PRODUCTION OF ANTIOXIDANT HYDROLYSATE FROM BOVINE CASEIN USING IMMOBILIZED *PENICILLIUM AURANTIOGRISEUM* PROTEASE ON MAGNETIC NANOPARTICLES

J. M. W. DUARTE NETO[1], J. C. MACIEL[2], L. B. CARVALHO Jr[1], C. A. LIMA[3] e A. L. F. PORTO[1]

[1] Universidade Federal de Pernambuco, Laboratório de Imunopatologia Keizo Asami
[2] Universidade Federal de Roraima, Conselho Superior
[3] Universidade de Pernambuco, Campus Garanhuns
E-mail para contato: analuporto@yahoo.com.br

ABSTRACT – Peptides with bioactive properties can be released from the precursor proteins during food processing or digestion, and act as antioxidative compounds. Although, it has been proven that hydrolysate exerts higher antioxidant activity than purified peptides. The process of enzyme immobilization proved to be more practical and advantageous for catalysis than using free enzyme. The aim of this study was to use a protease produced by *Penicillium aurantiogriseum* immobilized on polianilin-coated magnetic nanoparticles, for bovine casein hydrolysis and to evaluate the hydrolysate antioxidant properties. The 60-80% saline fraction of crude extract was used in immobilization. Commercial casein from bovine milk 1% (w/v) was hydrolyzed by 10 mg/mL of immobilized protease. The casein hydrolysates were used in ABTS and H_2O_2 scavenging activity assays, where Trolox was used as standard. In the ABTS scavenging assay, the hydrolysate (1% w/v) revealed a 1341 µmol of Trolox equivalent activity and in the hydrogen peroxide scavenging assay, the hydrolysate (using 0.1% w/v) presented a 547.82 µmol of Trolox equivalent activity. In both assays the hydrolysates presented a more than 2.5 times greater activity than non-hydrolyzed casein, which validates the process capacity of developing casein derived natural antioxidant ingredients with potential for functional foods.

1. INTRODUCTION

Reactive oxygen species (ROS) is a important component of our defense system, helping the host against microbial infection, but when overexpressed and accumulated beyond the cell antioxidant capacity, can affect energy production, survival, cell growth and numerous signaling pathways (Duan et al. 2014; Kim et al. 2013). The excessive ROS can be related to diabetes, chronic inflammation, hypertension, atherosclerotic cardiovascular disease, aging and cancer (Guo et al. 2014).

In food system, oxidative products as well as free radicals can be produced by lipids oxidation, especially in polyunsaturated fatty acids rich foods. It ends up compromising the taste of food, decreasing its shelf-life (Intarasirisawat et al. 2013). Synthetic antioxidants are commonly used in the food industry due to its stronger antioxidant activity, however have been proven to have potential health risks, so its use should be kept under strict control. Since natural antioxidants, such as tocopherols, can have higher production costs and lower efficiency, together with the fact of the growing concern of the consumed food additives, there is a growing demand for the identification of new natural sources of food antioxidants. This scenario, along with the well-known safety of natural peptide sequences, has led to an increasing interest in food-derived antioxidant peptides (Girgih et al. 2013).

The casein peptides have the potential to exert numerous health improving effects. Examples of bioactive peptides derived from casein include casein phosphopeptides, can play a role in the transport and absorption of certain minerals, among other properties, glycomacropeptides that bind toxins, which have properties to behave as casoxins, immunomodulators, opioid antagonists, while casomorphins can behave as agonist opioid receptor (Muro Urista et al. 2011). Caseins are available in large amounts at a high degree of purity and at low price in market which, make them attractive in the search for bioactive peptides (Corrêa et al. 2011). Several plants, animals, and microbial proteases are employed for peptides production.

Although, it has been proven that hydrolysate can exerts higher antioxidant activity than purified peptides. When it comes to the area of nutritional sciences application of non-purified protein hydrolysate can have certain benefits over those of purified peptides since the absorption of oligopeptides can be increased in the presence of sugar and amino acids (Sarmadi and Ismail, 2010).

Microorganisms proteases are more often used in industrial hydrolysis because of their well-known methods of rapid growth, the limited cultivation space required and the easy genetic manipulation to generate new enzymes with improved properties (Abidi et al. 2013).The Penicillium genus and its related mesophilic, thermophilic and acid-toletants species, are capable of producing extracellular proteases and due to its nutritionally undemanding nature they can grow in a range of diverse conditions, characteristics of industrial interest (Graminho et al. 2013). The *Penicillium aurantiogriseum* was described as a large amount producer of extracellular collagenolytic protease (Lima et al. 2011).

Enzyme immobilization has proved to add catalysis advantages. Immobilized enzymes can be easily separated from the products of hydrolysis, allowing continuous processes and reuse of the enzyme. Besides immobilization process can improve enzyme temperature resistance, protect against inhibitors action and autolysis (Tavares et al. 2013). The immobilization on magnetic particles is becoming preferable to other supports for industrial use due to easier recovery of the immobilized enzyme (Neri et al. 2008).

The objectives of the present study were to use the protease produced by *Penicillium aurantiogriseum* immobilized on magnetic nanoparticles on commercial bovine casein hydrolysis to evaluate its antioxidant properties, aiming the potential use of this enzyme in the production of antioxidant functional food ingredients derived from casein.

2. MATERIAL AND METHODS

2.1. Microorganism and Protease production

The *Penicillium aurantiogriseum* dierchx (URM4622) used was obtained in University Recife Mycologia (URM), inscribed in the Commonwealth Mycological Institute (CMI). The soybean flour medium was used for protease production as described by Lima et al. 2011, composed of 1.65% (w/v) filtered soybean flour (SF), 0.1% (w/v) NH_4Cl, 0.06% (w/v) $MgSO_4.7H_2O$, 0.435% (w/v) K_2HPO_4, 0.01% (w/v) glucose and 1.0% (v/v) mineral solution, pH 7.2. The mineral solution content was 100 mg $FeSO_4 \cdot 7H2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 100 mg $ZnSO_4 \cdot H_2O$ and 100 mg $CaCl_2 \cdot H_2O$, per 100 mL of distilled water.

The inoculum was prepared suspending the spores in 3 mL of a 0.9% (w/v) NaCl and 0.01% (v/v) Tween 80 solution previously sterilize, with 10^6 spores/mL, counted in Neubauer chamber. After inoculation, production were set up in 250 mL Erlenmeyer flasks containing 50 mL of the soybean flour medium and the fermentation occurred at 24 °C and 200 rpm over 72 h. At the end of fermentation, the broth obtained was vacuum filtered through 0.45 µm pore diameter nitrocellulose membranes to remove the mycelia. The filtrate was used as an enzyme source (referred to as the crude extract). The crude extract was precipitated by 60-80% ammonium sulphate saturation at 4°C. The obtained precipitates (partial purified enzyme) were dissolved in 0.05M Tris-HCl buffer (pH 9), dialysed over water and used in the immobilization process.

2.2. Protease immobilization

To produce the magnetite core nanoparticles, 5 mL of 1.1 M $FeCl_3 .6H_2O$ and of 0.6 M $FeCl_2 .4H_2O$ was added in 50 mL of distilled, under vigorous stirring, 5.0 M NaOH was added dropwise up to pH 10 and the temperature was increased up to 50°C for 30 min. The particles were thoroughly washed with distilled water until pH 7 was reached. After dried up (at 50 ºC) and macerated. Then 0.5 g of nanoparticles were added to 50 mL of 0.1 M $KMnO_4$ solution at 25°C for 1 h. After washed up, the magnetic-$KMnO_4$ nanoparticles were immersed into 50 mL of 0.5 M aniline solution diluted in 1.0 M HNO_3. Polymerization occurred at 4°C for 1 h. After that, the PANI coated nanoparticles were successively washed with distilled water, 0.1 M citric acid and rewashed with distilled water, and finally dried up at 50 °C and kept at 25°C.

For immobilization, the magnetic nanoparticles coated with polyaniline (PANI), was activated with a 2% (v/v) glutaraldehyde solution for 7.3 h. After washed ten times with distilled water and three times with 0,2 M Tris-HCl buffer pH 6.3, the activated magnetic nanoparticles were mixed with the partially purified enzyme solution, at a concentration of 200 µg/mL for 2 h, to immobilize. Then, the nanoparticles were washed over 30 min with 0.1M glycine solution, under gentil rotation, and other two times with distilled water. The washed up were used to measure non-binding proteins and estimate the immobilized amount.

2.3. Preparation of casein hydrolysates

Commercial casein from bovine milk, Sigma-Aldrich, was dissolved into 0.05M Tris-HCl buffer (pH 9) in a concentration of 1% (w/v). 100 µL of protease, in a concentration of 1.5 mg/mL, was added in a 20 mL of casein solution, in a 50 mL Erlenmeyer flask in water bath under 37 ºC. The hydrolisys occurred for 2.3 h, 2 mL aliquots were collected at 5, 15, 25, 35, 45, 65, 95, 125 and 155 min. A control was aliquotted just after adding the enzyme. All aliquots were heated at 80 ºC for 20 min, to stop the hydrolysis. Finally, the hydrolysates were lyophilized and stored at -18 ºC for further use. The same process was done using approximately the same amount of immobilized protease.

2.4. ABTS radical scavenging activity

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was measured using the method described by (Re et al. 1999). Using 7 mM of ABTS solution and 140 mM of potassium persulphate solution. The working solution was prepared by mixing 5 mL of ABTS solution with 88 µL of potassium persulphate solution and allowing them to react for 10 h at room temperature in dark. The solution was then diluted by mixing 1mL of ABTS working solution with approximately 50 mL of ethanol in order to obtain an absorbance of 0.7 units at 734 nm using a Ultrospec® 3000 spectrophotometer (Amersham). Fresh ABTS solution was prepared daily. Sample (30 µL) with different concentrations (1%, 0.5% and 0.1% w/v) was mixed with 3 mL of ABTS working solution and the mixture was left for 5 min at 25 ºC in dark, the same ABTS working solution with distilled water was used as blank sample and the non-hydrolyzed casein was used as reference sample. The absorbance was then measured at 734 nm. A standard curve of Trolox ranging from 100 to 1500 mM was prepared.

2.5. Hydrogen peroxide scavenging activity

The Hydrogen peroxide scavenging activity was assayed according to the method of Kittiphattanabawon et al. 2012. 850 µL from sample was mixed with 150 µL of 43 mM hydrogen peroxide in 0.1 M phosphate buffer, pH 7.4. After 5 min of reaction at 25 ºC, the absorbance was recorded at 230 nm. In the blank sample we used the same phosphate buffer without hydrogen peroxide and also used non-hydrolyzed casein as reference sample. Trolox (0–10 mM) was used as standard. The activity was expressed as µmol Trolox equivalents (TE)/mg sample.

3. RESULTS

Casein hydrolysates were reported to have highest concentration of histidine, lysine, proline and tyrosine, and all these amino acids have been previously found to act as free radical scavengers (Pihlanto, 2006). Scavenging activities of bovine casein hydrolysates were determined using two radicals: ABTS and H_2O_2, results presented in Table 1. The radical ABTS is reduced with concomitant conversion to a colourless product in the presence of antioxidants

with hydrogen-donating or chain-breaking properties. The hydrolysis of bovine casein with *P. aurantiogriseum* protease tended to enhance the antioxidant activity of hydrolysates measured by the ABTS assay. The antioxidant activity of the milk proteins hydrolysates seems to be inherent to the characteristic amino acid sequences of peptides derived, depending on the used protease specificity, although the relationship between peptide structure and activity or the antioxidant mechanism is yet not fully understood (Pihlanto, 2006). The increased antioxidant activity through hydrolysis suggests that this process contributed to antioxidant activity by releasing previously inactive peptides encrypted in the sequence of bovine casein. The 1% w/v hydrolysate demonstrated an equivalent activity to 1341 µmol of Trolox, and reaching 44.35% of ABTS inhibition. Intact bovine casein also shown to possess antioxidant properties, as measured by the ABTS method, although, hydrolysates demonstrated a 2.72 times higher activity than the non-hydrolyzed casein, as observed in Table 1.

The hydrogen peroxide, as reactive oxygen species (ROS), can cause oxidative stress and damage of biomolecule in the cell, leading to cell death and serious chronic diseases. Hydrogen peroxide, a weak oxidizing agent, is implicated indirectly in lipid oxidation. Hydrogen peroxide is a reactive non radical, which can permeate biological membranes and be converted to more reactive species as hydroxyl radical and singlet oxygen (Intarasirisawat et al. 2013). The casein hydrolysate exhibited strong hydrogen peroxide scavenging activity. 0.1 % w/v of the hydrolysate showed to be equivalent to 547.82 µmol of Trolox, indicating an H_2O_2 scavenging activity of 644.5 µmol of trolox/mg of sample. Although the non-hydrolyzed casein also presented a scavenging activity, hydrolysate sample showed a 3.44 times higher activity.

Furthermore, many factors can influence antioxidant activity of bioactive peptides as well. The antioxidant and biological activities can be affected by the operational conditions applied to isolate proteins, degree of hydrolysis, type of protease and peptide concentration. However, it has been postulated that the overall antioxidative activity must be ascribed to the integrative effects of these actions rather than to the individual actions of peptides (Sarmadi and Ismail, 2010).

4. CONCLUSION

Commercial bovine casein hydrolysis using *Penicillium aurantiogriseum* protease immobilized on magnetic nanoparticles coated with PANI was successfully done. The hydrolysates had their ROS scavenging properties tested, revealing a 1341 µmol of Trolox equivalent ABTS scavenging activity using 1% w/v hydrolysate concentration and 547.2 µmol of Trolox equivalent hydrogen peroxide scavenging activity using 0.1% w/v hydrolysate concentration. In both assays the hydrolysates presented a more than 2.5 times greater activity than non-hydrolyzed casein, confirming the potential use of *Penicillium aurantiogriseum* protease in antioxidant casein-derived functional foods production.

5. TABLES

Table 1. ABTS and H_2O_2 radical scavenging activities of casein hydrolysate.

Peptides (w/v)	**ABTS**		**H2O2**	
	Equivalent [Trolox] (µM)	(% inhibition)	Equivalent [Trolox] (µM)	µmol Trolox/mg sample
1%	1341	44.35		
0.5 %	771	23.65		
0.1%	297.67	6.47	547.82	644.5
0.05%			293.42	690.4
0.01%			47.42	557.88
Casein (w/v)				
0.1%	254.33	4.9	149.42	175.79

6. REFERÊNCIAS

ABIDI, F.; AISSAOUI, N.; GAUDIN, J-C.; CHOBERT, J-M.; HAERTLÉ, T.; MARZOUKI, M.N. MS Analysis and Molecular Characterization of Botrytis cinerea Protease Prot-2. Use in Bioactive Peptides Production. *Appl. Biochem. Biotechnol.*, v. 170 (2), p. 231-47, 2013.

CORRÊA, A.P.F.; DAROIT, D.J.; COELHO, J.; MEIRA, S.M.M.; LOPES, F.C.; SEGALIN, J.; RISSO, P.H.; BRANDELLI, A. Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. *J. Sci. Food. Agric*., v.91, p. 2247–54, 2011.

DUAN, X.; OCEN, D.; WU, F.; LI, M.; YANG, N.; XU, J.; CHEN, H.; HUANG, L.; JIN, Z.; XU, X. Purification and characterization of a natural antioxidant peptide from fertilized eggs. *Food. Res. Int.* v. 56, p. 18–24, 2013.

GIRGIH, A.T.; HE, R.; MALOMO, S.; OFFENGENDEN, M.; WU, J.; ALUKO, R.E. Structural and functional characterization of hemp seed (Cannabis sativa L.) protein-derived antioxidant and antihypertensive peptides. *J. Funct. Foods*., v.1, p. 1–11, 2013.

GUO, Y.; ZHANG, T.; JIANG, B.; MIAO, M.; MU, W. The effects of an antioxidative pentapeptide derived from chickpea protein hydrolysates on oxidative stress in Caco-2 and HT-29 cell lines. *J. Funct. Foods*., p. 1–8, 2014.

GRAMINHO, E.R.; DA SILVA, R.R.; DE FREITAS CABRAL, T.P.; ARANTES, E.C.; DA ROSA, N.G.; JULIANO, L.; OKAMOTO, D.N.; DE OLIVEIRA, L.C.G.; KONDO, M.Y.; JULIANO, M.A.; CABRAL, H. Purification, characterization, and specificity determination

of a new serine protease secreted by *Penicillium waksmanii*. *Appl. Biochem. Biotechnol.*, v. 169, p. 201–14, 2013.

INTARASIRISAWAT, R.; BENJAKUL, S.; WU, J.; VISESSANGUAN, W. Isolation of antioxidative and ACE inhibitory peptides from protein hydrolysate of skipjack (*Katsuwana pelamis*) roe. *J. Funct. Foods*., v. 5, p. 1854–1862, 2013.

KIM, E-K.; OH, H-J.; KIM, Y-S.; HWANG, J-W.; AHN, C-B.; LEE, J.S.; JEON, Y-J.; MOON, S-H.; SUNG, S.H.; JEON, B-T.; PARK, P-J. Purification of a novel peptide derived from *Mytilus coruscus* and in vitro/in vivo evaluation of its bioactive properties. *Fish. Shellfish. Immunol*., v. 34, p. 1078–84, 2013.

KITTIPHATTANABAWON, P.; BENJAKUL, S.; VISESSANGUAN, W.; SHAHIDI, F. Gelatin hydrolysate from blacktip shark skin prepared using papaya latex enzyme: Antioxidant activity and its potential in model systems. *Food. Chem*., v. 135, p. 1118–26, 2012.

LIMA, C.A.; MARQUES, D.A.V.; BARROS NETO, B.; LIMA FILHO, J.L.; CARNEIRO-DA-CUNHA, M.G.; PORTO, A.L.F. Fermentation medium for collagenase production by *Penicillium aurantiogriseum* URM4622. *Biotechnol. Prog*., v. 27, p. 1470–7, 2011.

LIMA, C.A.; FILHO, J.L.L.; NETO, B.B.; CONVERTI, A.; CUNHA, M.G.C.DA.; PORTO, A.L.F. Production and Characterization of a Collagenolytic Serine Protease by *Penicillium aurantiogriseum* URM 4622: A Factorial Study. *Biotechnol. Bioprocess. Eng*., 2011.

MURO URISTA, C.; ÁLVAREZ FERNÁNDEZ, R.; RIERA RODRIGUEZ, F.; ARANA CUENCA, A.; TÉLLEZ JURADO, A. Review: Production and functionality of active peptides from milk. *Food. Sci. Technol. Int*., v. 17, p. 293–317, 2011.

NERI, D.F.M.; BALCÃO, V.M.; COSTA, R.S.; FERREIRA, E.M.F.C.; TORRES, D.P.M.; RODRIGUES, L.R.; JR, L.B.C.; TEIXEIRA, J.A. B-Galactosidase from Aspergillus oryzae immobilized onto different magnetic supports : A comparative experimental and modelling study of the galactooligosaccharides production. p. 1036–1041, 2008.

PIHLANTO, A. Antioxidative peptides derived from milk proteins. *Int. Dairy. J.*, v. 16, p. 1306–1314, 2006.

RE, R.; PELLEGRINI, N.; PROTEGGENTE, A.; PANNALA, A.; YANG, M.R.C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free. Radical. Biol. Med.*, v. 26, p. 1231–1237, 1999.

SARMADI, B.H.; ISMAIL, A. Antioxidative peptides from food proteins: a review. *Peptides*, v. 53, p. 1949–56, 2010.

TAVARES, A.P.M.; RODRÍGUEZ, O.; FERNÁNDEZ-FERNÁNDEZ, M.; DOMÍNGUEZ, A.; MOLDES, D.; SANROMÁN, M.; MACEDO, E. Immobilization of laccase on modified silica: stabilization, thermal inactivation and kinetic behaviour in 1-ethyl-3-methylimidazolium ethylsulfate ionic liquid. *Bioresour. Technol.*, v. 131, p.405–12, 2013.