CHEMICAL ENGINEERING TRANSACTIONS
VOL. 75, 2019
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Sauro Pierucci, Laura Piazza

ISBN 978-88-95608-72-3; **ISSN** 2283-9216
DOI: 10.3303/CET1975032

Activity and Stability in the Presence of a Non-Ionic Surfactant of a Protease for Hard Surface Cleaning in Food Industry

Otilia Herrera-Márquez*, Mercedes Fernández-Serrano, Marcia Pilamala, María B. Jácome, Encarnación Jurado-Alameda, Germán Luzón

Department of Chemical Engineering, University of Granada. Avda. Fuentenueva, s/n, 18071. Granada (Spain)
oherrera@ugr.es

A protease intended for use as a detergent additive has been studied in terms of activity as a function of temperature and stability at storage (40 ºC and 60 ºC during 15 days) and under typical washing conditions (60 ºC during 1 h) in the presence of an alkylpolyglucoside (APG), a non-ionic surfactant. Under storage at 60 ºC, the protease completely lost its activity before two days, while at 40 ºC the rate of deactivation increased with the increase in the concentration of APG. Under washing conditions the protease undergoes a complete deactivation after 60 minutes; nevertheless, the presence of the APG seems not to influence on enzyme stability. Results show that this protease is not suitable for cleaning processes at high temperatures. Furthermore, its addition to liquid detergent formulations will need a stabilization step that will ensure its activity along storage and at operation conditions.

1. Introduction

Cleaning and disinfection processes constitute a critical phase in the operation of the food industry (Fryer and Asteriadou, 2009). The intensity of cleaning treatments leads to the generation of wastewater with a high content of detergents, which constitute an unfavorable influence on water quality and the survival of aquatic organisms (Han et al., 2013). That is why the development of sustainable cleaning methods is currently under study, being examples of this research the addition of microparticles (Jurado-Alameda et al., 2015) or ozone (Jurado-Alameda et al., 2016) to detergent formulations.

Enzymatic detergents bring multiple advantages, such as lower temperature requirements and a decrease in the addition of harmful chemicals with an increase in soil removal (Bravo-Rodríguez et al., 2006). Within food installations, enzyme-based CIP (Cleaning In Place) systems have been tested with satisfactory cleaning efficiency and adequate cost-benefit balance (Boyce et al., 2010). Specifically, proteases have become a key ingredient in detergent formulations, facilitating the cleaning of proteinaceous deposits arising after typical operations of the food industry such as drying or heating, and being able to replace NaOH as the main ingredient in industrial cleaning (Grasshoff, 2002).

The enzymes contained in detergent formulations must maintain their activity and stability in the presence of typical formulation ingredients, such as surfactants, builders, bleaches, and other enzymes. Stability is crucial not only under washing conditions but also upon storage, being lower in liquid formulations because of the higher exposition to denaturants and easiness of undesirable reactions (Stoner et al., 2004). In this work, the activity and stability at storage of a protease intended for use as a detergent additive have been studied under washing conditions and at storage under accelerated aging conditions in the presence of an alkylpolyglucoside, a non-ionic surfactant. The remaining activity over time has been determined in order to ascertain the actual suitability of this enzyme to be included in cleaning formulations intended for CIP processes in the food industry.

Paper Received: 1 May 2018; Revised: 21 August 2018; Accepted: 5 November 2018

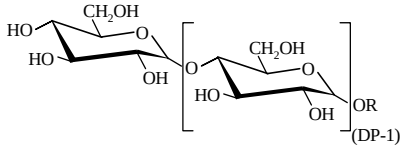
Please cite this article as: Herrera Marquez O., Fernandez Serrano M., Pilamala Bonilla M., Jacome Sandoval M.B., Jurado Alameda E., Luzon Gonzalez G., 2019, Activity and Stability in the Presence of a non Ionic Surfactant of a Protease for Hard Surface Cleaning in Food Industry, Chemical Engineering Transactions, 75, 187-192 DOI:10.3303/CET1975032

2. Materials and methods

2.1 Materials

The enzyme Bioproteasa L-800, specifically developed for detergency applications, was supplied by Biokatal (Barcelona, Spain). The abbreviation PR will be used to refer to this protease onwards. It shows an optimal activity at a pH range from 7 to 11, with a temperature of optimal performance around 60 ºC with slight variations depending on the pH.
The stability of this protease has been studied in the presence of an alkylpolyglucoside (abbreviated APG), a non-ionic surfactant supplied by Kao Corporation S.A. (Barcelona, Spain), whose main characteristics are listed in Table 1.

Table 1. Properties of the surfactant assayed

APG	
Surfactant	Alkylpolyglucoside
Trade name	Glucopon© 650 EC
Structural formula	
Alkyl chain length R	C_8-C_{14}
Polymerization degree DP	1.3[a]
Water content (%)	50.4[a]
CMC, mg/L (25 ºC)	153[b]
Molecular weight	397[a]

[a] Bravo-Rodríguez et al. (2005) [b] Ríos (2014)

2.2 Enzymatic activity

The activity of the protease Bioproteasa L-800 has been measured by a modification of Anson's method proposed by Sigma-Aldrich (2019). Hemoglobin from bovine blood was used for substrate preparation (2% w/v in urea 6 M), adjusting the pH of the solution to 8.0 with HCl 1 N before use. 0.5 mL of enzymatic solution was added to 2.5 mL of hemoglobin substrate, with a reaction time of 20 min. This activity assay was carried out at 40, 50 and 60 ºC. The hydrolysis reaction was stopped adding 5 mL of trichloroacetic acid (5% w/v). Samples were centrifuged for 5 min at 8000 g (Universal 320R, Hettich). Afterward, the solubilized proteins in the supernatant were assayed using the Folin-Ciocalteau phenol reagent (Folin and Ciocalteu, 1927) and absorbance was measured at 750 nm. One unit of protease activity will hydrolyze hemoglobin to produce color equivalent to 1.0 µmol of tyrosine per minute under the assay conditions. All activity assays were made in triplicate.

2.3 Stability under storage conditions

Two detergent solutions were prepared, both with a PR concentration of 3% w/w and with an APG concentration of 10% and 40% w/w, ranging within the usual concentrations of liquid detergents (Pongsawasdi and Murakami, 2010). These solutions were prepared in 0.1 M phosphate buffer, pH=8.0, and were agitated in a vortex for 2 min to ensure homogeneity. The stability test was carried out keeping these solutions at 40 °C and 60 ºC for a maximum of 15 days, taking samples periodically and determining their enzymatic activity immediately. After appropriate dilution, the activity assay was carried out at 40 ºC as described in 2.2. Residual protease activity was expressed as % of relative activity as compared to control, which was considered as 100%. All activity tests were made in triplicate.

2.4 Stability under washing conditions

A PR solution (0.06 g/L) with and without APG (at a concentration of 0.5 g/L) was used in the stability test under washing conditions. The enzyme and surfactant concentrations were chosen according to the habitual concentrations found in the washing liquor during the cleaning processes of hard surfaces (Gormsen, Marcussen and Damhus, 1998). These solutions were prepared in 0.1 M phosphate buffer pH=8.0, and were maintained at 60 ºC, taking samples periodically from 0 to 60 min. After appropriate dilution, activity assays were carried out at 40 ºC as described in 2.2. Residual enzymatic activity was expressed as % of relative activity as compared to activity at t=0, which was considered as 100%. All activity tests were made in triplicate.

3. Results and discussion

3.1. Enzymatic activity

According to the information provided by the supplier, the protease used in this work is stable in the temperature range commonly used in washing processes of hard surfaces. To determine the relationship between protease activity and temperature, activity tests were carried out at 40, 50 and 60 ºC at the experimental conditions described in 2.2. The enzymatic solution used for activity assays was prepared at a concentration of 0.01 g/L at pH 8.0 in 0.1 M phosphate buffer. Figure 1 shows that the activity of the protease has a linear relationship with the temperature at the operation range studied.

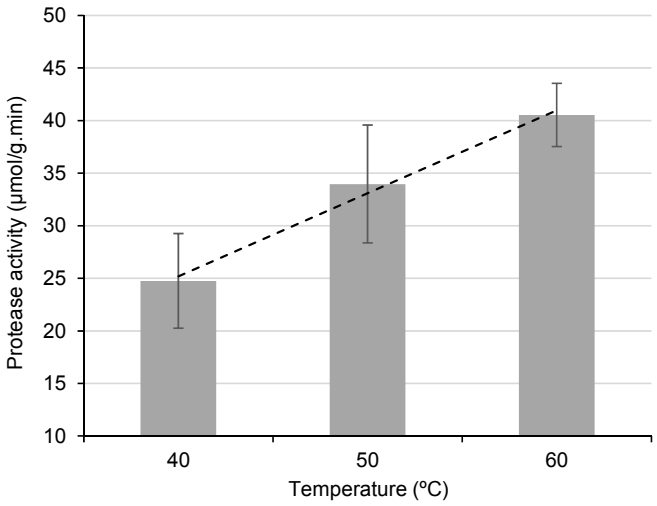


Figure 1. Protease activity as a function of temperature (pH of 8.0, protease concentration of 0.01 g/L, hemoglobin 2% w/v in urea 6 M as a substrate, incubation time of 20 min). The error bars represent ±SD of 3 replicates.

3.2. Stability under storage conditions

In recent years, different research works have been based on the synthesis and purification of proteases and the study of their stability against surfactants and another detergent additives, as well as against commercial detergent formulations (Dadshahi et al., 2016; Hammami et al., 2017). However, there is a lack of information on long-term stability of proteases at storage in the presence of surfactants at usual concentrations found in liquid detergents.

The protease studied undergoes a significant deactivation after only a few days of storage in the conditions of enzyme and surfactant concentrations stated in 2.3. In fact, at 60 ºC practically no residual enzymatic activity was found after only two days of storage. The results of the stability test under accelerated aging conditions (40 ºC up to 15 days) of the Bioproteasa L-800 in the presence of the non-ionic surfactant APG are shown in Figure 2. These results of relative activity can be adjusted to a second-order deactivation kinetic model:

$$-\frac{de}{dt} = k_2 e^2 \quad (1)$$

Integrating and relating the active enzyme, *e*, to relative enzymatic activity *a*, the expression for relative activity over time results in:

$$\frac{1}{a} = \frac{1}{a_O} + k_2 t \quad (2)$$

Trend lines of the deactivation models for these solutions are shown in Figure 2, while Table 2 shows the values obtained for the kinetic constants and their regression coefficients.

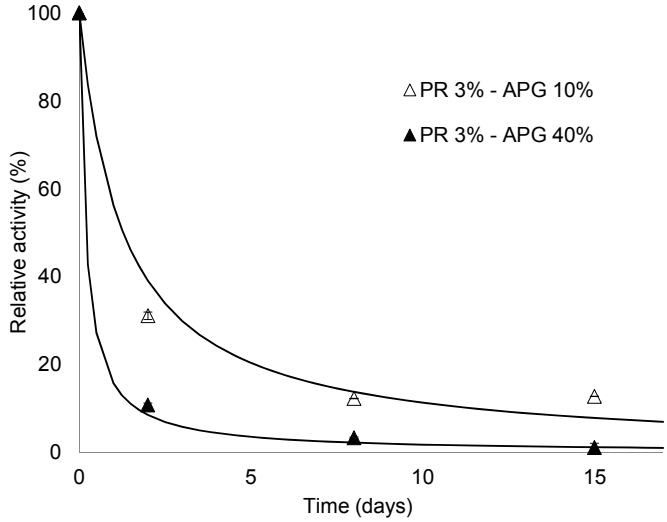


Figure 2. Stability of detergent solutions with protease (PR, 3% w/w) and APG (10% and 40% w/w) at 40 ºC. pH 8. Lines represent linear regression to a second-order deactivation kinetic model. The error bars represent ±SD of 3 replicates of activity assays.

Table 2. Kinetic constants and regression coefficients for the second-order deactivation model in stability study of detergent solutions with the protease at accelerated aging conditions.

Solution	Temperature (ºC)	k_2 (min^{-1})	R^2
PR 3% - APG 10%	40	0.78	0.95
PR 3% - APG 40%	40	5.34	0.97

These results suggest that the increasing concentration of APG in the detergent formulations may induce a faster deactivation. In a previous work of von Rybinski and Hill (1998) the presence of APG stabilized various enzymatic solutions under storage conditions, although the concentration of surfactant used in that work was only 5% w/w. Furthermore, protease was the enzyme in which a lower effect on stability was observed with the addition of APG, which suggests that the presence of APG at high concentrations does not contribute to the stability of the protease in liquid detergents.

3.3. Stability under washing conditions

The study of the stability of proteases in solutions of non-ionic surfactants has been usually carried out at temperatures below 40 ºC (Hammami et al., 2017; Jellouli et al., 2011). Specifically, Zhang and Zhang (2016) demonstrated that a commercial protease kept its activity in the presence of APG up to 5 h at 25 ºC. Nevertheless, stability studies at higher temperatures are rarely found despite they are frequently used in CIP (Cleaning In Place) processes in the food industry. Figure 3 shows the time profiles of the relative activity of the Bioproteasa L-800 in phosphate buffer pH 8.0 and in the presence of the surfactant APG at 60 ºC, both enzyme and surfactant in concentrations usually found in the washing liquor during cleaning processes. As it can be seen, the protease undergoes a complete deactivation after only 60 min, being the relative activity profiles adjusted to a first-order deactivation kinetic model where the concentration of active enzyme *e* follows the equation:

$$-\frac{de}{dt} = k_1 e \tag{3}$$

Relating *e* to relative enzymatic activity *a* and after integration the expression for relative activity over time becomes

$$a = a_o \exp(-k_1 t) \tag{4}$$

The values of kinetic constants and correlation parameters are shown in Table 3.

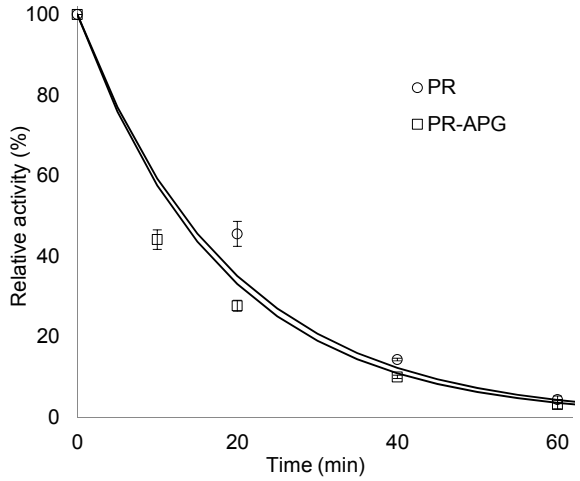


Figure 3. Stability of the protease (PR, 0.06 g/L) over time and in the presence of APG (0.5 g/L) at 60 ºC. pH of 8.0. Lines represent linear regression to a first-order deactivation kinetic model. The error bars represent ±SD of 3 replicates of activity assays.

Table 3. Kinetic constants and regression coefficients for first-order deactivation model in stability study of detergent solutions with a protease under washing conditions.

Solution	Temperature (ºC)	k_1 (min^{-1})	R^2
PR (0.06 g/L)	60	0.052	0.99
PR (0.06 g/L) - APG (0.5 g/)	60	0.055	0.99

The protease relative activity profiles and the values of the kinetic constants obtained suggest that, unlike that obtained in the stability at storage study, the presence of the surfactant APG does not have a significant effect on the stability of the enzymatic solution.

4. Conclusions

Stability study at storage at 40 ºC shows that the increasing concentration of APG in the detergent formulation contributes to a faster deactivation of the protease studied. At washing conditions, with significantly lower enzyme and surfactant concentrations, the presence of the APG does not have influence on the deactivation profile, but the enzyme undergoes a complete deactivation after 60 min. Relative activity results over time can be adjusted to kinetic models, showing a second-order deactivation under storage conditions and a first-order deactivation under washing conditions. Results at 60 ºC suggest that this protease is not suitable to be used in cleaning process that take place at high temperatures. Stability tests results at 40 ºC, on the other hand, suggest the need to stabilize this protease within the detergent formulation, adding stabilizers or using newer methods such as encapsulation.

Acknowledgments

We thank Biokatal (Barcelona, Spain) for providing the enzyme Bioproteasa L-800. This work has been financed by the Ministerio of Economía y Competitividad, Spain (Research Project ref. CTQ2015-69658-R).

References

Boyce, A., Piterina, A. V., Walsh, G., 2010, Assessment of the potential suitability of selected commercially available enzymes for cleaning-in-place (CIP) in the dairy industry, Biofouling, 26, 837–850. https://doi.org/10.1080/08927014.2010.522705

Bravo-Rodríguez, V., Jurado-Alameda, E., Martínez-Gallegos, J.F., Reyes-Requena, A., García-López, A.I., 2006, Thermal deactivation of a commercial α-amylase from Bacillus licheniformis used in detergents, Biochemical Engineering Journal, 27, 299–304. https://doi.org/10.1016/j.bej.2005.08.018

Bravo-Rodriguez, V., Jurado Alameda, E., Reyes Requena, A., García López, A.I., Bailón-Moreno, R., Cuevas Aranda, M., 2005, Determination of average molecular weight of commercial surfactants:

Alkylpolyglucosides and fatty alcohol ethoxylates. Journal of Surfactants and Detergents, 8, 341–346. https://doi.org/10.1007/s11743-005-0366-y

Dadshahi, Z., Homaei, A., Zeinali, F., Sajedi, R.H., Khajeh, K., 2016, Extraction and purification of a highly thermostable alkaline caseinolytic protease from wastes Penaeus vannamei suitable for food and detergent industries, Food Chemistry, 202, 110–115. https://doi.org/10.1016/J.FOODCHEM.2016.01.104

Folin, O., Ciocalteu, V., 1927, On tyrosine and tryptophane determinations in proteins, The Journal of Biological Chemistry, 73, 627–650.

Fryer, P.J., Asteriadou, K., 2009, A prototype cleaning map: A classification of industrial cleaning processes, Trends in Food Science and Technology, 20, 255–262. https://doi.org/10.1016/j.tifs.2009.03.005

Gormsen, E., Marcussen, E., Damhus, T., 1998, Enzymes, Chapter in: Showell, M.S. (Ed.), Powdered Detergents, Surfactant Science Series, 71, CRC Press, Taylor & Francis Group, USA, 137–164.

Grasshoff, A., 2002, Enzymatic Cleaning of Milk Pasteurizers, Food and Bioproducts Processing, 80, 247–252. https://doi.org/10.1205/096030802321154736

Hammami, A., Hamdi, M., Abdelhedi, O., Jridi, M., Nasri, M., Bayoudh, A., 2017, Surfactant- and oxidant-stable alkaline proteases from Bacillus invictae: Characterization and potential applications in chitin extraction and as a detergent additive, International Journal of Biological Macromolecules, 96, 272–281. https://doi.org/10.1016/J.IJBIOMAC.2016.12.035

Han, W., Zhou, G., Blower, S., 2013, Study on the Treatment of Surfactant Wastewater by Anaerobic Biological Filter, Advanced Materials Research, 671–674, 2754–2759. https://doi.org/10.4028/www.scientific.net/AMR.671-674.2754

Jaiswal, N., Prakash, O., 2011, Immobilization of Soybean α-amylase on Gelatin and its Application as a Detergent Additive, Asian Journal of Biochemistry, 6, 337–346.

Jellouli, K., Ghorbel-Bellaaj, O., Ayed, H. Ben, Manni, L., Agrebi, R., Nasri, M., 2011, Alkaline-protease from Bacillus licheniformis MP1: Purification, characterization and potential application as a detergent additive and for shrimp waste deproteinization, Process Biochemistry, 46, 1248–1256. https://doi.org/10.1016/J.PROCBIO.2011.02.012

Jurado-Alameda, E., Vicaria, J.M., Herrera-Márquez, O., Olivares, V., Sosa, G., 2016, Study on the use of ozonation catalyzed by nanoparticles for ecological cleaning processes, Chemical Engineering Transactions, 47, 259-263. https://doi.org/10.3303/CET1647044

Jurado, E., Herrera-Márquez, O., Plaza-Quevedo, A., Vicaria, J.M., 2015, Interaction between non-ionic surfactants and silica micro/nanoparticles. Influence on the cleaning of dried starch on steel surfaces, Journal of Industrial Engineering and Chemistry, 21, 1383-1388. https://doi.org/10.1016/j.jiec.2014.06.011

Mitidieri, S., Souza Martinelli, A.H., Schrank, A., Vainstein, M.H., 2006, Enzymatic detergent formulation containing amylase from Aspergillus niger: a comparative study with commercial detergent formulations. Bioresource Technology, 97, 1217–24. https://doi.org/10.1016/j.biortech.2005.05.022

Pongsawasdi, P., Murakami, S., 2010, Carbohydrases in detergents, Chapter In: Hagen, E.T. (Ed.), Detergents: Types, Components and Uses, Biochemistry Research Trends Series, Nova Science Publishers, USA, 71-95.

Ríos, F., 2014, Environmental impact of commercial surfactants: biodegradability, toxicity and ozonation, Ph.D. Thesis, University of Granada, Granada, Spain.

Roy, J.K., Mukherjee, A.K., 2013, Applications of a high maltose forming, thermo-stable α-amylase from an extremely alkalophilic Bacillus licheniformis strain AS08E in food and laundry detergent industries, Biochemical Engineering Journal, 77, 220–230. https://doi.org/10.1016/j.bej.2013.06.012

Roy, J.K., Rai, S.K., Mukherjee, A.K., 2012, Characterization and application of a detergent-stable alkaline α-amylase from Bacillus subtilis strain AS-S01a. International Journal of Biological Macromolecules, 50, 219–29. https://doi.org/10.1016/j.ijbiomac.2011.10.026

Sigma-Aldrich, 2019, Enzymatic Assay of Proteinase K with Hemoglobin Substrate <www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-proteinase-k-hemoglobin-substrate.html> accessed 3.1.19.

Stoner, M.R., Dale, D.A., Gualfetti, P.J., Becker, T., Manning, M.C., Carpenter, J.F., Randolph, T.W., 2004, Protease autolysis in heavy-duty liquid detergent formulations: effects of thermodynamic stabilizers and protease inhibitors, Enzyme and Microbial Technology, 34, 114–125. https://doi.org/10.1016/J.ENZMICTEC.2003.09.008

von Rybinski, W., Hill, K., 1998, Alkyl Polyglycosides—Properties and Applications of a new Class of Surfactants, Angewandte Chemie International Edition, 37, 1328–1345. https://doi.org/10.1002/(SICI)1521-3773(19980605)37:10<1328::AID-ANIE1328>3.0.CO;2-9

Zhang, J., Zhang, J., 2016, Study on the interaction of alkaline protease with main surfactants in detergent. Colloid and Polymer Science, 294, 247–255. https://doi.org/10.1007/s00396-015-3777-3