Vol. 18, No. 3 (2019) 1063-1071
Revista Mexicana de Ingeniería Química

TIME-EFFICIENT pH-BASED METHOD TO QUANTIFY THE ACTIVITY OF ALKALINE AND HALO-ALKALINE PROTEASES

MÉTODO RÁPIDO BASADO EN pH PARA CUANTIFICAR LA ACTIVIDAD DE PROTEASAS ALCALINAS Y HALOALCALINAS

R.B. Martínez-Pérez[1,3,†], M.A. Camacho-Ruiz[2,†], I. Estrada-Alvarado[3], V. Armenta-Pérez[1], R.M. Camacho-Ruiz[1*]

[1]*Biotecnología Industrial, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ), Zapopan 45019, Jalisco, México.*
[2]*Departamento de Fundamentos del Conocimiento, CUNorte, Universidad de Guadalajara, Colotlan 46200, Jalisco, México.*
[3]*Departamento de Biotecnología y Ciencias Agroalimentarias, Instituto Tecnológico de Sonora, Cd Obregón 85000, Sonora, México.*

Received: September 26, 2018; Accepted: November 27, 2018

Abstract

Proteases from haloalkaliphilic microorganisms are biocatalyst with a great interest to be studied in biotechnological implications. Nevertheless, available methods to assay haloalkaliphilic proteases on protein substrates are few, are carry out to end point and implies the use of complicated steps. This work proposes the development and validation of a real time protease assay. We found pairs of indicator-buffer useful to carrying out the tests at pH of 7.4, 8.2, 9.7 and 10.9. Sodium caseinate, proposed as substrate, was soluble in all the conditions tested. The addition of salts to the reaction medium was explored; being feasible to carry out test up to 4 M of NaCl and 2 M of KCl. The linearity, accuracy, quality and sensitivity of the proposed method were validated. Quality measurements were obtained, with a *Z Factor* greater than 0.5, in tests performed using 40 ng×mL^{-1} of enzyme, in addition to a linear response to the biocatalyst concentration. The method is developed in microplate format, what makes it a time efficient method to be used for alkaline and halophilic proteases but also for non halophilic ones.

Keywords: protease activity, haloalkaline, protease screening, casein, pH-indicator.

Resumen

Las enzimas proteolíticas de microorganismos haloalcalifílicos son biocatalizadores de gran interés para ser estudiadas en implicaciones biotecnológicas. Sin embargo, los métodos disponibles para el ensayo de proteasas haloalcalinas sobre sustratos proteicos son escasos, se llevan a cabo a punto final e implican diversos pasos para cuantificar la actividad enzimática. Este trabajo plantea el desarrollo y validación de un método de monitoreo de actividad de proteasas en tiempo real. Se encontraron pares de tampones-indicadores útiles para la realización de los ensayos en niveles de pH de 7.4, 8.2. 9.7 y 10.9. El caseinato de sodio, propuesto como sustrato, fue soluble en todas las condiciones probadas. Se exploró la adición de sales al medio de reacción, siendo factible realizar ensayos hasta 4 M de NaCl y 2 M de KCl. Se validó la linealidad, exactitud, calidad de las mediciones y sensibilidad del método planteado. Se lograron mediciones de buena calidad, con un *Factor Z* mayor a 0.5, en ensayos realizados utilizando desde 40 ng×mL^{-1} de enzima, además de una respuesta lineal a la concentración del biocatalizador. Otra ventaja del método es que, al desarrollarse en formato microplaca, permite realizar múltiples ensayos en poco tiempo tanto de proteasas clásicas como de proteasas alcalinas y halofílicas.

Palabras clave: actividad proteasa, haloalcalino, cribado de proteasas, caseína, indicador de pH.

1 Introduction

Time efficient and sensitive methods are essential in biotechnology in different areas as virology, cancer research or fermentation process (Wiegmann *et al*., 2018; Kang *et al*., 2018; Zhou *et al*., 2017) among others. Enzymatic activity measurements are essential in biotechnological process. One of the most used enzymes are proteases, which are used at elevated temperatures, pH and ionic strength. Proteases are enzymes that hydrolyze peptide bonds of polypeptides or proteins to release small peptides and amino acids (Rawlings *et al*., 2012). They represent one of the most valuable industrial enzymes, extensively exploited

* *Corresponding author. E-mail*: rcamacho@ciatej.mx; † These authors have contributed equally.
https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n3/Martinez
issn-e: 2395-8472

commercially in food, pharmaceutical, leather, bakery, textile, silk, brewing, photography, bioremediation, dairy, biosynthesis and detergent industry (Coelho *et al*., 2016; Contesini *et al*., 2018; Tovar-Jimenez *et al*., 2017). These kinds of enzymes are ubiquitous and are found in a wide diversity of sources such as plants, animals, and microorganisms (Rao *et al*., 1998).

Microorganisms like halophiles or alkalophiles are adapted to more than one environmental stressor and allows them to survive under saline and alkaline conditions and produce alkaline proteases. These kinds of proteases with high stability (salt, pH, temperature, among others) are efficient biocatalyst to be used in varied biotechnological implications and are in high demand (Raval *et al*., 2014).

Due to the importance of proteolytic enzymes, a great number of methods are available to assay protease activity on protein and synthetic peptide substrates (Charney *et al*., 1947; Götze *et al*., 2011; Bremmer *et al*., 2012). In the last decade, scientists dedicated to the discovery, production, optimization and purification of alkaline, halo-alkaline and halo-tolerant proteases have been using casein as substrate to quantify protease activity, an example is the thermostable alkaline protease from *Yarrowia lipolytica* (Hernández-Martínez *et al*., 2017; Lopez-Flores *et al*., 2016) and, to a lesser extent, have also used other substrates such as azo-casein, hemoglobin and chromogenic peptides (Supplementary Fig. 1). Proteolytic assays using protein substrates such as casein, azo-casein and hemoglobin are carried out by discontinuous sampling and they require multiple steps (Coelho *et al*., 2016; Cupp-Enyard 2008). One of the drawbacks associated with the use of such methods is that the unreacted substrate must be precipitated with a strong acid (TCA 5-10%). It is necessary to incubate the sample for a while to allow the precipitate to form (usually 1 hour at room temperature or with cold shock at 4 or -20 °C) and centrifuge to remove the precipitate. Generally, it is also required to neutralize the assay solution before quantifying the peptides released. Another major problem with these methods is that discontinuous sampling does not allow detection of lag phase or reaction saturation easily, so the enzymatic activity could be underestimated.

On the other hand, although chromogenic peptides allow the protease activity assays, these substrates are hydrolyzed by site specific proteases and are not useful for non-specific enzymes discovery.

In this study a new assay compatible with high throughput screening to measure the activity of halo-alkaline and alkaline proteases was developed. In this method the hydrolysis of casein was followed indirectly by using pH-indicators.

2 Materials and methods

2.1 Chemicals and enzymes

MOPS (M1254 Sigma Aldrich), TRIS (T4661 Sigma Aldrich), CHES (C8210 Sigma Aldrich), CAPS (C2632 Sigma Aldrich), phenol red (114529 Sigma Aldrich), cresol red (114472 Sigma Aldrich), phenolphtalein (105945 Sigma Aldrich), mordant orange 1 (195073 Sigma-Aldrich), sodium caseinate from bovine milk (D8654 Sigma Aldrich), DMSO (D8418 Sigma Aldrich), protease from *Bacillus licheniformis* (P4860 Sigma-Aldrich) and protease from *Bacillus sp*. (P3111 Sigma-Aldrich). Savinase® (Ultra 16L) was purchased from Novozymes and Curtizyme® was purchased from ENMEX.

2.2 pH-based method procedure

At the time of the test, the substrate mixture for the enzymatic reaction was prepared in a volume of 10 mL by mixing distilled water, 25 μL of buffer, 500 μL of sodium caseinate, 60 μL of pH-indicator, and convenient volume of salt stock solution according to desirable concentration of salt. A buffer-indicator pair was used for each pH of reaction according to Table 1, and corresponding pH was adjusted to substrate mixture. Then, 20 μL of conveniently diluted enzyme was deposited in a microwell of a 96-well microplate. Additionally, 20 μL of culture media or diluted buffer according to the pH of reaction (at the concentration indicated in Table 1) was deposited in a separated microwell to serve as the blank of the reaction. Immediately, 100 μL of substrate mixture were added to each microwell with an eight-channel pipette and microplate was positioned in an X-mark microplate reader. Readings were performed every 30 seconds for a time span of 5 to 15 minutes at the corresponding maximum wavelength for each indicator (Table 1). Kinetics were carried out at 37 °C with a shake of 5 seconds before each reading and the assays were performed in triplicate (Supplementary Fig. 2). Hydrochloric acid calibration curves were made at all conditions tested for the quantification of protease activity. One unit of protease activity is defined as the amount, expressed in micromoles of hydrochloric acid, of amino groups released from casein per minute.

Table 1 Characteristics of buffers and pH-indicator pairs.

pH	Buffer	Buffer pK_a	Indicator	Indicator pK_a	λmax	Indicator (mM)	Buffer (mM)	Indicator color
7.4	MOPS	7.2	Phenol red	7.9	558	0.35	2.5	Red to yellow
8.2	TRIS	8.06	Cresol red	8.3	574	0.2	2.5	Red to yellow
9.7	CHES	9.5	Phenolphthalein	9.7	553	0.5	2.5	Fuchsia to colorless
10.9	CAPS	10.4	Mordant orange 1	11.1	493	0.5	2.5	Red to yellow

Protease activity was calculated as equation (1).

$$U \times mL^{-1} = \frac{(\text{Reaction rate} - \text{Blank rate}) \times 120 \times \text{Factor dilution})}{\text{Standard slope} \times 20} \quad (1)$$

Were *Reaction rate* and *Blank rate* are given in OD×min^{-1} and corresponds to absorbance decrease of reaction catalyzed by enzyme and changes in absorbance due to background signal, respectively. *Standard slope* corresponds to slope of hydrochloric acid calibration curve in mM×OD^{-1}. Factor 120 is the volume (μL) used in protease assay. Factor 20 is the volume (μL) of enzyme tested.

2.3 *Linearity, sensibility and quality of pH-based method*

The linear relationship of the detection of enzymatic activity as a function of the enzyme concentration was analyzed. For this, serial dilutions of Savinase were tested in the presence of 2 M NaCl, 2 M KCl or without salt, at all pH proposed. Effect of Savinase amount on the steady-state reaction rate was measured. Lower limit of detection was expressed as the mean of background signal plus three standard deviation of background signal.

In addition, quality of assays as a function of the enzyme concentration was estimated using the following *Z factor* equation (2) (Zhang *et al*., 1999):

$$ZFactor = 1 - \frac{3(\sigma\text{Reaction rate} + \sigma\text{Blank rate}}{|\mu\text{Reaction rate} - \mu\text{Blank rate}|} \quad (2)$$

Where μ and σ represent the means and the standard deviations of enzymatic reactions, *Reaction rate* and *Blank rate* represent absorbance decrease of reaction catalyzed by enzyme (positive control) and changes in absorbance due to background signal (negative control), respectively. The *μReaction rate -μBlank rate* is the separation between the readings for the controls. For a robust assay, it is necessary to have *Z Factor* greater than 0.5, Z < 0 is a sign of poor quality (Zhang *et al*., 1999).

2.4 *Analysis of the accuracy of pH-based method, effect of ionic strength*

Accuracy was tested through a comparison of simultaneous assays using pH-based method and classical Folin's reagent in the same conditions. For this, the effect of the ionic strength (0-4 M NaCl concentration on the protease activity of Savinase (0.58 μg×mL^{-1}) and protease from *Bacillus licheniformis* (2.26 μg×mL^{-1}) was tested with both methods at pH 9.7 and 37 ºC. Assays with Folin's reagent were performed according to Cupp-Enyard (2008) with slight modifications: a 0.5 % (w×v^{-1}) sodium caseinate solution in 25 mM CHES buffer was used as substrate and the reaction volume was 600 μL.

2.5 *Analysis of pH range of assay*

To corroborate the effectiveness of the pH-based method to testing alkaline proteases, Savinase activity was measured at pH 7.4, 8.2, 9.7 and 10.9 using buffer and indicator pairs described in Table 1.

3 Results and discussion

3.1 *Biochemical foundation of the method*

A spectrophotometric method was developed in microplate format for the measurement of protease activity at different ionic strength and alkaline pH. This method allows the quantitative analysis of numerous samples simultaneously. The fundament of the method consists in measuring indirectly the protein structure peptide bond cleavage in a weakly buffered solution by using a pH-indicator. When a peptide bond is cleaved by a protease, a proton is released if the pH of the reaction media is alkaline (Adler-Nissen 1993) (Fig. 1a). Then, a pH-indicator is protonated, and its color was change due to a change on its structure (Fig. 1b). Enzymatic reaction can be monitored at certain time intervals by measuring the absorbance at a given wavelength, which depends on the pH-indicator used.

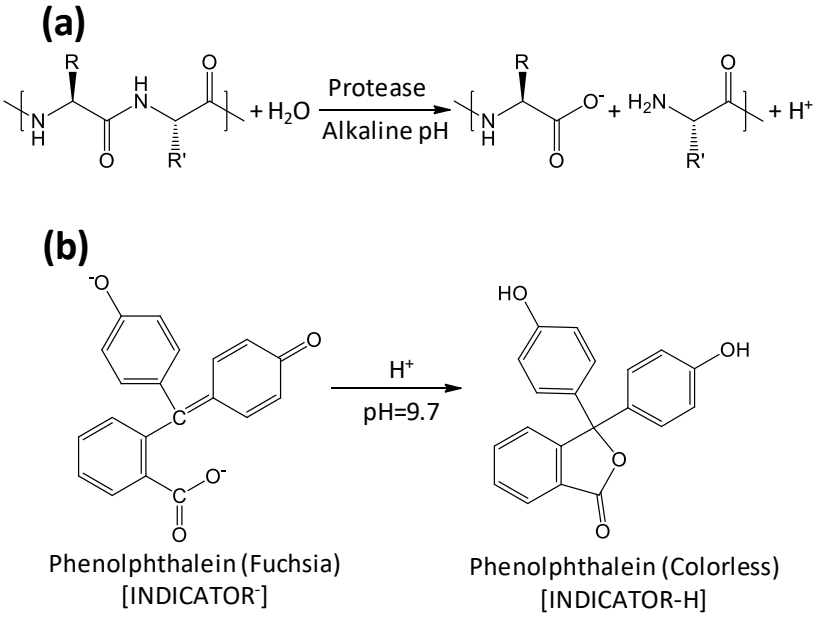

Fig. 1 Principle of pH-based method. Reaction model of microplate protease assay (a). Example of Phenolphthalein colour change due to protonation of the molecule (b).

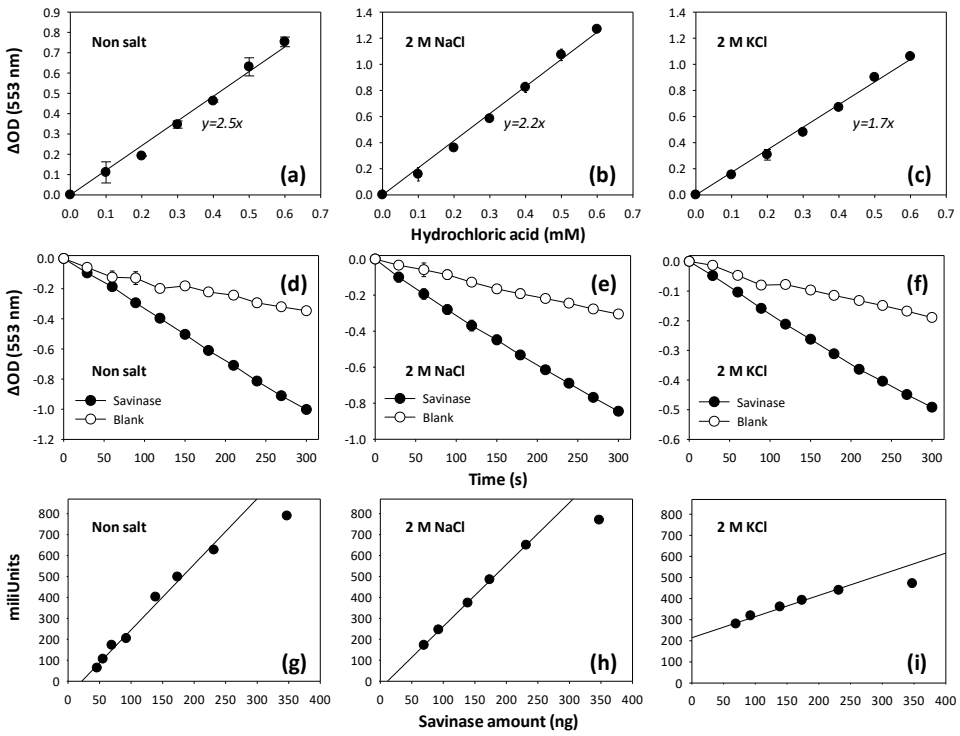

Fig. 2 pH-based method performance. Hydrochloric acid calibration curves (a, b, c), enzyme reaction progress curve during casein hydrolysis with different salt concentration and 139 ng of enzyme mL^{-1} (d, e, f), and effect of enzyme amount on the steady-state of reaction rate (g, h, i). Assays were carried out at pH 9.7 and kinetics were done using Savinase enzyme.

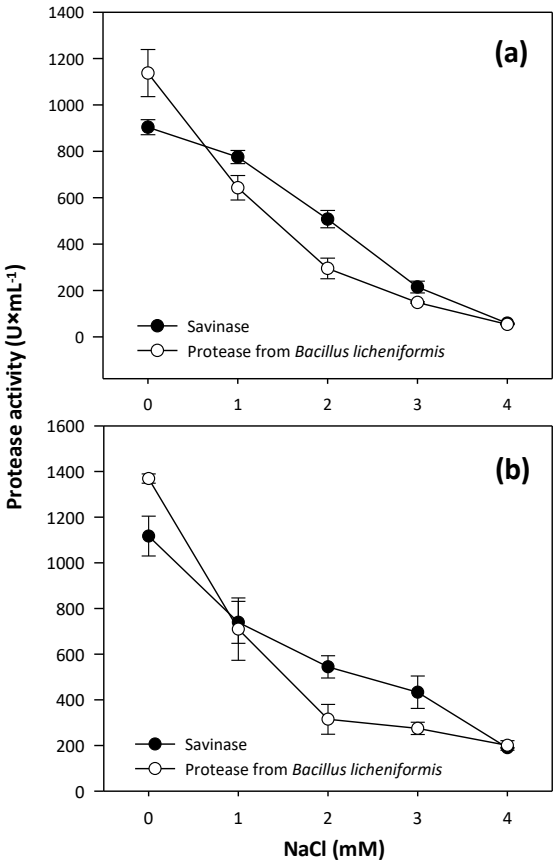

Fig. 3 Comparison of Folin's reagent (a) and pH-based method (b). Effect of ionic strength in protease activity. Kinetics were carried out at pH 9.7 and 37ºC with Savinase (0.58 μg×mL^{-1}) or protease from *Bacillus licheniformis* (2.26 μg×mL^{-1}).

Biochemical foundation of the method is based in the acid dissociation constant (pK_a) of the reagents. For this reason, the pH level of the reaction media and the pK_a of the indicators and buffers play a fundamental role for the good development of the methodology as well as the degree of dissociation of amino groups. For a linear response pK_a of indicator and buffer used as well as reaction pH should be similar (Rosenberg *et al*., 1989; Janes *et al*., 1998) In addition, to detect the released protons, pH of reaction media must be equal to or greater than pK_a of the reaction products (Camacho-Ruiz *et al*., 2015). As reported by Adler-Nissen (1986), the amino groups of the peptides that are produced during proteolysis of casein maintain a pK_a of about 7.3 to 7.6, at 30 to 40 °C. Therein, buffer-indicator pairs were selected which were useful for performing assays in a pH range ranging from 7.5 to 10.9.

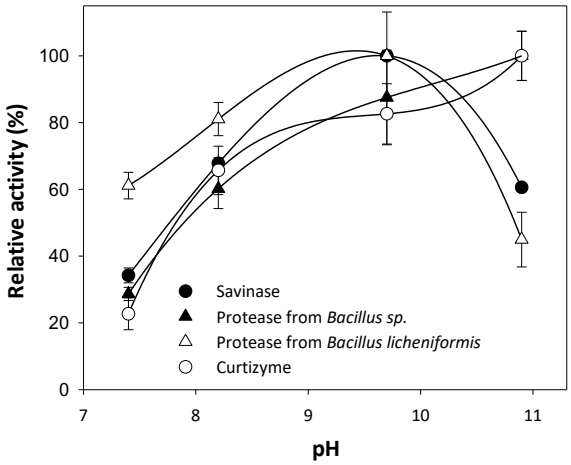

Fig. 4 pH activity profile of alkaline proteases using protease pH-based method with 2 M NaCl. The solid line represents a polynomial regression of the data (R^2 > 0.9).

On the other hand, the sensitivity of the method depends on the concentration of the buffer and indicator. By increasing the concentration of the indicator and decreasing the concentration of the buffer, greater sensitivity will be achieved. The concentration of the indicator is link on its solubility in water and its absorbance value observed is related to the maximum wavelength h. The concentration of the buffer is a compromise between sensitivity and buffering of pH changes. Having small pH changes is important because the kinetic properties depend on the pH of the reaction. For this method, a concentration of 2.5 mM buffer was empirically selected, which ensures small changes in pH (<0.05 pH units for a change in absorbance of 100 mOD under the conditions of the method; Supplementary Fig. 3).

In addition, assays were performed to determine the maximum wavelength and the appropriate indicator concentration (Supplementary Fig. 4). Data Table 1 shows a summary of the pK_a values and concentrations of each selected buffer-indicator pair, as well as the maximum wavelength and indicator turn color.

3.2 Substrate solubility and stability

For the development of the pH-based method it was proposed the use of sodium caseinate, which is a standard substrate for proteases (Alkanhal *et al*., 1985). This substrate was selected because its solubility in water even in the presence of high salt concentrations over a wide alkaline pH range.

At the concentration used for the tests (0.5% $w \times v^{-1}$), complete solubility of sodium caseinate was observed in all pH level and salt concentrations tested. However, a system to determine activity at pH 10.9 in solutions containing 2 M NaCl or more could not be established because Mordant Orange 1 precipitated, but in all other test conditions, the indicators were completely soluble.

Once the substrate and indicator were solubilized, these remained soluble throughout the assay. However, it is not recommended to conserve the prepared reaction solution for more than half an hour because, over time, some of the indicators lost their coloration on their own. It is therefore suggested to prepare the reaction solution fresh before use. In addition, the substrate stock solution must be kept frozen in aliquots to avoid degradation.

It should be mentioned that at the time of reaction solution preparation, it is necessary to adjust the pH of the same for the accomplishment of a correct test. The solutions with the correct pH will become reddish or fuchsia depending on the indicator used (Supplementary Fig. 5).

3.3 System calibration

To quantify enzymatic activity, calibration curves were performed for each of the test conditions. Examples of standard curves of hydrochloric acid at pH 9.7 in three different salinity conditions are shown in Fig. 2a, 2b, 2c. As can be seen, a linear relationship between acid concentration and absorbance of the solution was obtained. In all cases a determination coefficient R^2 of 0.9 was observed considering concentrations of hydrochloric acid from 0 up to at least 0.6 mM.

It should be noted that it is necessary to perform standard curves for each of the test conditions; this will reduce the error due to interference of the reaction medium with pK_a values.

3.4 Enzyme reaction progress curve

The commercial protease Savinase from Novozymes was used to carry out the validation of the method due to its biochemical characteristics that allow it to be active in all the evaluated test conditions. Being that Savinase is a commonly used protease in liquid and powder laundry formulations recommended for washing solutions with a pH ranging from 6.5 to 11.

Enzymatic kinetics were performed using Savinase at all proposed pH level for the method (Supplementary Fig. 6) and at different salt concentrations, observing enzyme activity in each of the reaction conditions. Some examples of typical kinetics of Savinase at pH 9.7 in the presence and absence of 2 M NaCl or 2 M KCl are shown in Fig. 2d, 2e, 2f. Protease activity was observed by a decrease in the absorbance of the reactant medium, which must be greater than the decrease caused by the background signal (blanks). For reactions at pH 9.7 lower detection limit of decrease in absorbance rate of 40 $mOD \times min^{-1}$ was estimated.

In all cases it was possible to measure an initial rate of at least 5 min of reaction, depending on the concentration of enzyme. In addition, enzymatic activity for each condition was quantified. For kinetics of Savinase at pH 9.7 1117±87 $U \times mL^{-1}$ without salt, 544±49 $U \times mL^{-1}$ with 2 M NaCl and 306±20 $U \times mL^{-1}$ with 2 M KCl were obtained.

3.5 Detection system linearity

A linear relationship between enzyme amount and enzyme activity is desirable for a good precision of the method. The length of the linearity interval depends on the reaction being carried out in the steady state. For this, it is necessary to keep the substrate in excess and measure initial velocity when less than 10% of the substrate has been hydrolyzed (Brooks *et al*., 2004).

Detection system linearity of pH-based method was validated. In Fig. 2g, 2h, 2i it is shown the effect of Savinase amount on enzyme activity at pH 9.7 with and without salt. A linearity interval was established by which the estimated initial velocities for each enzyme concentration correspond to the maximum velocity. The detection lower limit was observed using 40 ng of Savinase. Moreover, the maximum limit of detection was different according to the salt present on reaction media. Using either of the two conditions, without salt or with 2 M NaCl, a system saturation was observed when 240 ng of Savinase was used; whereas, with 2 M KCl it was observed from 150 ng. However, linearity interval will vary depending on the enzyme and pH tested.

It is worth mentioning that the tests carried out at the lower limit of detection reached a *Z Factor* value between 0 and 0.5, which corresponds to marginal tests. In addition, as the amount of enzyme increases, the *Z Factor* increases, which was greater than 0.5 starting from the tests carried out with 60 ng of Savinase both for the tests carried out without salt as well as for the tests carried out with 2 M NaCl or 2 M KCl (Supplementary Fig. 7). A spectrophotometric assay is considered excellent when the *Z Factor* is

greater than 0.5, due to which it is suggested that pH-based method is a good method to detect halo-alkaline and alkaline proteolytic activity. However, calculating the *Z Factor* is recommended for each enzymatic assay in order to verify the quality of the measurements.

3.6 Comparison of pH-based method and classical Folin's reagent to measure protease activity

To verify accuracy, pH-based method was compared with the classical Folin's reagent technique. Biochemical foundation of this technique consists in the quantification of amino acids and peptide fragments released by proteases through the formation of a blue chromophore constituted by the aromatic residues and a phosphotungtic-phosphomolibdenum complex (Blainski *et al*., 2013). Absorbance value is compared with a standard curve of tyrosine to calculate enzymatic activity. In Folin's reagent technique one unit of protease activity is defined as the amount in micromoles of tyrosine equivalents released from casein per minute (Cupp-Enyard, 2008).

Unlike this, pH-based method does not measure the units of activity in tyrosine equivalents, but quantifies the amount of cleavage peptide bonds in HCl equivalent. However, both methods allow realizing the sodium caseinate proteolysis tests in different ionic strength, so it was decided to carry out a comparative study of both techniques.

Savinase and protease from *Bacillus licheniformis* activities were measured as a function of ionic strength. Assays were carried out under the same conditions for both methods. A 0.5 % ($v \times v^{-1}$) sodium caseinate substrate solution was used in a salt-free reaction medium and with 1 M to 4 M of NaCl at pH 9.7. Activity profiles are shown in Fig. 3, both Savinase and protease from *Bacillus licheniformis* had the maximum activity in the absence of salt, almost as the saline concentration increases the protease activity decreases.

This phenomenon was observed when using either technique. Although the definition of protease activity Units is different for the Folin's reagent technique compared to the pH-based method, a similar estimation was observed for both methods in tests conducted at pH 9.7.

On the one hand, the enzymatic activity was measured in Anson Units using the Folin's reagent (Anson 1938; Cupp-Enyard 2008), while using the pH-based method the Units of enzymatic activity are indirectly estimated as liberated amino groups. However, it cannot be said that there is an equivalence of units, but only that there is a good approximation. Using the pH-based method it was possible to detect a lag phase of approximately 5 min in some kinetics performed at elevated ionic strength (3 M NaCl or more). This lag phase was also observed using the Folin's reagent technique, but in this case, it was necessary to perform several assays with different incubation periods. The activity profiles shown in Fig. 3 were determined by considering the maximum velocity achieved by monitoring the kinetics for 10 min.

3.7 Using pH-based method for alkaline proteases, pH effect

An assay was performed to measure the effect of pH on the enzymatic activity of four alkaline proteases using the pH-based method. For this, Savinase, protease from *Bacillus sp*., protease from *Bacillus licheniformis* and Curtizyme activities were measured at all pH level proposed. As expected, maximum activity for Savinase and protease from *Bacillus licheniformis* was observed at pH 9.7, whereas maximum activity for protease from *Bacillus sp*. and Curtizyme was observed at pH 10.9 (Fig. 4).

However, it is important to clarify that possibly the enzymatic activity measured at pH 7.4 and pH 8.2 is being underestimated due to the pK_a of the amino groups of the peptides released. According to Adler-Nissen (1986), in the determinations made at pH 7.4 approximately 50% of the amino groups released would be detected, while at pH 8.2 more than 85 % are detected. Nevertheless, it is necessary to determine the degree of dissociation of the amino groups under the test conditions of the pH-based method to calculate the correction factor $1/\alpha$ proposed by Adler-Nissen.

On the other hand, it is estimated that in the tests carried out at pH 9.7 and 10.9 approximately 100% of the amino groups released are detected. Based on the foregoing, it is recommended to preferably use the test conditions at pH≥8.2 to ensure greater sensitivity of the pH-based method.

Conclusions

Protease assays than can be used in a high throughput screening platform are desirable. In particular, the development of useful methods for haloalkaline proteases are of great interest. The method developed

uses casein as substrate, is performed in one step and can be followed in real time. Is time efficient, compared to classical methods and can be used in microplate format with good quality and sensitivity. Is useful in the prospection of new proteases from haloalkaliphilic microorganisms because high concentration of salt could be used in the assay 4M NaCl and pH from 7.4 to 10.9. Besides the method can be used in purification process due to large scale samples that could be processed with low requirements of reactants, as well as in the monitoring of protease activity in enzyme production cultures where proteolysis is undesirable (Ramos-Ibarra *et al*., 2017).

Acknowledgements

This work was funded by Consejo Nacional de Ciencia y Tecnología (CONACYT).

References

Adler-Nissen J. (1986). *Enzymic Hydrolysis of Food Proteins*. Elsevier Applied Science, UK.

Alkanhal H.A., Frank J.F. and Christen J.L. (1985.) Measurement of microbial protease activity using a pH-stat titration. *Journal of Food Protection 4*, 351-354.

Anson, M.L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *The Journal of General Physiology 22*, 79-89.

Blainski A., Lopes G.C. and de Mello J.C. (2013). Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium Brasiliense* L. *Molecules 18*, 6852-65.

Bremmer S.C., Chen J., McNeil A.J. and Soellner M.B. (2012). A general method for detecting protease activity via gelation and its application to artificial clotting. *Chemical Communications 48*, 5482-5484.

Brooks H.B., Geeganage S., Kahl S.D., Montrose C., Sittampalam S., Smith M.C. and Weidner J.R. (2004). Basics of enzymatic assays for HTS. In: *Assay Guidance Manual*, (G.S. Sittampalam, N.P. Coussens, *et al*., eds.), Pp. 63-76. Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda.

Camacho-Ruiz M.A., Mateos-Díaz J.C., Carriere F. and Rodriguez J.A. (2015). A broad pH range indicator-based spectrophotometric assay for true lipases using tributyrin and tricaprylin. *Journal of Lipid Research 56*, 1057-1067.

Charney J. and Tomarelli R.M. (1947). A colorimetric method for the determination of the proteolytic activity of duodenal juice. *Journal of Biological Chemistry 171*, 501-505.

Coêlho D.F., Peron P., Freitas F., Gava P., Silveira E. and Basile E. (2016). Azocasein substrate for determination of proteolytic activity: Reexamining a traditional method using Bromelain Samples. *BioMed Research International 2016*, 1-6.

Contesini F.J., Melo R.R. and Sato H.H. (2018). An overview of *Bacillus* proteases: from production to application. *Critical Reviews in Biotechnology 38*, 321-334.

Cupp-Enyard C. (2008). Sigma's non-specific protease activity assay-casein as a substrate. *Journal of Visualized Experiments 19*, 899.

Götze S. and Saborowski R. (2011). Nanodrop fluorometry adopted for microassays of proteasomal enzyme activities. *Analytical Biochemistry 413*, 203-205.

Hernandez-Martinez R., Sancho-Solano A., Loera-Corral O., Rojo-Dominguez A., Regalado-González C., Huerta-Ochoa S. and Prado-Barragán L.A. (2011). Purification and characterization of a thermostable alkaline protease produced by *Yarrowia lipolytica. Revista Mexicana de Ingeniería Química 10*, 333-341.

Janes L.E., Löwendahl A.C. and Kazlauskas R.J. (1998). Quantitative screening of hydrolase libraries using pH indicators: Identifying active and enantioselective hydrolases. *Chemistry A European Journal 4*, 2324-2331.

Kang K., Peng L., Jung Y.J., Kim J.Y., Lee E.H., Lee H.J., Kim S.M., Sung S.H., Pan C.H. and Choi Y. (2018). High-throughput and direct measurement of androgen levels using turbulent

flow chromatography liquid chromatography-triple quadrupole mass spectrometry (TFC-LC-TQMS) to discover chemicals that modulate dihydrotestosterone production in human prostate cancer cells. *Biotechnology Letters 40*, 263-270.

López-Flores A.R., Luna-Urban C., Buenrostro-Figueroa J.J., Hernández-Martínez R., Huerta-Ochoa S., Escalona-Buendía H., Aguilar-González C.N. and Pardo-Barragan, L.A. (2016). Effect of pH, temperature and protein and carbohydrates source in protease production by *Yarrowia lipolytica* in solid culture. *Revista Mexicana de Ingeniería Química 15*, 57-67.

Ramos-Ibarra J.R., Miramontes C., Arias A., Arriola E., Guatemala G. and Corona-González R.I. (2017). Production of hydrolytic enzymes by solid-state fermentation with new fungal strains using orange by-products. *Revista Mexicana de Ingeniería Química 16*, 19-31.

Rao M.B., Tanksale A.M., Ghatge M.S. and Deshpande V.V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews 62*, 597-635.

Raval V.H., Pillai S., Rawal C.M. and Singh S.P. (2014). Biochemical and structural characterization of a detergent-stable serine alkaline protease from seawater haloalkaliphilic bacteria. *Process Biochemistry 49*, 955-962.

Rawlings N.D., Barrett A.J. and Bateman A. (2012). MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Research 40*, D343-D350.

Rosenberg R.M., Herreid R.M., Piazza G.J. and O'Leary M.H. (1989). Indicator assay for amino acid decarboxylases. *Analytical Biochemistry 181*, 59-65.

Tovar-Jimenez X., Muro-Urista C.R., Tellez-Jurado A., Mercado-Flores Y., Abreu-Corona A. and Arana-Cuenca A. (2017). Hydrolysate antimicrobial activity released from bovine whey protein concentrate by the aspartyl protease Eap1 of *Sporisorium reilianum. Revista Mexicana de Ingeniería Química 16*, 11-18.

Wiegmann V., Bernal Martinez C. and Baganz F. (2018). A simple method to determine evaporation and compensate for liquid losses in small-scale cell culture system. *Biotechnology Letters 40*, 1029-1036.

Zhang J.H., Chung T.D.Y. and Oldenburg K.R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *Journal of Biomolecular Screening Journal 4*, 67-73.

Zhou X., Shi H., Yang S. and Sun P. (2017). An efficient rapid system for assaying HBx-mediated transactivation. *Biotechnology Letters 39*, 1091-1099.