Food Engineering Progress
Vol. 16, No. 3. pp. 193~198 (2012.08)

산업식품공학
Food Engineering Progress

Application of Convenient Chromogen-based Assay to Measurement of Protease Activity

Nam-soo Kim*, Jin-Soo Maeng, Yong-Jin Cho, Chul-Jin Kim, and Chong-Tai Kim
Functional Materials Research Group, Korea Food Research Institute

Abstract

This study was carried out to apply an optimized convenient assay, exploiting azo dye-bound chromogenic substrates, to measurement of protease activity. When determined for responses at varying concentrations of two substrates, azocasein and azoalbumin, using 0.5 and 5.0 mg/mL each of bovine pancreas trypsin, 3% azocasein was found to be the most appropriate substrate solution to measure protease activity. Compared with a conventional casein-Folin phenol assay, the chromogen-based protease assay exploiting 3% azocasein showed better precision to have a coefficient of variability in seven repetitive measurements less than 1.11%. When various reagent-grade and industrial proteases that showed proteinase or peptidase activities were tested by this assay at increasing enzyme concentrations, typical shape of rectangular hyperbola in activity-enzyme concentration profiles was observed. In addition, the assay of this study was suitable for activity measurement in real samples that were prepared by hydrolyzing wheat gluten and anchovy fine powder with proteases.

Key words: application, convenient, chromogen-based assay, measurement, protease activity

Introduction

Proteolytic enzymes that degrade proteins to small peptides and amino acids belong to one of the most important classes of enzyme and account for nearly 60% of the industrial enzyme market in the world (Kumar & Takagi, 1999; El Enshasy et al., 2008). Up to now, they have been used in a number of biotechnological processes including fermentation, baking, cheese preparation and ripening, meat tenderization, production of protein hydrolyzates, washing of ultrafiltration and reverse osmosis membranes, and production of hospital foods, digestive aids and medical drugs (Sugiura et al., 1976; Gusek & Kinsella, 1987; Kumar & Takagi, 1999; Dayanandan et al., 2003).

Protease activity has been routinely determined by the measurements of fluorescence, UV and visible ray for the enzymatic reaction products (Guarise et al., 2006; Nakashima et al., 2008; Kostallas & Samuelson, 2010). Some other methods including the electrochemical assay based on a polyanion-sensitive membrane electrode (Abd-Rabboh et al., 2003), turbidimetric assay using a protease-responsive chaperone protein (Sao et al., 2009), ultrasound velocity measurement for enzyme hydrolyzates (Born et al., 2010), surface-enhanced Raman scattering (Yazgan et al., 2010) and bacteriophage-based infection method (Capek et al., 2010) have also been introduced to measure protease activity. Although the methods mentioned above are sensitive and suitable for the protease assays using biological samples from clinical diagnostics etc., they are, in most cases, complicated in measuring procedure, quite specific in application area and dependent on high-cost research facilities.

Therefore, need for a broad spectrum convenient assay that can be used to determine activities of various endo- and exo-proteases easily has been increasing for the food industry as a routine analytical measure for protease activity (Borda et al., 2004; Katsaros et al., 2009). In this study, a chromogen-based protease assay that is able to be conducted conveniently was optimized for substrate concentration. The established method was compared with a conventional protease assay, casein-Folin phenol assay, regarding repeatability of measurement, and then was applied to activity measurements of various reagent-grade and industrial proteases in buffer solutions, and in real samples.

Materials and Methods

Reagents and Materials

To develop the protease assay, trypsin (from bovine

*Corresponding author: Nam-soo Kim, Functional Materials Research Group, Korea Food Research Institute, Baekhyun 516, Bundang, Songnam 463-746, Korea
Tel: 82-31-780-9131; Fax: 82-31-709-9876
E-mail: k9130sen@hanmail.net
Received May 15, 2012; revised July 23, 2012; accepted July 23, 2012

pancreas, TPCK-treated) was used as a model enzyme. In addition to trypsin, trypsin acetylated (from bovine pancreas, synthetic), α-chymotrypsin (from bovine pancreas, TLCK-treated), ficin (from fig tree), papain (from papaya latex), thermolysin (from *Bacillus thermoproteolyticus* rokko), pepsin (from porcine gastric mucosa), Alcalase 2.4 L (subtilisin, from *B. licheniformis*), Protamex (from *B. licheniformis* and *B. amyloliquefaciens*), Flavourzyme 500 MG (aminopeptidase, from *Aspergillus oryzae*) and Marugoto E were used to prepare activity-enzyme concentration profiles. Out of them, reagent-grade proteases were obtained from Sigma-Aldrich (St. Louis, MO, USA), and Alcalase 2.4 L, Protamex and Flavourzyme 500 MG that were the products of Novozymes A/S (Bagsvaerd, Denmark) were obtained from a local supplier. Marugoto E was the product of Supercritical Technology Research Corporation (Hiroshima, Japan). As general protease substrates that are degraded by various proteases non-specifically, two chromogenic proteolytic substrates, azocasein and azoalbumin, were also purchased from Sigma-Aldrich. To carry out a comparative casein Folin-phenol assay, N,N-dimethylated casein, Lowry reagent and Folin & Ciocalteu's phenol reagent (2 N) of Sigma-Aldrich were used. All other chemicals to prepare buffer solutions and to conduct the enzyme reaction were the guaranteed reagents from various suppliers, and double distilled water was used throughout this study. Microfuge tubes having the capacity of 2 mL were used for the enzyme reaction and following centrifugation after trichloroacetic acid (TCA) treatment.

Enzyme assay

Chromogen-based assay

The chromogen-based protease assay was carried out referring to a previous report (Phillips et al., 1984) with some modification. Each 250 mL solutions (0.2-9.5%, w/v), dissolved in 0.1 M phosphate buffer (differing in pH according to the enzymes used), 200 μL of the same buffer solution and 50 μL of an enzyme solution dissolved in the same buffer solution were consecutively added to a 2 mL microfuge tube, and the mixture was incubated at 37°C for 2 hr. To the reaction mixture, 500 μL of 30% TCA were added to precipitate the unreacted substrate. After setting still at 4°C for 30 min, the precipitated mixture was centrifuged at 10,000×g for 15 min to obtain the supernatant including the released azo dye. Five hundred microliters of the supernatant were taken from the tube and were neutralized with 500 μL of 1 M NaOH. The sample solution was then measured for the absorbance at 440 nm to determine enzyme activity. For a blank run, the enzyme solution was first inactivated with 30% TCA, followed by the consecutive addition of the substrate and buffer solution. The mixture was then treated as described above. In this study, bovine pancreas trypsin was used as a model enzyme to optimize the substrate concentration for proteolysis. That is, trypsin was dissolved in 0.1 M phosphate buffer (pH 7.5) in 0.05 and 0.5 mg/mL, and the concentrations of two chromogenic substrates-azocasein and azoalbumin, dissolved in the same buffer solution, were varied from 0.2 to 9.5% (w/v) for the enzyme reaction.

Comparative conventional assay: casein-Folin phenol assay

A conventional casein-Folin phenol assay for protease activity was conducted as follows for comparison with the developed chromogen-based assay (Abe et al., 1987; Tsuchiya et al., 1987). To 5 mL of 1% N,N-dimethylated casein, dissolved in 0.1 M phosphate buffer (pH 7.5), was added 1 mL of an enzyme solution and the resulting mixture was incubated at 37°C for 2 hr. Five milliliters of 25% TCA were added to stop the reaction and the precipitated reaction mixture was centrifuged at 10,000×g for 30 min after setting still at 4°C for 1 hr. To 1 mL of the supernatant, were added 2.5 mL of Lowry reagent and 0.5 mL of 1 N Folin & Ciocalteu's phenol reagent. After swirling gently, the mixture was incubated at 37°C for 30 min and then was determined for the absorbance at 660 nm.

Activity-enzyme concentration profiles of various proteases as determined by the optimized method

Responses of the reagent-grade and industrial proteases were determined at increasing enzyme concentrations to prepare activity-enzyme concentration profiles. At this moment, the tested concentrations of the reagent-grade proteases were 0.05, 0.1, 0.5, 1 and 5 mg/mL. Meanwhile, those of the industrial proteases were 0.25, 0.5, 2.5, 5 and 25 mg/mL because these enzymes were normally less pure than the reagent-grade ones. Most of these solutions were prepared in 0.1 M sodium phosphate buffers at the optimum pH of the corresponding proteases. In contrast, pepsin solutions were prepared in 0.01 N HCl. The experimental design is summarized in Table 1.

Application of the optimized method to measuring protease activity in real samples

To apply the developed chromogen-based assay to activity measurement in real samples, four protein hydrolyzates were prepared exploiting wheat gluten (WG) and anchovy fine powder (AFP) as substrates, and Alcalase 2.4 L and Flavourzyme 500 MG as hydrolytic enzymes. The protein

Table 1. Experimental design for obtaining activity-enzyme concentration profiles of various proteases.

Enzyme	Reaction temperature (°C)	Reaction pH	Enzyme concentration (mg/mL)
Trypsin acetylated [a]	37	7.5	0.05, 0.1, 0.5, 1, 5
α-Chymotrypsin[b]	37	7.5	0.05, 0.1, 0.5, 1, 5
Ficin[b]	37	6.5	0.05, 0.1, 0.5, 1, 5
Papain[b]	37	6.5	0.05, 0.1, 0.5, 1, 5
Thermolysin[b]	37	7.5	0.05, 0.1, 0.5, 1, 5
Pepsin[b]	37	2.0 (0.01 N HCl)	0.05, 0.1, 0.5, 1, 5
Trypsin[b]	37	7.5	0.05, 0.1, 0.5, 1, 5
Alcalase 2.4 L[c]	37	7.5	0.25, 0.5, 2.5, 5, 25
Protamex[d]	37	7	0.25, 0.5, 2.5, 5, 25
Marugoto E[d]	37	7	0.25, 0.5, 2.5, 5, 25
Flavourzyme 500 MG[d]	37	6.5	0.25, 0.5, 2.5, 5, 25

[a] Synthetic.
[b] Lyophilized powder.
[c] Liquid.
[d] Crude powder.

hydrolyzates were produced as follow. WG and AFP were individually suspended in distilled water as 12% (w/v) concentration. After adjusting temperature of the suspensions to 37°C, Alcalase 2.4 L and Flavourzyme 500 MG were added to the suspensions in the basis of 0.7 and 4 g per 100 g substrate, respectively. After hydrolyzing at 37°C for 1 hr with a constant stirring at 300 rpm, the resulting mixtures were immediately centrifuged at 10,000×g at 10°C for 30 min. The supernatants were designated as wheat gluten hydrolyzates (WGHs) and anchovy fine powder hydrolyzates (AFPHs), respectively. For the WGHs and AFPHs obtained, protease activities were determined by the current method as described above.

Results and Discussion

Optimization of substrate concentration

It has been reported that chromogenic proteolytic substrates are normally used for assay development in the concentration range of 1.0-2.5% (Charney & Tomarelli, 1947; Phillips et al., 1984; Yoo et al., 2004; Hosseininaveh et al., 2009). To establish an optimum substrate concentration for the chromogen-based protease assay of this study, we first optimized substrate concentration. At all combinations of trypsin concentration and substrate species, typical rectangular hyperbolic saturation profiles were observed at increasing substrate concentrations (Fig. 1), as reported previously in the concentration-dependent degradation of N,N-dimethylated casein with a microbial protease (Kim, 1992). However, the substrate concentrations at which the saturation in enzyme activity was observed were different according to substrate species. When azocasein was used as the substrate, enzyme activity started to be saturated near 3% in substrate concentration irrespective of enzyme concentration. In contrast, enzyme activity was not saturated at 3% azoalbumin when 0.5 mg/mL trypsin was used as the enzyme solution (panel A of Fig. 1), which indicated that azocasein was more preferred substrate than azoalbumin. Therefore, the substrate solution for further study was selected as 3% azocasein to obtain higher responses, to ensure a substantially higher substrate concentration than Michaelis-Menten constant and simultaneously to avoid possible competitive inhibition by the chromogen-bound substrate (Borda et al., 2004). Compared with previously reported chromogen-based assays (Charney & Tomarelli, 1947; Phillips et al., 1984), our method that used 3% azocasein as the substrate solution contributed to the improvement in assay performance through the optimization of substrate species and its concentration.

Precision of optimized chromogen-based protease assay

The chromogen-based protease assay exploiting 3% azocasein was compared with a conventional casein-Folin phenol assay with respect to repeatability of measurement. For this purpose, seven repetitive measurements were conducted for both methods. As shown in Table 2, color development after the enzyme reaction was more pronounced and significantly different in 5% ($p < 0.05$) significant level in the case of the current assay, which seemed to indicate a good sensitivity. In addition, the blank value of the present assay

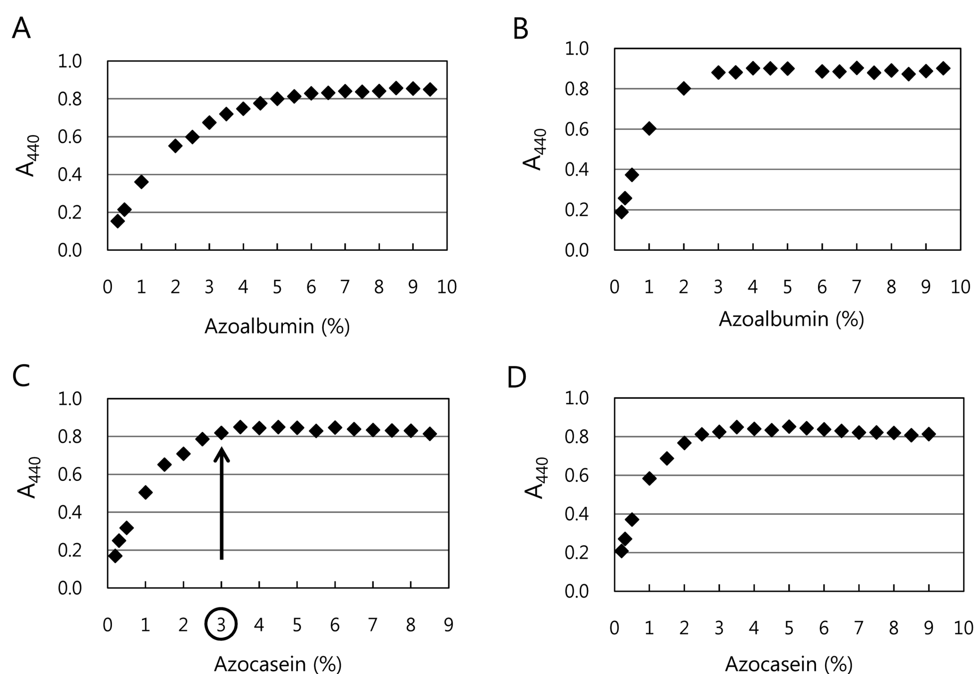

Fig. 1. Optimization of substrate concentration in chromogen-based protease assay using trypsin as a model enzyme. Enzyme concentrations (mg/mL): A, 0.5; B, 5.0; C, 0.5; D, 5.0.

Table 2. Comparison of precision in activity measurement of trypsin between the chromogen-based assay of this study and comparative casein-Folin phenol assay.

	Chromogen-based assay (A_{440})	Casein-Folin phenol assay (A_{660})
	Mean±SD	Mean±SD
Sample	1.3027±0.0145 [1] (n=7)	0.5941±0.0234 [2] (n=7)
CV (%)	1.11	3.94
Blank [a]	0.0514±0.0069 [2] (n=3)	0.2086±0.0141 [1] (n=3)

[a] Enzyme solution was first inactivated with 30% TCA before adding the substrate solution.
[1-2]Means within the same row with different Arabic numeral superscripts are significantly different at $p < 0.05$ by ANOVA. The absorbance values of sample and blank were separately treated by significance test.

was much smaller than that of the casein-Folin phenol assay, which was evidenced by the fact that these two values were significantly different in 5% ($p < 0.05$) significant level. Although the coefficient of variability (CV), a general index of repeatability of measurement, of 3.94% found for the comparative method was still good, that of 1.11% for the chromogen-based assay of this study was excellent considering that a CV value less than 5% is regarded as the criterion of good precision (Kim et al., 1996). Based on the above sensitivity, repeatability of measurement and simplicity in measuring procedure, we presumed that the current method is convenient and efficient in determining protease activity.

Measurement of protease activity

The optimized chromogen-based assay of this study was applied to measuring concentration-dependent activities of various proteases in buffer solutions. As shown in Fig. 2, general shape of rectangular hyperbola having the first- and zero-order regions was observed for all tested reagent-grade and industrial proteases (Marangoni, 2003). However, the responses of the proteases at a fixed enzyme concentration were quite species-specific, possibly depending on enzyme purity and turnover rate. These indicate that the present method is suitable for routine activity measurements of various proteases that have different action mechanism. Moreover, the CVs for the activities of different buffered enzyme solutions were present, in most cases, far below 5%, which also showed a good precision of the current method.

Protease activity in real samples

Four WGHs and AFPHs that were prepared by the separate treatments with Alcalase 2.4 L and Flavourzyme 500 MG were measured for protease activity with the present method in seven replications. As shown in Table 3, the protein

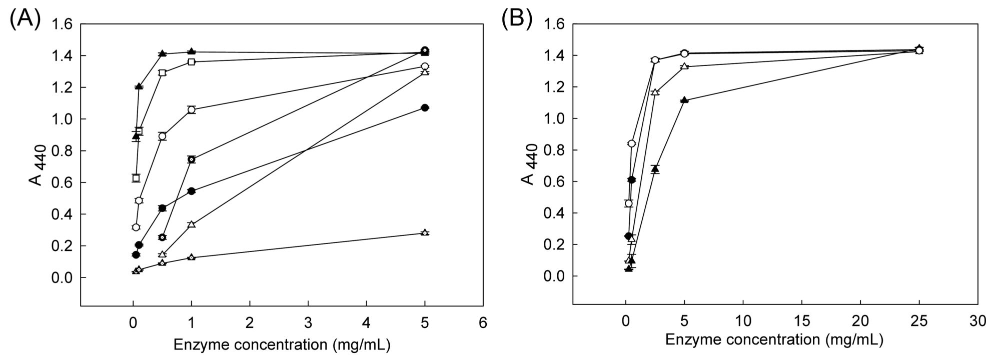

Fig. 2. Effects of enzyme concentrations on activity of reagent-grade (A) and industrial (B) proteases as measured by the chromogen-based protease assay of this study. Symbols : (A) -○-, trypsin acetylated; -●-, α-chymotrypsin; -⊙-, ficin; -△-, papain; -▲-, thermolysin; -△-, pepsin; -□-, trypsin. (B) -○-, Alcalase 2.4 L; -●-, Protamex; -△-, Marugoto E; -▲-, Flavourzyme 500 MG. Measurements were done in triplicate and error bars were inserted.

Table 3. Enzyme activities in protein hydrolyzates as measured by the chromogen-based protease assay of this study.

	WGH [a]		AFPH [b]	
	Alcalase 2.4 L [c]	Flavourzyme 500 MG [d]	Alcalase 2.4 L [c]	Flavourzyme 500 MG [d]
A_{440} [e] (Mean±SD)	0.6467±0.0375 [1] (n=7)	0.4425±0.0510 [2] (n=7)	0.6404±0.0801 [1] (n=7)	0.4339±0.0439 [2] (n=7)

[a] Wheat gluten hydrolyzate.
[b] Anchovy fine powder hydrolyzate.
[c] Treatment was done in the basis of 0.7-g enzyme/100-g substrate.
[d] Treatment was done in the basis of 4-g enzyme/100-g substrate.
[e] $A_{440\ sample} - A_{440\ blank}$. For a blank run, each protein hydrolyzate was first inactivated with 30% TCA before adding 3% azocasein as the substrate solution for activity measurement.
[1-2] Means within the same row with different Arabic numeral superscripts are significantly different at $p < 0.05$ by ANOVA.

hydrolyzates prepared by Alcalase 2.4 L treatment showed bigger protease activities than those prepared by Flavourzyme 500 MG, irrespective of substrate species, which seemed to indicate that Alcalase 2.4 L, an endo-type protease, was more effective in azocasein hydrolysis during the assay than Flavourzyme 500 MG, an exo-type protease (Nchienzia et al., 2010; Cabanillas et al., 2012). This fact was confirmed by significance test in 5% ($p < 0.05$) significant level.

The mean protease activity of the WGH treated with Alcalase 2.4 L was the same as that of the AFPH treated with the same enzyme and a similar result was found regarding Flavourzyme 500 MG treatment, which was also statistically supported in 5% ($p < 0.05$) significant level. These results seemed to be in the right because the same amount of Alcalase 2.4 L and Flavourzyme 500 MG were used for the production of WGHs and AFPHs. Considering the results in Table 3, the chromogen-based assay of this study was likely to be a reliable analytical measure to determine protease activities in the protein hydrolyzates prepared from different proteinaceous substrates.

In conclusions, the chromogen-based protease assay of this study seems to be suitable for a routine activity measurement of most reagent-grade and industrial proteases that have endo- or exo-type action mechanism, considering its simple and reproducible properties. Also, the method was applicable to activity measurement in real samples such as enzymatic protein hydrolyzates.

Acknowledgement

This study was carried out as one part of the research project of Development of High Pressure Process for Foods, Korea Food Research Institute, Korea.

References

Abd-Rabboh HSM, Nevins SA, Durust N, Meyerhoff ME. 2003. Electrochemical assay of protease activities based on polycation/polyanion complex as substrate and polyion sensitive membrane electrode detection. Biosens. Bioelectron. 18: 229-236.

Abe K, Kondo H, Arai S. 1987. Purification and properties of a cysteine proteinase from germinating rice seeds. Agric.

Biol. Chem. 51: 1509-1514.

Borda D, Indrawati, Smout C, Van Loey A, Hendrickx M. 2004. High pressure thermal inactivation of a plasmin system. J. Dairy Sci. 87: 2351-2358.

Born K, Manns A, Dzeyk K, Lutz-Wahl S, Gau D, Fischer L. 2010. Evaluation of ultrasound velocity measurements for estimating protease activities using casein as substrate. Biotechnol. Lett. 32: 249-253.

Cabanillas B, Pedrosa MM, Rodríguez J, Muzquiz M, Maleki SJ, Cuardano C, Burbano C, Crespo JF. 2012. Influence of enzymatic hydrolysis on the allergenicity of roasted peanut protein extract. Int. Arch. Allergy Imm. 157: 41-50.

Capek P, Kirkconnel KS, Dickerson TJ. 2010. A bacteriophage-based platform for rapid trace detection of proteases. J. Am. Chem. Soc. 132: 13126-13128.

Charney J, Tomarelli RM. 1947. A colorimetric method for the determination of the proteolytic activity of duodenal juice. J. Biol. Chem. 171: 501-505.

Dayanandan A, Kanagaraj J, Sounderraj L, Govindaraju R, Rajkumar GS. 2003. Application of an alkaline protease in leather processing: an ecofriendly approach. J.Clean. Prod. 11: 533-536.

El Enshasy H, Abuoul-Enein A, Helmy S, El Azaly Y. 2008. Optimization of the industrial production of alkaline protease by *Bacillus licheniformis* in different production scales. Aust. J. Basic Appl. Sci. 2: 583-589.

Guarise C, Pasquato L, De Filippis V, Scrimin P. 2006. Gold nanoparticles-based protease assay. P. Natl. Acad. Sci. USA 103: 3978-3982.

Gusek TW, Kinsella JE. 1987. Purification and characterization of the heat-stable serine proteinase from *Thermomonospora fusca* YX. Biochem. J. 246: 511-517.

Hosseininaveh V, Bandani A, Hosseininaveh F. 2009. Digestive proteolytic activity in the Sunn pest, *Eurygaster integriceps*. J. Insect Sci. 9: 1-11.

Katsaros GI, Katapodis P, Taoukis PS. 2009. High hydrostatic pressure inactivation kinetics of the plant proteases ficin and papain. J. Food Eng. 91: 42-48.

Kim N. 1992. Purification and Characterization of an Extracellular Proteinase from *Serratia marcescens*. PhD Thesis, Seoul National University, Seoul, Korea, p. 51.

Kim N, Haginoya R, Karube I. 1996. Characterization and food application of an amperometric needle-type L-lactate sensor. J. Food Sci. 61: 286-290.

Kostallas G, Samuelson P. 2010. Novel fluorescence-assisted whole-cell assay for engineering and characterization of proteases and their substrates. Appl. Environ. Microb. 76: 7500-7508.

Kumar CG, Takagi H. 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol. Adv. 17: 561-594.

Marangoni AG 2003. Characterization of enzyme activity. In: Enzyme Kinetics, A Modern Approach. John Wiley & Sons, New Jersey, USA, pp. 44-60.

Nakashima K, Maruyama T, Kamiya N, Goto M. 2008. Spectrophotometric assay for protease activity in ionic liquids using chromogenic substrates. Anal. Biochem. 374: 285-290.

Nchienzia HA, Morawicki RO, Gadang VP. 2010. Enzymatic hydrolysis of poultry meal with endo-and exopeptidases. Poultry Sci. 89: 2273-2280.

Phillips PK, Prior D, Dawes B. 1984. A modified azoalbumin technique for the assay of proteolytic enzymes for use in blood group serology. J. Clin. Pathol. 37: 329-331.

Sao K, Murata M, Fujisaki Y, Umezaki K, Mori T, Niidome T, Katayama Y, Hashizume M. 2009. A novel protease activity assay using a protease-responsive chaperone protein. Biochem. Bioph. Res. Co. 383: 293-297.

Sugiura M, Suzuki M, Ishikawa M, Sasaki M. 1976. Pharmaceutical studies on aminopeptidase from *Aspergillus japonica*. I. Chem. Pharm. Bull. 24: 2286-2293.

Tsuchiya K, Arai T, Seki K, Kimura T. 1987. Purification and some properties of alkaline proteinase from *Cephalosporium* sp. KM 388. Agric. Biol. Chem. 51: 2959-2965.

Yazgan NN, Boyaci IH, Temur E, Tamer U, Topcu A. 2010. A high sensitive assay platform based on surface-enhanced Raman scattering for quantification of protease activity. Talanta 82: 631-639.

Yoo JI, Lee YS, Song C-Y, Kim BS. 2004. Purification and characterization of a 43-killodalton extracellular serine proteinase from *Cryptococcus neoformans*. J. Clin. Microbiol. 42: 722-726.