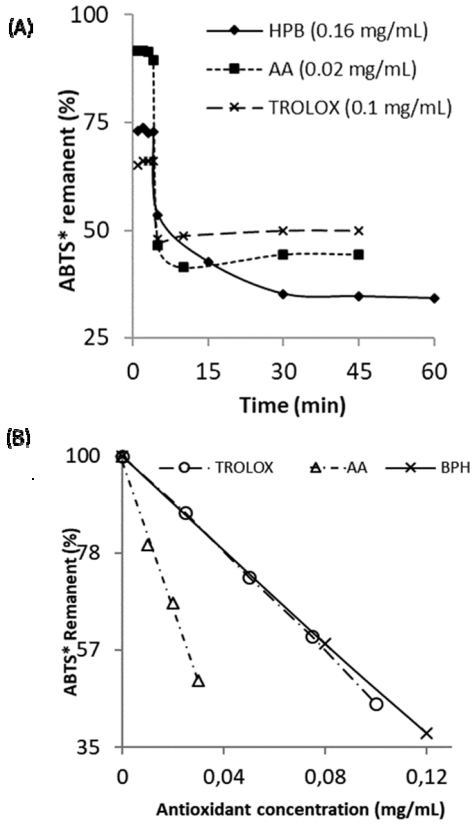

Fig. (1). Consumption of ABTS derived radicals following the addition of Trolox, AA and HPB, (**A**) as a function of the time and (**B**) as a function of the antioxidant concentration.

Table 3. Factorial design 3^2 of the enzymatic hydrolysis of bovine plasma.

Assays	Eo/So (AU/g)	So (g/L)	Time (min)
1	0.98	42	11,69
2	0.61	32	19,89
3	0.98	22	6,07
4	0.61	42	21,97
5	0.98	32	8,62
6	0.61	32	16,52
7	0.25	42	*
8	0.61	32	13,52
9	0.61	22	16,29
10	0.61	32	17,98
11	0.25	22	26,79
12	0.61	32	14,74
13	0.25	32	49,47

* With these conditions for So and Eo/So, it was not possible a DH equal 20%.

Table 4. Analysis of variance (Valor P) factorial design.

Source (X_i)	Value-P
Model	0.0001
Eo/So	<0.0001
So	0.1492
Eo/SoxSo	0.0021
Eo/So^2	0.0004
Eo/So^2xSo	0.0085
Lack of fit	0.5276
R^2	0.9745
Adjusted R^2	0.9533

Moreover, the same binding capacity of ABTS* radicals versus the concentration of HPB, AA and Trolox was analyzed. It was determined that the IC50 for HPB (0.097 mg/mL) is similar to IC50 of Trolox (0.092 mg/mL) and 3.23 times the IC50 of AA (0.030 mg/mL). Trolox is a synthetic antioxidant of reference with an excellent activity [27-30]. The HPB is a natural hydrolysate that can be used as an antioxidant in different processes, especially in the food industry.

3.2. Effects of So and Eo/So on $t_{DH20\%}$

All hydrolyses were conducted until a DH of 20 % was achieved, which is the DH with the highest antioxidant capacity (Table **2**) to obtain the peptide fractions of interest [31, 19]. The factorial design was developed in order to evaluate the effects of So and Eo/So on the $t_{DH20\%}$ as shown in Table **3**, with the experimental runs being performed randomly and the values of the response variable being obtained in each run. The results of the ANOVA of the factorial design are presented in Table **4**, where the value P is assigned to each factor, indicating statistical significance over the response variables. The analysis of variance ANOVA (Table **4**) indicated that the model is significant and that it describes appropriately the response under the operating conditions.

The usefulness of that model is to fix and to control the start operating conditions for the reaction, and to predict the response from the polynomial equation and to obtain a plot that describes the behavior of the hydrolysis regarding the So and Eo/So.

The ANOVA indicates that the Eo/So is the most significant factor for the reaction time, given that the linear and quadratic terms show a highly significant effect with $p<0.001$. In the experimental work range, a cubic effect and interaction between Eo/So and So show a statistically significant effect, with $p<0.0001$, while the So alone has no statistically significant effect, which indicates that this variable alone does not influence a possible enzymatic inhibition during the reaction.

In the same way, Table **4** shows a significant effect of interactions between Eo/SoxSo and Eo/So^2xSo in the enzymatic hydrolysis of bovine plasma within the experimental range. This means that with different substrate concentrations and the same enzyme/substrate ratio, the time to achieve the DH was different. This result indicates that a complex relationship exists between S_0 and $t_{DH20\%}$.

Table 5. The best treatment predicted for the process of hydrolysis and experimental time.

Factor	Optimal Value	Predicted Time (min)	Experimental Time (min)
Eo/So (AU/g So)	0.89	9,6	11,1 ± 1,1
So (g/L)	42		

Data were fitted into a third-order polynomial equation (Eq.16). The analysis of variance (ANOVA) showed that the representative polynomial fit the experimental data very well, with an R^2 of 0.9745. This indicates that the obtained model describes the influence of the independent variables on the time of the enzymatic hydrolysis of bovine plasma protein at a satisfactory percentage of 97%.

$$t_{DH20\%} = -65.981 + \left(5.169 \times \frac{Eo}{So}\right) + \left(4.690 \times So\right) - \left(0.286 \times \frac{Eo}{So} \times So\right) - \left(0.088 \times \frac{Eo^2}{So}\right) + \left(0.004 \times So\right) \quad (16)$$

Fig. (**2**) shows the graphed behavior of the response according to the two factors (So and Eo/So). A strong effect of Eo/So can be seen in comparison with the effect of So in accordance with the adjusted model. An inverse relationship between the Eo/So and the response can also be seen, which is to be expected because the higher the concentration of enzyme, the greater the catalytic effect. Therefore, less time is required to reach a DH of 20 %.

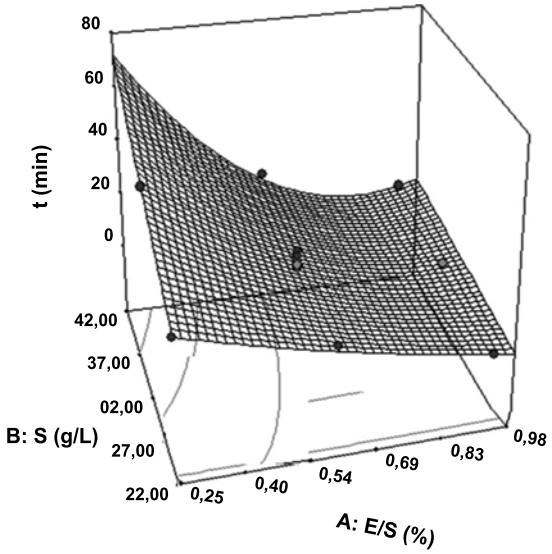

Fig. (2). The response surface for the effects of So and Eo/So on the $t_{DH20\%}$.

3.3. Optimization of the Production Process

The model of Equation 12 was established in order to predict the value of the factors that minimize the $t_{DH20\%}$ and maximize the So, which is important to achieve greater commercial viability of the process of enzymatic hydrolysis of bovine plasma factors.

Table **5** shows the local minimum value of time and the values of the factors defined. These results can be verified in Fig. (**2**), where the lowest reaction time is achieved for the values of So and Eo/So shown (Table **5**), in the experimental region.

To confirm the validity of the proposed mathematical model, three experimental runs under the best conditions were performed. The results presented in Table **5** are the average of these values. The comparison between the predicted time and the experimental time has an absolute error of +1,5 min, indicating that the experimental time is longer than the time that was predicted by the model. However, these results serve to corroborate that the data simulated by the model are close to the experimental data, so the model can be used to predict times of hydrolysis in order to establish conditions for the Eo/So and the So.

The optimal conditions of reaction time (11,1 ± 1,1 min) compared to other works about the same hydrolysis of bovine plasma protein with subtilisin is nearly 35 times lower than other researchers [13]. Additionally, these results show that the predicted conditions are adequate for the hydrolysis of bovine plasma protein, with conditions that are reported and compared with experimental results. It is a simple methodology in terms of implementation to understand the effect of the input variables on the system and to achieve smaller hydrolysis time, without using the largest amount of enzyme, which is reflected in the process costs.

3.4. The Kinetic Modeling of Protein Enzymatic Hydrolysis

The experimental design shows an important effect of interactions between the So and the Eo/So on the estimation of $t_{DH20\%}$.

In Fig. (**3A**), the curves of enzymatic hydrolysis are shown using different concentrations of substrate and a constant enzyme/substrate ratio, indicating that the reaction is slower when the substrate concentration increases without effect in the initial reaction rate at different substrate concentrations. This result showed that concentrations between 22 and 42 g/L, the inhibition by substrate was so significant in the production of a hydrolysate of bovine plasma. Due to the higher substrate, the rate decreases and the $t_{DH20\%}$ is higher. Fig. (**3B**) shows that a higher enzyme/substrate ratio causes the reaction to occur faster and consistent with its normal behavior.

According to the time-course for the hydrolysis curves shown in Fig. (**3**), the values of the reaction kinetic constants k_1, k_2, K_m, and K_{is} (Table **6**) were obtained and calculated

using a non-linear regression analysis (applying MATLAB software) according to the exponential equation (Eq. 15).

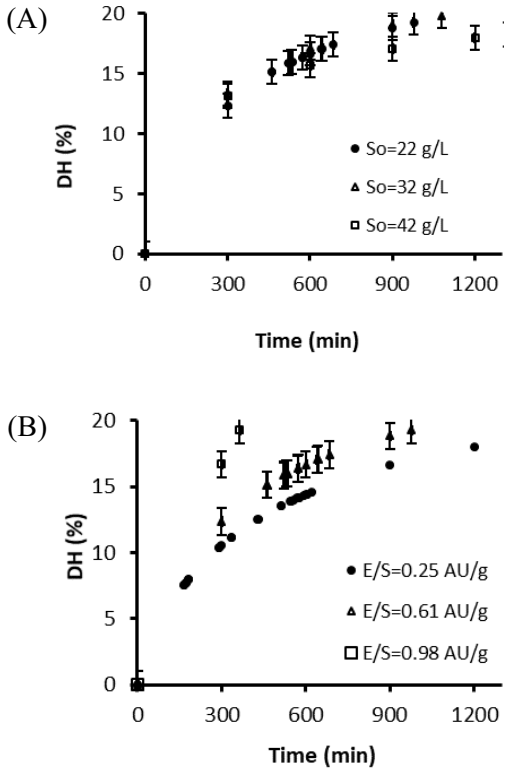

Fig. (3). Hydrolysis curves for **A**) different substrate concentrations with an Eo/So: 0.61 AU/g substrate (Up) and **B**) different enzyme/substrate ratios with the So: 22 g/L (Down).

It has been observed (Table **6**) that k_2 is 2.5 times greater than k_1, that means the dominant reaction in the system is the production of the hydrolysate, it is in accordance with the statement of Michaelis Menten of a reaction in the stable state.

Table 6. Determination of kinetic parameters k_1, k_2, K_{is}, and K_m of Eq. 8.

Kinetic Constants	Value
k_1	8,2467
k_2 (g^2/L.min)	20,3215
K_m (g/L)	0,1003
K_{is} (g/L)	0,1816

Then, the possible Eq. 15 is presented as:

The values of K_{iS} and K_m are low as compared with the value substrate concentration. If K_m is the value that equals the concentration of the substrate when the reaction rate reaches a value equal to half of the maximum speed, therefore, it indicates that the reaction is really fast, which can be observed with the value of reaction time obtained. In addition, if K_m represents the affinity that the enzyme has for the substrate, then the affinity of subtilisin by bovine protein is high.

The values of K_{iS} are low compared with the value of substrate concentration in this reaction which is a small effect of inhibition by the substrate. The constant k_2 is the actual kinetic constant of the reaction, also known as the number of the substrate molecule where a molecule of the enzyme can convert into a product. In the hydrolysis of bovine albumin serum, Qi and He (2006) obtained K_M of 0.0748 g/L, K_{iS} of 7.961 g/L and a k_2 of 38.439 g^2/L.min, but they worked with substrate concentration less than 2.5 g/L and with another enzyme [32]. In agreement with other research, on enzymatic hydrolysis of bovine plasma with a substrate concentration maximum of 10 g/L and the values of K_m lower than the value of K_{iS}, they also concluded to obtain the inhibition by substrate effects in the hydrolysis [33].

A good arrangement (R^2: 0.9932) demonstrated that Eq.17 can represent the proposed reaction kinetic model of the bovine plasma protein-subtilisin system to tested conditions, then the kinetic behavior of the reaction may be due to the substrate inhibition (Fig. **4**).

3.5. Hydrolysis Curve Fitting and Kinetic Model Application

The differential equation (Eq. 17) was solved through numerical methods (ode 15s) using these values for the kinetic constants (Table **6**) at different working conditions (varying Eo/So). The theoretical hydrolysis curves that correspond to the different values of the So such as 22 g/L, 32 g/L, and 42 g/L (Eo/So= 0.25, 0.61, and 0.98 AU/g) were obtained experimentally.

The fitting results and the experimental data are shown in Fig. (**4**). The sum of squares due to the error (SSE) between the calculated values and the experimental data was less than 0.019 corresponding to an R^2 of 0.9932, which demonstrated again that the proposed reaction mechanism and phenomenological kinetic model are reasonable.

The obtained hydrolysis curves are typical in the enzymatic hydrolysis of food proteins [18, 34], such that the reaction begins with a rapid kinetic behavior and then tends to reach a steady-state phase. Previous investigations [11] have indicated that a decrease in the rate of hydrolysis reaction generally responds to the following four factors:

$$\frac{d(DH)}{dt} = \frac{20.3215 \times e_o}{0.1003 + S_o + S_o / 0.1816} \exp\left(- \frac{8.2467 \times S_o^2 \times 0.1003}{0.1003 + S_o + S_o^2 / 0.1816} (DH) \right) \quad (17)$$

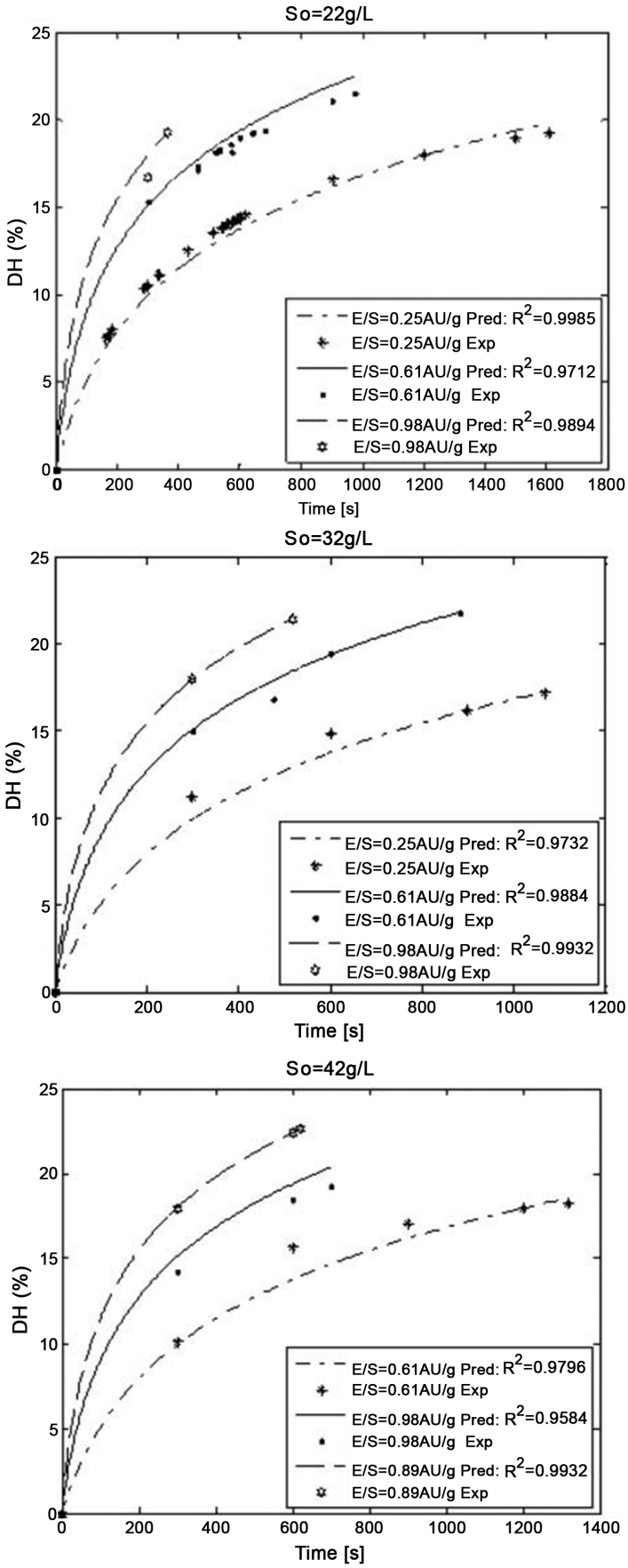

Fig. (4). Fitted and simulated curves of hydrolysis of bovine plasma protein under optimal conditions.

(a) A decrease in the concentration of the peptide bonds susceptible to hydrolysis by proteases,

(b) inhibition of the enzyme by substrate,

(c) the thermal denaturation of the enzyme [14, 35] and

(d) the possible inhibition of the enzyme by hydrolysis products [18].

These three curves show that when the substrate concentration is increased, the time to achieve a specific DH also increases. Besides, the substrate concentration changes rate [d(DH)/dt] with the decreased time, which was the base of the proposed model (Eq. 17).

In Fig. (**4**), for each substrate concentration, the DH increases when an enzyme/substrate ratio is increased. This condition permits that a greater soluble protein concentration can hydrolyze as far as DH values are obtained [36], considering that high enzyme concentrations reduce the loss of denaturalized protein which is precipitated after hydrolysis as the reaction increases the formation of small peptides that represent more COO^- and NH_3^+ groups [37, 38].

Meanwhile, the kinetic model can also be used to predict the $t_{DH20\%}$ in the bovine plasma protein-subtilisin system at different substrate and enzyme concentration values under eligible pH and temperature conditions.

In Table **6**, it can be seen that, according to the values of kinetic parameters, the hydrolysis reaction of bovine plasma protein favored the cleavage of peptide bonds to produce the HPB in a smaller time because the k_2 value is higher than the other kinetic parameters. Although K_{is} is similar to K_m, it is higher among both. These results may indicate that the first hypothesis is the factor acting most strongly in this case, according to the phenomenological kinetic model demonstrated.

CONCLUSION

The time to achieve a DH of 20% (tDH20%) at the hydrolysis of protein of bovine plasma is affected significantly by the enzyme-substrate ratio, which can be calculated through an equation polynomic with a determination coefficient (R2) of 0,9745. It is possible to predict the kinetics of hydrolysis reaction only with the control of the initial substrate concentration and the initial concentration of the enzyme through an exponential mathematical relationship. This equation shows the subtilisin undergoing an inhibition by substrate under the experimental conditions of this work. The production of the hydrolysate of HPB with antioxidant capacity accompanied by an IC50 similar to a commercial antioxidant such as ascorbic acid and Trolox, had to be achieved in a time period of 11,1 ± 1,1 min.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

The authors acknowledge the financial support of the University of Antioquia, Ruta N Corporation and COLCIENCIAS, with its program of support to the national scientific community through of national doctoral program and its call No. 757 of 2016 for the financial support in the execution of the project.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Isaza, J.M.; Londoño, L.M.; Restrepo, D.; Cortes, M.; Suárez, H. Producción y propiedades funcionales de plasma bovino hidratado en embutido tipo salchichón. *Rev. Colomb. Cienc. Pecu.,* **2010**, *23*(2), 199-206.

[2] Friedman, M. Nutritional value of proteins from different food sources. A review. *J. Agric. Food Chem.,* **1996**, 44, 6-29. [http://dx.doi.org/10.1021/jf9400167]

[3] Wanasundara, J.; Ronald, P.D.; Pegg, B.; Phyllis, J.S. *Value added applications for plasma proteins from the beef processing industry*; Can Meat Sci Assoc, **2003**, pp. 10-15.

[4] Wu, Z.; Pan, D.; Zhen, X.; Cao, J. Angiotensin I-converting enzyme inhibitory peptides derived from bovine casein and identified by MALDI-TOF-MS/MS. *J. Sci. Food Agric.,* **2013**, *93*(6), 1331-1337. [http://dx.doi.org/10.1002/jsfa.5894] [PMID: 23015408]

[5] Park, K.; Hyun, C. Antigenotoxic effects of the peptides derived from bovine blood plasma proteins. *Enzyme Microb. Technol.,* **2002**, *30*, 633-638. [http://dx.doi.org/10.1016/S0141-0229(02)00024-8]

[6] Naso, L.G.; Lezama, L.; Valcarcel, M.; Salado, C.; Villacé, P.; Kortazar, D.; Ferrer, E.G.; Williams, P.A.M. Bovine serum albumin binding, antioxidant and anticancer properties of an oxidovanadium(IV) complex with luteolin. *J. Inorg. Biochem.,* **2016**, *157*(157), 80-93. [http://dx.doi.org/10.1016/j.jinorgbio.2016.01.021] [PMID: 26828287]

[7] Adoui, F.; Boughera, F.; Chataigne, G.; Chihib, N-E.; El Hameur, H.; Dhulster, P.; Zidoune, M.N.; Nedjar-Arroume, N. A simple method to separate the antimicrobial peptides from complex peptic casein hydrolysate and identification of a novel antibacterial domains within the sequence of bovine alfa-casein. *Int. Rev. Chem. Eng.,* **2013**, *5*(2), 179-187.

[8] Benítez, R.; Ibarz, A.; Pagan, J. Protein hydrolysates: Processes and applications. *Acta Bioquim. Clin. Latinoam.,* **2008**, *42*, 227-236.

[9] Kechaou, E.S.; Dumay, J.; Donnay-Moreno, C.; Jaouen, P.; Gouygou, J-P.; Bergé, J-P.; Amar, R.B. Enzymatic hydrolysis of cuttlefish (Sepia officinalis) and sardine (Sardina pilchardus) viscera using commercial proteases: Effects on lipid distribution and amino acid composition. *J. Biosci. Bioeng.,* **2009**, *107*(2), 158-164. [http://dx.doi.org/10.1016/j.jbiosc.2008.10.018] [PMID: 19217554]

[10] Margot, A.; Flaschel, E.; Renken, A. Empirical kinetic models for tryptic whey-protein hydrolysis. *Process Biochem.,* **1997**, *32*, 217-223. [http://dx.doi.org/10.1016/S0032-9592(96)00060-X]

[11] Marquez, M.; Vazquez, M. Modeling of enzymatic protein hydrolysis. *Process Biochem.,* **1999**, *35*(1), 111-117. [http://dx.doi.org/10.1016/S0032-9592(99)00041-2]

[12] He, Z.M.; Qi, W.; He, M.X. A novel exponential kinetic model for casein tryptic hydrolysis to prepare active peptides. *Chin. J. Chem. Eng.,* **2002**, *10*, 562-566.

[13] Seo, H-W.; Jung, E-Y.; Go, G-W.; Kim, G-D.; Joo, S-T.; Yang, H-S. Optimization of hydrolysis conditions for bovine plasma protein using response surface methodology. *Food Chem.,* **2015**, *185*, 106-111. [http://dx.doi.org/10.1016/j.foodchem.2015.03.133] [PMID: 25952847]

[14] Marquez, M.C.; Fernandez, V. Enzymic hydrolysis of vegetable proteins: Mechanism and kinetics. *Process Biochem.,* **1993**, *28*(7), 481-490. [http://dx.doi.org/10.1016/0032-9592(93)85032-B]

[15] Sousa, R., Jr; Lopes, G.P.; Tardioli, P.W.; Giordano, R.L.C.; Almeida, P.I.F.; Giordano, R.C. Kinetic model for whey protein hydrolysis by alcalase multipoint - immobilized on agarose gel particles. *Braz. J. Chem. Eng.,* **2004**, *21*(02), 147-153. [http://dx.doi.org/10.1590/S0104-66322004000200003]

[16] Salami, M.; Yousefi, R.; Reza, M.; Dalgalarrondo, M.; Chobert, J-M.; Haertlé, T.; Hadi, S.; Akbar, A.; Niasari-Naslaji, A.; Akbar, A. Kinetic characterization of hydrolysis of hydrolysis of camel and bovine milk proteins by pancreatic enzymes. *Int. Dairy J.,* **2008**, *18*, 1097-1102. [http://dx.doi.org/10.1016/j.idairyj.2008.06.003]

[17] Ruan, Ch.; Chi, Q.Y.J.; Zhang, R.D. Kinetics of hydrolysis of egg white protein by pepsin. *Czech J. Food Sci.,* **2010**, *28*, 355-363. [http://dx.doi.org/10.17221/228/2009-CJFS]

[18] Valencia, P.; Pinto, M.; Almonacid, S. Identification of the key mechanisms involved in the hydrolysis of fish protein by Alcalase. *Process Biochem.,* **2014**, *49*, 258-264. [http://dx.doi.org/10.1016/j.procbio.2013.11.012]

[19] Figueroa, O.A.; Zapata, J.E.; Gutierrez, G.A. Modelamiento de la cinética de hidrólisis enzimática de proteínas del plasma bovino. *Rev EIA,* **2012**, *17*, 71-84.

[20] Guadix, A.; Guadix, E.M.; Páez-Dueñas, M.P.; González-Tello, P.; Camacho, F. Technological processes and methods of control in the hydrolysis of proteins. *Ars Pharm,* **2000**, *41*(1), 79-89.

[21] Adler-Nissen, J. *Enzymatic hydrolysis of food proteins*; Elsevier Applied Science Publishers: London, UK, **1986**.

[22] Liu, Q.; Baohua, K.; Jiang, L.; Cui, X.; Lui, J. Free radical scavening activity of porcine plasma protein hydrolysates determined by electron spin resonance spectrometer. *Lebensm. Wiss. Technol.,* **2009**, (42), 956-962. [http://dx.doi.org/10.1016/j.lwt.2008.12.007]

[23] Gómez, L.J.; Figueroa, O.A.; Zapata, J.E. Actividad antioxidante de hidrolizados enzimáticos de plasma bovino obtenidos por efecto de Alcalasa 2.4 L. *Inf. Tecnol.,* **2013**, *24*, 33-42. [http://dx.doi.org/10.4067/S0718-07642013000100005]

[24] Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.,* **1999**, *26*(9-10), 1231-1237. [http://dx.doi.org/10.1016/S0891-5849(98)00315-3] [PMID: 10381194]

[25] Dunn, I.J.; Heinzle, E.; Ingham, J.; Pfenosil, J.E. *Biological Reaction Engineering. Dynamic Modelling Fundamentals with Simulation Examples,* 2nd ed; Wiley-VCH: Federal Republic of Germany, **2003**. [http://dx.doi.org/10.1002/3527603050]

[26] Gonzàlez-Tello, P.; Camacho, F.; Jurado, E.; Páez, M.P.; Guadix, E.M. Enzymatic hydrolysis of whey proteins: I. Kinetic models. *Biotechnol. Bioeng.,* **1994**, *44*(4), 523-528. [http://dx.doi.org/10.1002/bit.260440415] [PMID: 18618786]

[27] Berg, R.; Haenen, G.; Berg, H.; Bast, A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem.,* **1999**, *66*, 511-517. [http://dx.doi.org/10.1016/S0308-8146(99)00089-8]

[28] Arts, M.J.; Haenen, G.R.; Voss, H-P.; Bast, A. Antioxidant capacity of reaction products limits the applicability of the Trolox Equivalent Antioxidant Capacity (TEAC) assay. *Food Chem. Toxicol.,* **2004**, *42*(1), 45-49. [http://dx.doi.org/10.1016/j.fct.2003.08.004] [PMID: 14630129]

[29] Pisoschi, A.M.; Cheregi, M.C.; Danet, A.F. Total antioxidant capacity of some commercial fruit juices: Electrochemical and spectrophotometrical approaches. *Molecules,* **2009**, *14*(1), 480-493. [http://dx.doi.org/10.3390/molecules14010480] [PMID: 19158657]

[30] Jung, M.Y.; Min, D.B. ESR study of the singlet oxygen quenching and protective activity of Trolox on the photodecomposition of riboflavin and lumiflavin in aqueous buffer solutions. *J. Food Sci.,* **2009**, *74*(6), C449-C455. [http://dx.doi.org/10.1111/j.1750-3841.2009.01230.x] [PMID: 19723181]

[31] Gómez, L.J.; Zapata, J.E. Effects of hydrolysis and digestion *in vitro* on the activity of bovine plasma hydrolysates as inhibitors of the angiotensin I converting enzyme. *Braz. Arch. Biol. Technol.,* **2014**, *57*, 386-393. [http://dx.doi.org/10.1590/S1516-89132014005000004]

[32] Wei, Q.; Zhimin, H. Enzymatic hydrolysis of protein: Mechanismand kinetic model. *Front. Chem. China,* **2006**, *3*, 308-314.

[33] Figueroa, O.A.; Zapata, J.E.; Sánchez, C.P. Optimization of enzymatic hydrolysis of proteins Bovine plasma. *Inf. Tecnol.,* **2016**, *27*(2), 39-52. [http://dx.doi.org/10.4067/S0718-07642016000200006]

[34] Lamsal, B.; Jung, S.; Johnson, L. Rheological properties of soy protein hydrolysates obtained from limited enzymatic hydrolysis. *LWT-Food Sci Techn,* **2007**, *40*(7), 1215-1223. [http://dx.doi.org/10.1016/j.lwt.2006.08.021]

[35] Demirhan, E.; Apar, D.K.; Özbek, B. Sesame cake protein hydrolysis by alcalase: Effects of process parameters on hydrolysis, solubilisation and enzyme inactivation. *Korean J. Chem. Eng.,* **2011**, *28*, 195-202. [http://dx.doi.org/10.1007/s11814-010-0316-2]

[36] Klompong, V.; Benjakul, S.; Kantachote, D.; Shahidi, F. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. *Food Chem.,* **2007**, *102*, 1317-1327. [http://dx.doi.org/10.1016/j.foodchem.2006.07.016]

[37] Witono, Y.; Windrati, W.S.; Taruna, I.; Afriliana, A.; Assadam, A. Characteristics and sensory analysis of ketchup and sauce products from "Bibian" fish hydrolysate. *Am. J. Food Sci Techn,* **2014**, *2*(6), 203-208. [http://dx.doi.org/10.12691/ajfst-2-6-6]

[38] Witono, Y.; Taruna, I.; Windrati, W.S.; Azkiyah, L.; Sari, T.N. Wader (Rasbora jacobsoni) protein hydrolysates: Production, biochemical and functional properties. *Agric. Agric. Sci. Procedia,* **2016**, *9*, 482-492. [http://dx.doi.org/10.1016/j.aaspro.2016.02.167]