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# Effects of Ultrasonic and High-Pressure Homogenization Pretreatment on the Enzymatic Hydrolysis and Antioxidant Activity of Yeast Protein Hydrolysate

***Z. S. Moosavi*** [a], ***S. Mirdamadi*** [b*], ***M. Mirzaei*** [c*], ***M. Larypoor*** [d]

[a] M. Sc. of the Department of Microbiology, North Branch of Islamic Azad University, Tehran, Iran.
[b] Professor of the Department of Biotechnology, Iranian Research Organization for Science & Technology (IROST), Tehran, Iran.
[c] Assistant Professor of the Department of Food Science and Technology, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran.
[d] Assistant Professor of the Department of Microbiology, North Branch of Islamic Azad University, Tehran, Iran.

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ABSTRACT: Protein hydrolysate is highly regarded as a source of naturally occurring antioxidant peptides. The purpose of this study was to investigate the effect of Ultrasonic (Frequency, 20 KHz; Amplitude, 50%; Time, 30 min) and high-pressure homogenization (Power, 1500 bar; Rated flow, 10 dm/h) pretreatment on the enzymatic hydrolysis and antioxidant properties of yeast protein hydrolysate obtained from *Kluyveromyces marxianus*. Trypsin and chymotrypsin were used for protein hydrolysis. Respectively, 73.22%, 23.01% of the total protein was released through sonication and high-pressure homogenization processes. The progress of the enzymatic hydrolysis was evaluated based on the number of free amino groups measured by the O-phetaldialdehyde (OPA) method. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities assays were used to evaluate the antioxidant activity. Sonication pretreatment caused a higher degree of hydrolysis by chymotrypsin compared to high-pressure homogenization. Samples pretreated by high-pressure homogenization exhibited significantly ($P<0.05$) higher DPPH and ABTS radicals scavenging activity when hydrolyzed by trypsin and higher ABTS radical scavenging activity when hydrolyzed by chymotrypsin. The degree of hydrolysis increased with increasing hydrolysis time. The chymotrypsin was significantly ($P<0.05$) more effective than trypsin in the hydrolysis of protein. High-pressure homogenization pretreatment and trypsin hydrolysis were considered as the best method for producing yeast protein hydrolysate with DPPH (297.36 µMTE/mg protein) and ABTS (1189.02 µMTE/mg protein) radicals scavenging activities.

**Keywords**: *Antioxidant Activity, Enzymatic Hydrolysis, High-Pressure Homogenization, Kluyveromyces marxianus, Sonication.*

## Introduction

Today, diet and lifestyle play a significant role in our lives. The awareness of the importance of healthy diets led to the development of new, safe and healthy foods. Oxidation is an important reason for the food spoilage and can be the cause of food deterioration and shorten the shelf-life. It is also the leading cause of many diseases, such as cardiovascular disease, diabetes, inflammatory disease, and ageing. The synthetic antioxidants are subject to strict regulation because of the potential health

*Corresponding Author: Mirdamadi@irost.ir, Mahtam86@gmail.com

risk (Hettiarachy *et al*., 1996). Therefore, there is a massive demand for natural antioxidants as an alternative to synthetic compounds (Zeece *and Kelly,* 2008; Rao *et al*., 2012). Natural food-derived peptides with specific bioactivity were therefore aroused a great deal of interest among the researchers and were defined as the specific protein fragments that exhibit bioactivity including antihypertensive, antimicrobial and antioxidant activity (Hartman and Meisel, 2007).

Enzymatic hydrolysis is the process of breaking down the peptide bonds of the parent protein (Souissi *et al*., 2007; Nasri 2017) and is widely used to extract bioactive peptides from various food protein sources. The progress of enzymatic hydrolysis is influenced by different factors, including the type of enzymes, time of hydrolysis, enzyme to substrate ratio and protein pretreatment. Even for a specific enzyme, the variety of possible cleavage sites is vast, and pretreatment of protein can be considered as a new approach to reduce undesirable cleavages and increase the hydrolysis desired peptide bonds. Conformational changes within the protein structure could improve the accessibility of the enzyme to desired peptide bonds (Leeb *et al*., 2011) .

Yeast cells containing 45-55% protein has been considered as a protein source for producing bioactive peptides (Mirzaei *et al*., 2015; Revillion *et al*., 2003; Hernawan & Fleet, 1995). *Kluyveromyces marxianus* is a GRAS (Generally Regarded As Safe) and important yeast that has shown great potential for producing single cell protein (SCP).

Our previous research had indicated the antioxidant activity of yeast protein hydrolysate prepared by trypsin and chymotrypsin (Mirzaei *et al*., 2015; Mirzaei *et al*., 2016; Mirzaei *et al*., 2017).

This research aimed to compare two different pretreatments including high-pressure homogenization and sonication, for their influence on the progress of enzymatic hydrolysis as well as the antioxidant activity of the final product through possibility unfolding the protein structure. Also, finding the relationship between the progress of enzymatic hydrolysis and antioxidant activity was the subject of this research. Autolysis treatment was considered as a standard and reference method for yeast cell disruption and protein hydrolysis.

## Materials and Methods

- *Materials*

Chemical were purchased from Sigma-Aldrich Chemical Company, America. *K.marxianus* (PTCC 5195) was obtained from Persian Type Culture Collection (PTTC), culture media were obtained from Merck Chemical Company, Germany.

- *Culture conditions*

The yeast cells were cultured on yeast mold (YM) at 28°C, 150 rpm for 24 hr. Cells were harvested through centrifugation at 3000g for 15 min. The yeast pellet was washed three times with distilled water and stored at -20°C.

- *Autolysis treatment*

Collected cells (1.25 gr) were suspended in 50 ml distilled water containing 0.75 ml ethyl acetate and incubated at 52°C, 120 rpm, pH of 5 for 72 hr (Beehalova & Beran, 1986; Conway *et al*., 2001; Wang *et al*., 2003). Samples were takenand enzymes were inactivated by heating at 85°C for 15 minutes. The autolysates were centrifuged (11500 g, 15 min), and the supernatant was stored at -20 °C for further analysis.

- *Processing of yeast cells under high hydrostatic pressure (HHP) and sonication treatment*

High-pressure homogenization treatment was performed with a homogenizer model GEA Niro Soavi Panda PLUS 2000 with a pressure of 1500 bar, flow rate of 10 dm/h,

minimum sample volume of 30 ml, dimensions of 81×54×44 cm, and using distilled water as the pressure medium. Sonication treatment was performed with a sonicator model Bandelin sonoplus HD 2200 with operating voltage of 230 V, frequency of 20 kHz and total time of 30 min at 2 min intervals. Finally, the suspensions were centrifuged at 11500 g for 15 min, and the supernatant was stored at -20°C.

- *Enzymatic hydrolysis*

The protein solution (4 mg/ml) prepared by sonication and high-pressure homogenization was treated by two enzymes including trypsin (Sigma, 1000-2000 Unit/mg solid ) and chymotrypsin (Sigma, 60 Unit/mg protein) with an enzyme/substrate ratio of 1/10, at 37°C for 5 hr. The enzymes were inactivated by heating at 85 °C for 15 min at the end of hydrolysis process

- *Protein assay*

The concentration of protein was determined by Lowry's method (Lowry *et al*., 1951), modified by Hartree (1972). Bovine serum albumin (BSA) was used as the protein standard.

- *Evaluation of the degree of hydrolysis (DH)*

The degree of hydrolysis was determined according to the method of Church (1983) based on the reaction of α-amino nitrogen with O-Phtaldialdehyde (Salami *et al*., 2008; Jamdar *et al*., 2010; Sun & Levis, 2011). Free amino groups were calculated using the standard curve of L-leucine (0-4mg/ml) as mmol leu/mg protein.

- *Measurement of antioxidant activity*

Antioxidant activity was measured based on DPPH and ABTS radical scavenging activity.

- *DPPH radical scavenging activity test*

The scavenging effect on the DPPH free radical was conducted by measuring the decrease in absorbance of DPPH solution after reaction with the peptide solution according to a modified method of Son *et al*., (2002). The radical scavenging activity (%) was calculated by:

DPPH radical scavenging activity (%) = $Abs_{517nm}$ control – $Abs_{517nm}$ sample *100 / $A_{517nm}$ control

- *ABTS radical scavenging activity test*

The ability to scavenge ABTS free radicals was measured from the decrease in absorbance due to suppression of the colour formation according to the method described by Re *et al*. (1999).

The extent of radical scavenging activity was calculated by using the following equation:

ABTS radical scavenging activity (%) = $Abs_{734nm}$ control – $Abs_{734nm}$ sample *100 / $Abs_{734nm}$ control

- *Statistical analysis*

All data were means of three replications and represented as the mean ±standard deviation (SD) and subjected to analysis variance (ANOVA) using SPSS program version 24. The significant differences ($P<0.05$) between the results were identified, and $P<0.05$ was considered as significant.

**Results and Discussion**

- *Protein release through high-pressure homogenization, sonication and autolysis treatment of yeast cell*

In this study, *K. marxianus* was used as a protein source. Through sonication and high-pressure homogenization, 73% and 23.01% of total protein were released. The total protein of yeast cells was previously measured by Kjeldahl method and reported in our last research (Mirzaei *et al*., 2015). The results of protein extraction during the

autolysis process is presented in Table 1. The rate of protein extraction increased during the first 24 hr ($P<0.05$), followed by a slight increase up to 72 hr of autolysis.

The results showed that autolysis completed after 17 hr, and it was more effective compared to physical treatments. Yeast cells contain a complex system of intracellular proteases and peptidases and their specific protease inhibitors (Beehalova and Beran, 1986) that play essential roles in autolysis of yeast cells and protein extraction. Other researchers reported a complete release of protein after 24-72 hr autolysis and maximum autolysis happened during the first 24 hr (Tangular & Erten 2008; Mirzaei *et al.*, 2015).

- *Effects of high-pressure homogenization, sonication pretreatment, and autolysis on enzymatic hydrolysis*

The protein solution samples prepared by two different sonication and high-pressure homogenization method were examined for the progress of enzymatic hydrolysis by trypsin and chymotrypsin. The extent of protein hydrolysis was measured by assessing the degree of hydrolysis (DH), which is the most widely used indicator of protein hydrolysis (Bougatef *et al*., 2010). Results are presented in Figures 1.

As expected, in all samples, the DH increased with hydrolysis time, showing a gradual release of peptide fragments during protein hydrolysis. The results indicated the progress of trypsin and chymotrypsin hydrolysis for 90 min. After that, no significant ($P>0.05$) changes were observed (results are not shown). The same results were also reported by Diniz and Martin (1997), Guerad *et al.*, (2002); Kristinsson & Rasco (2000b); Kritinsson & Rasco (2000a); Ovissipour *et al.*, (2009 a) and Ovissipour *et al*., (2009 b). A decrease in hydrolysis rate over longer time may be due to denaturation of the protease enzyme and reducing its biological activity (Guerard *et al*., 2002), reducing the peptide bonds available for proteolytic enzymes over the time, and contribution of enzyme inhibitors (Souissi *et al*., 2007; Wasswa *et al.*, 2007).

Also, our results indicated no significant difference ($P>0.05$) in the progress of trypsin hydrolysis in protein solutions prepared by sonication and high-pressure homogenization. But this difference was significant ($P<0.05$) for protein solution hydrolyzed by chymotrypsin as sonication pretreatment caused a higher degree of hydrolysis. This effect may be contributed to unfolding the protein structure and exposing more cleavage sites to enzymes due to sonication treatment as Qiufang *et al*. (2017) previously reported, or aggregation of protein due to high-pressure homogenization as Funtenberger *et al*. (1995) and Zhang *et al.* (2012) reported. According to different

**Table 1.** Average of protein extraction during autolysis of *K. marxianus.* Results are presented as mean ± SD of three replications

| | Time (hr) | Average of Protein Extraction (mg / g yeast dry weight) | Percentage of Protein Extraction |
|---|---|---|---|
| ***Kluyveromyces marxianus*** | 0 | 2.64±0.1 | 24.44 |
| | 4 | 5.33±0.2 | 49.37 |
| | 7 | 6.71±0.2 | 62.158 |
| | 17 | 10.85±0.2 | 100 |
| | 21 | 11.32±0.3 | 100 |
| | 24 | 12.23±0.3 | 100 |
| | 31 | 11.44±0.3 | 100 |
| | 48 | 11.48±0.4 | 100 |
| | 72 | 12.16±0.1 | 100 |

researches, an increase in the accessibility of the enzyme to possible peptide bonds within the limited denaturated protein happens but hydrolysis rate will decrease in highly denaturated protein due to polymerization (Leeb *et al*., 2011).

High-pressure homogenization could result in the formation of new disulfide bonds and producing protein aggregation that causes many cleavage sites to remain hidden inside the protein structure and leads to the complicated interaction between protein and enzyme. High-pressure treatment exhibited a significant increase in the hydrolysis of the different protein (Penas *and Gomez* 2004; Vilela *et al*., 2006; Zeece *and Kelly* 2008). The opposite results were also reported by other researchers (Iametti *et al*., 1998; Han *et al.*, 2007; Yin *et al*., 2008). The improvement of hydrolysis by high-pressure homogenization depends on sources of proteins, conditions of pressure treatment, and types of enzymes (Balasubramaniam, 2016).

According to our results, chymotrypsin was more effective compared to trypsin in the hydrolysis of yeast extract obtained from sonication treatment and high-pressure treatment (Figure 1). This might be contributed to the higher number of the potential hydrolytic site targeted by chymotrypsin ( Mirzaei *et al*., 2015).

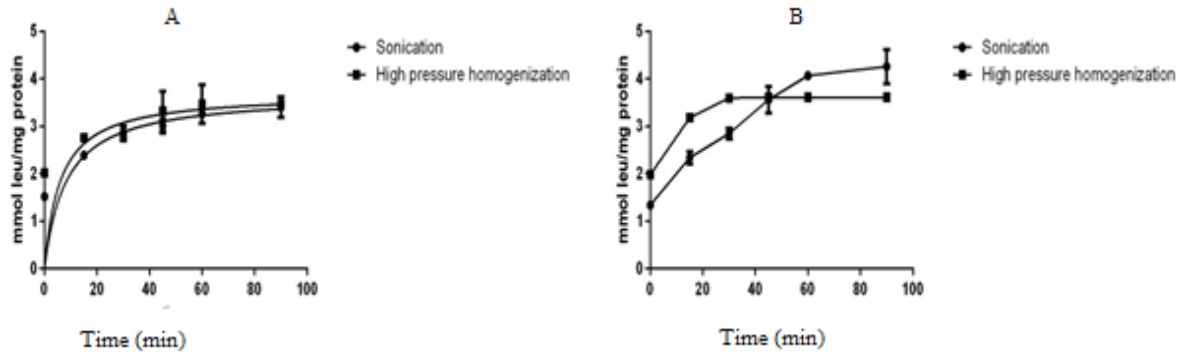


**Fig. 1.** The progress of trypsin (A) and chymotrypsin (B) hydrolysis in yeast extract obtained from sonication and high-pressure homogenization treatment. Results are presented as mean ± SD of three replications.

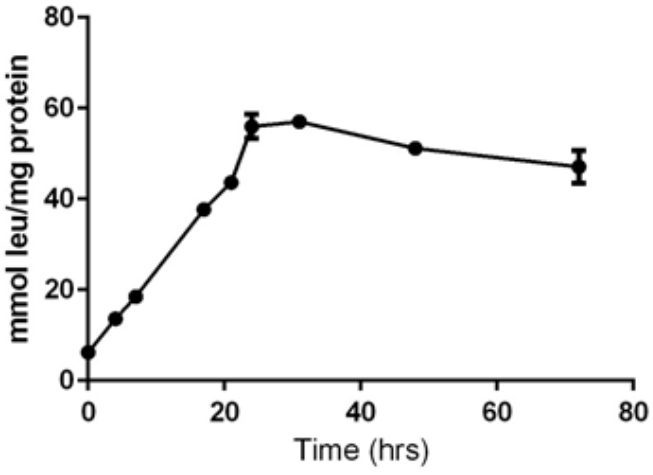


**Fig. 2.** The progress of autolysis in *K.marxianus*. Results are presented as mean ± SD of three replications.

Figure 2 shows the progress of autolysis over 80 hr. During the first 31 hr, the rate of autolysis is high and continues to decrease after that. During autolysis, the amount of extract protein increases significantly such that the increase in DH might be attributed to the protein content of the hydrolysate and proteinases and peptidases that have been released into the autolysates that continue their action during the autolysis period as was previously reported by Lukondeh *et al*. (2003).

- *Effects of high-pressure homogenization, sonication pretreatment, and autolysis on the antioxidant activity*

The antioxidant assays based on DPPH and ABTS radical scavenging activity as the most popular methods of evaluating total antioxidant capacity were used for assessing the antioxidant activity of yeast protein hydrolysates (Martysiak-Zurowska & Wenta, 2012).

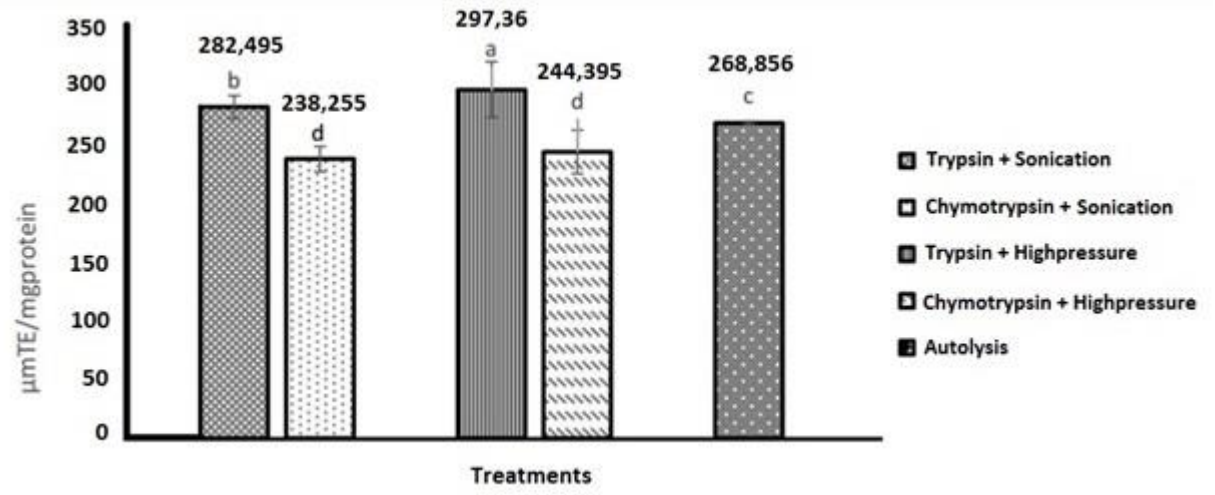


**Fig. 3.** The DPPH radical scavenging activity of yeast protein hydrolysate pretreated through sonication and high-pressure homogenization and hydrolyzed by trypsin and chymotrypsin enzymes. Autolysis is considered as a standard and reference method for producing yeast extract.

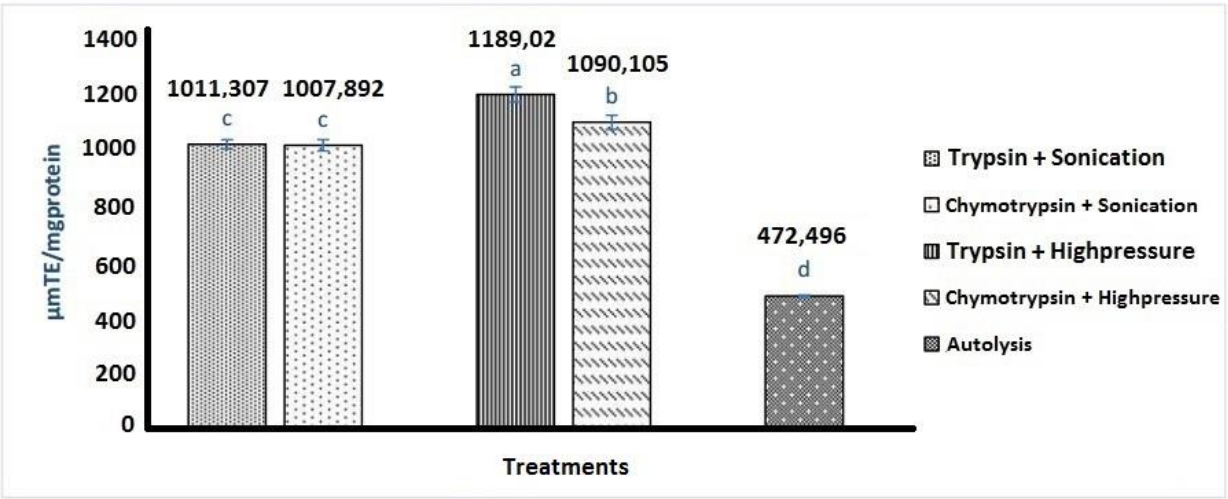


**Fig. 4.** ABTS radical scavenging activity of yeast protein hydrolysate pretreated by sonication and high-pressure homogenization and hydrolyzed by trypsin and chymotrypsin enzymes. Autolysis is considered as a standard and reference method for producing yeast extract.

According to the results presented in Figures 3 and 4, samples pretreated by high-pressure homogenization exhibited significant higher DPPH and ABTS radicals scavenging activity when hydrolyzed by trypsin and higher ABTS radical scavenging activity when hydrolyzed by chymotrypsin. This observation could be contributed to exposure to new cleavage sites and greater exposure of aromatic or hydrophobic residues, thus increasing the antioxidant activity (Van der Plancken *et al.*, 2005). As it was reported previously, high-pressure homogenization changes protein structure primarily by rupturing or forming non-covalent bond-electronic interactions, hydrophobic interactions, and hydrogen bonds. In addition to pressure intensity and holding time, the pH, ionic strength, and solvent conditions can significantly affect high-pressure modification of food proteins (Balasubramaniam, 2016). Sing *et al*. (2014) also reported that high pressure processing can modify the functional properties of the protein by changing its structure and causing protein denaturation, aggregation or gelation, depending on the protein system and applied pressure. Overall high pressure treatment caused an increase in antioxidant activity of egg protein hydrolysed by trypsin (Singh and Ramaswamy, 2014). Other researchers also reported that pretreatment of of β-lactoglobulin by high voltage electrical treatment and pulsed electric field improve the susceptibility of protein to enzyme and induce the formation of active site in protein molecule which leads to release of bioactive peptides (Mikhaylin *et al*., 2017)

The results indicated that the trypsin was a more active enzyme compared to chymotrypsin for production of peptides with DPPH and ABTS radicals scavenging activity. This result was identical for both samples pretreated with sonication and high-pressure homogenization. The roles of trypsin in producing small size antioxidant peptides with exposed hydrophobic side chains were reported, previously by Memarpoor-Yazdi *et al*. (2013).

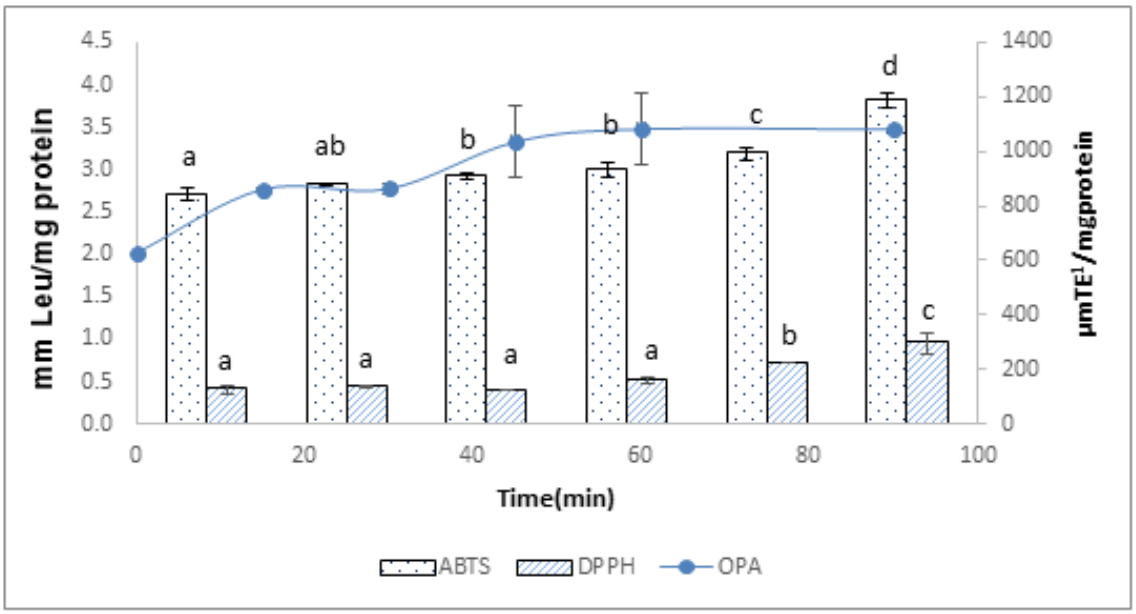


**Fig. 5.** Considering DPPH and ABTS radicals scavenging activity of yeast protein hydrolysate pretreated by high-pressure homogenization and hydrolyzed by trypsin hydrolysis. Data are the mean value of three replications and are represented as mean ±SD.

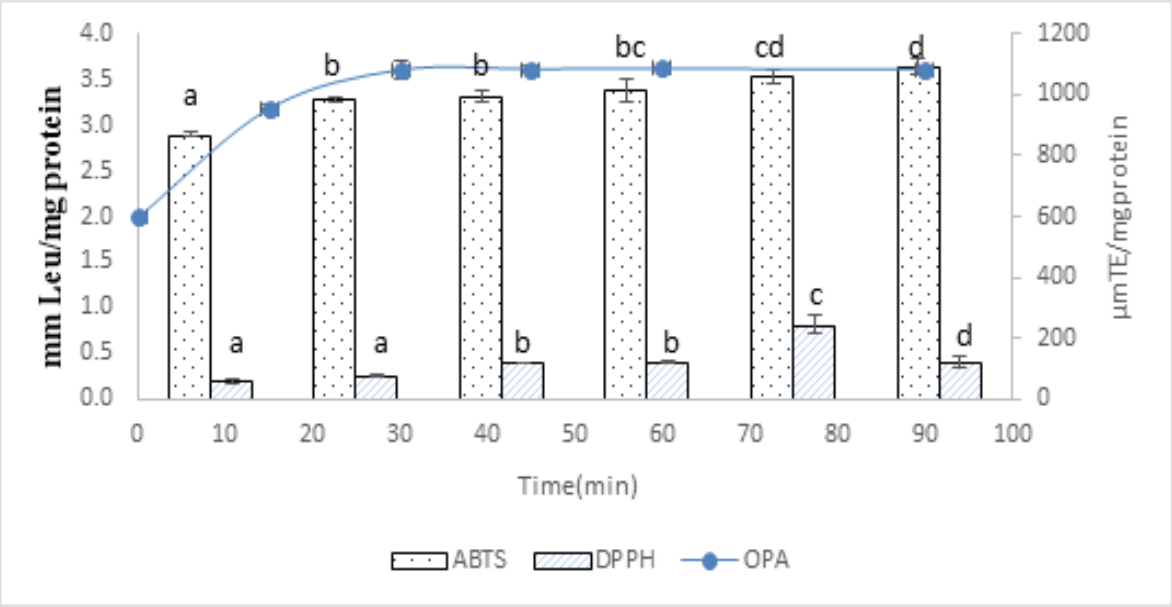


**Fig. 6.** Considering DPPH and ABTS radicals scavenging activity of yeast protein hydrolysate pretreated by high-pressure homogenization and hydrolyzed by chymotrypsin. Data are the mean value of three replications and are represented as mean ±SD.

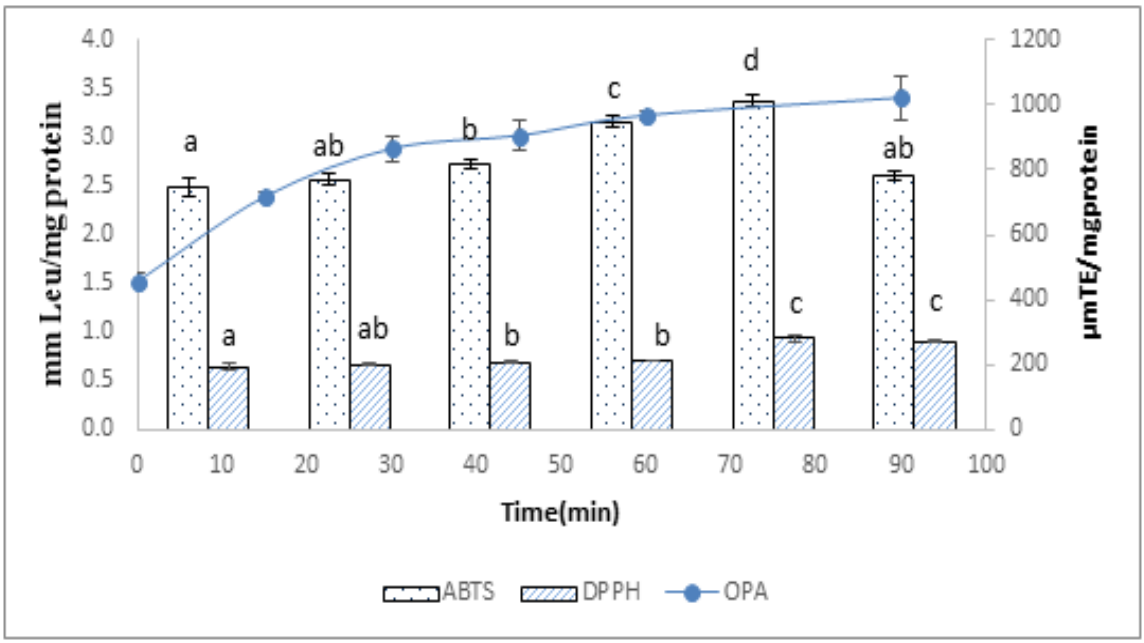


**Fig. 7.** Considering DPPH and ABTS radicals scavenging activity of yeast protein hydrolysate pretreated by sonication and hydrolyzed by trypsin. Data are the mean value of three replications and are represented as mean ±SD.

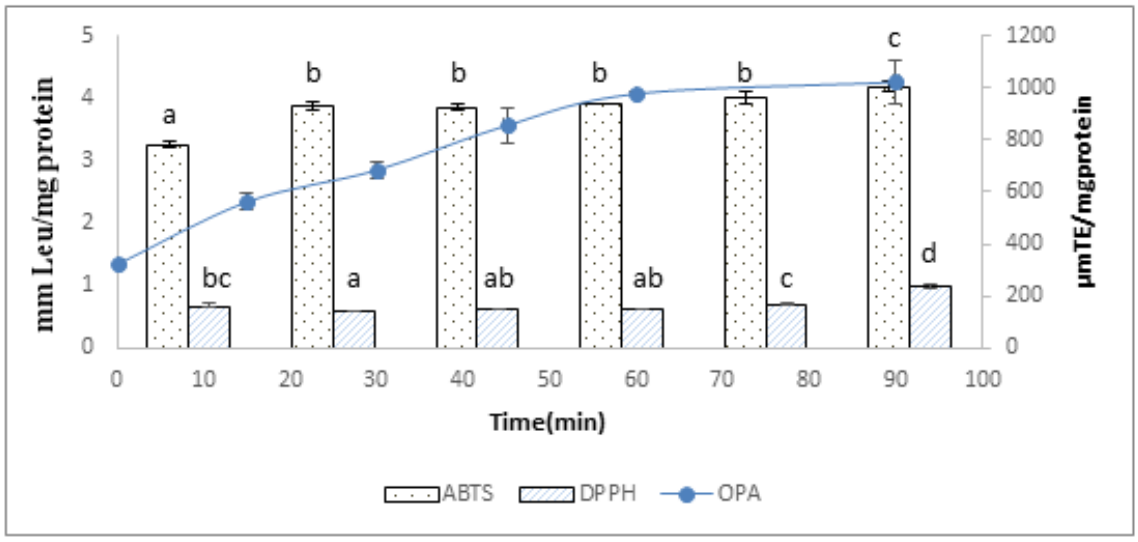


**Fig. 8.** Considering DPPH and ABTS radicals scavenging activity of yeast protein hydrolysate pretreated by sonication treatment and hydrolyzed by chymotrypsin. Data are the mean value of three replications and are represented as mean ±SD.

Besides, the relation between the progress of enzymatic hydrolysis and antioxidant activity was considered in all hydrolysate and autolysate samples. As it can be found in Figures 5, 6, 7 and 8, enzymatic hydrolysis by trypsin and chymotrypsin led to increasing the DPPH and ABTS radicals scavenging activity of yeast protein hydrolysates simultaneous with increasing the OPA values. These results were the same for both samples pretreated through high pressure and sonication treatments and are indications of producing antioxidant peptides in the effects of enzymatic hydrolysis. The observed decrease in DPPH and ABTS radicals scavenging activity of samples prepared by respectively homogenization-chymotrypsin hydrolysis and sonication-trypsin hydrolysis after 90 min and autolysis can be justified by the intensity of hydrolysis and thus hydrolysis of peptides, which have previously had antioxidant activity. Our results confirmed the reports of other researches that antioxidant activity does not necessarily increase by hydrolysis (Jun *et al*., 2004; Wang *et al*., 2012; Wiriyaphan *et al*., 2012; Mirzaei *et al*., 2015). Jun *et al*. (2004) also reported that pepsin hydrolysate of yellowfin sole frame with the lowest DH value exhibited the highest antioxidant activity compared to those hydrolyzed by alcalase and trypsin.

## Conclusion

Altogether, our results indicated the importance of protein pretreatment and the type of enzymes on the progress of enzymatic hydrolysis and antioxidant activity of yeast protein hydrolysate. The results showed that pretreatment of *Kluyveromyces marxianus* cells by high-pressure homogenization before enzymatic hydrolysis by trypsin is an effective method to produce antioxidant peptides from yeast protein.

## Acknowledgment

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