# Antioxidant, ACE-Inhibitory and antibacterial activities of *Kluyveromyces marxianus* protein hydrolysates and their peptide fractions

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**ABSTRACT:**
**Background:** There has been evidence that proteins are potentially excellent source of antioxidants, antihypertensive and antimicrobial peptides, and that enzymatic hydrolysis is an effective method to release these peptides from protein molecules. The functional properties of protein hydrolysates depends on the protein substrate, the specificity of the enzymes, the conditions used during proteolysis, degree of hydrolysis, and the nature of peptides released including molecular weight, amino acid composition, and hydrophobicity.

**Context and purpose of this study**: The possibility of using strain of *Kluyveromyces marxianus* as a source of yeast extract with antioxidant, antibacterial, and ACE-inhibitory activity was the subject of this research.

**Results:** Autolysis and enzymatic hydrolysis were completed respectively, after 96 h and 5 h. Overall, trypsin (18.52% DH) and chymotrypsin (21.59% DH) treatments were successful in releasing antioxidant and ACE inhibitory peptides. Autolysate sample (39.51% DH) demonstrated poor antioxidant and ACE inhibitory activity compared to trypsin and chymotrypsin hydrolysates. The chymotrypsin 3-5 kDa (301.6±22.81 μM TE/mg protein) and trypsin < 3 kDa (280.16±39.16 μM TE/mg protein) permeate peptide fractions showed the highest DPPH radical scavenging activity. The trypsin <3 kDa permeate peptide fraction showed the highest ABTS radical scavenging (1691.1±48.68 μM TE/mg protein) and ACE inhibitory ($IC_{50}$=0.03±0.001 mg/mL) activities. The fraction (MW=5-10 kD) obtained after autolysis treatment showed antibacterial activity against *St. aureus* and *Lis. monocytogenes* in well diffusion screening. The minimum inhibitory concentration (MIC) value was 13.3 mg/mLagainst *St. aureus* and *Lis. monocytogenes* calculated by turbidimetric assay and it showed bactericidal activity against *St. aureus* at 21.3 mg/mL protein concentration.

**Conclusions:** Altogether, the results of this study reveal that *K. marxianus* proteins contain specific peptides in their sequences which can be released by enzymatic hydrolysis and autolysis.

**Key words:** *Kluyveromyces marxianus*, Antioxidant activity, ACE-inhibitory, Antimicrobial activity, Protein hydrolysate, Bioactive peptide.

## INTRODUCTION

The nutritional properties of food proteins have been investigated for many years; food proteins depend on their amino acid content. Recently, there has been some evidence that proteins are potentially excellent sources of antioxidants, antihypertensive, and antibacterial peptides. Furthermore, the possibility of releasing the biologically active peptides from food proteins has gained a lot of interest due to their effects on numerous physiological functions of the organisms. They exhibit antimicrobial, antioxidative, hypocholesterolemic, immunomodulatory, and opioid activities, as well as blood pressure lowering effects (ACE-inhibitory) and enhancement of mineral absorption [1, 2]. Enzymatic hydrolysis is an effective method for releasing these peptides from protein molecules [3]. Protein hydrolysates have been used in food supplements, infant foods, sport nutrition products, and functional foods, as well as for the nutritional management of consumers with digestive problems [2]. Oxidation is an important reason of food spoilage. Furthermore, it was confirmed that generation of radicals during oxidation is related to the progress of many diseases including cardiovascular disease, diabetes, inflammatory disease and aging [4]. Consequently, protection against free radicals is a key factor in preventing diseases and deterioration of foods. The use of synthetic antioxidants in food products is under strict regulation. Therefore, the search for natural and safe antioxidants from various natural sources has been gaining much interest [5].

Hypertension is one of the risk factors for cardiovascular diseases. It is well known that the renin-angiotensin system plays an important role in the regulation of blood pressure. The angiotensin-converting enzyme (ACE) catalyzes the hydrolysis of the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II [6].

As many pathogenic bacteria have acquired resistance toward antibiotics, it is therefore important to seek for possible new natural antibiotics e.g. antibiotic peptides or cationic peptides [7]. There has been some evidence that proteins can yield antioxidants, antihypertensive and antimicrobial peptides, and enzymatic hydrolysis is an effective method for releasing these peptides from protein molecules [3].

The antioxidant and antihypertensive and antibacterial activities of protein hydrolysates depend on the protein substrate, the specificity of the enzymes, the conditions used during proteolysis, degree of hydrolysis, and the nature of the peptides released, including molecular weight, amino acid composition, and hydrophobicity [8]. Numerous animal and plant food proteins, mainly milk proteins, casein and whey, egg and meat muscle proteins, have been used for producing bioactive peptides. Based on current literature, food proteins are selected as sources of bioactive peptides based on a value-added use of abundant underutilized proteins or protein-rich food industry by-products, and containing specific peptide sequences or amino acid residues of particular pharmacological interest [9].

In recent years, *Kluyveromyces marxianus* has been used for the large-scale production of single cell protein (SCP) [10]. It has been discovered that yeast protein has a high proportion of hydrophobic and basic residues and may have the potential to prepare protein hydrolysates with antioxidant, antimicrobial and antihypertensive activities [11].

To the best of our knowledge, there is no report on the biological activity of yeast extract obtained from *K. marxianus*. The possibility of using strain of *K. marxianus* as a source of yeast extract with antioxidant, ACE-inhibitory and antimicrobial activity, in addition to considering the activity of different peptide size fractions, were the subject of this research.

## MATERIALS AND METHODS
### Materials
*Kluyveromyces marxianus* (PTCC 5195) was obtained from Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST). Chymotrypsin (EC 3.4.21.1, containing 55 unit/mg solid), trypsin (EC3.4.21.4; containing 1300 BAEE unit/mg solid), ortho-phthaldialdehyde (OPA), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and 2,2'–azinobis (3-ethyl-benzothiazoline-6-sulfonate)(ABTS$^+$), furanacrylolyl tripeptide (FAPGG) and 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2- carboxylic acid (trolox) were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). Other chemicals were of analytical grade.

### Culture conditions
*K. marxianus* was aerobically grown in Yeast Mold (YM) culture at 28 °C and 150 rpm in an shaking incubator (IRC-1-U ). Cells were harvested in middle of logarithmic phase by centrifugation at 3000×g for 10 min. The yeast pellet was washed with distilled water three times and stored at -20 °C.

### Preparation of yeast autolysate samples
The yeast cells were collected and washed with distilled water. Suspensions of 2.5% dry yeast cells in 50 mL distilled water was incubated at 52 °C, pH 5 [12] with agitation at 120 rpm for up to 96 h. Samples were withdrawn at every 24 h, heated at 85 °C for 15 min to terminate enzyme activity [13], and cooled down to 4 °C. Cellular debris was removed by centrifugation at 11500×g for 10 min at 4 °C.

### Preparation of samples by enzymatic treatment
To obtain protein extract, suspensions of 2.5% dry yeast cells in 50 mL distilled water were disrupted by a sonicator (Part NO. S-4000) at a fixed power of 600 W, frequency of 20 kHz and amplitude of 50%. Total cycle time for ultrasonic treatment was 10 min. The pulse duration and pulse intervals were each one minute. The jar was immersed in an ice–water bath to avoid increasing temperature during sonication. The cellular debris and particles were removed by centrifugation at 11500×g for 10 min. The intrinsic yeast cell enzymes in the supernatant were inactivated by heating at 85 °C for 15 min. The protein concentration of supernatant was measured and adjusted to 4 mg/mL using phosphate buffer (50 mM, pH 7.8).

The solution of extracted protein was subjected to trypsin and chymotrypsin hydrolysis. The ratio of protein substrate to each enzyme was 10:1. After the 5-h incubation at 37 ºC, the enzymatic hydrolysis was stopped by heating in water (85 ºC, 15 min). The control sample was considered as a sample which contains just intrinsic yeast cell enzymes [14].

**Protein assay**

The total protein content of yeast cells was measured by the Kjeldahl method via multiplication of the total nitrogen by 6.25 [15]. The soluble protein content of supernatant during autolysis and physical-enzymatic treatment was determined by Lowry's method, modified by Hartree [16] using bovine serum albumin (BSA) as the protein standard [16, 17].

**Evaluation the degree of hydrolysis**

This assay is used for reaction of  free α-amino nitrogen with o-phthaldialdehyde (OPA) [18-20]. A fresh OPA solution was prepared daily as follows: 25 mL of 100 mM sodium tetrahydroborate, 2.5 ml of 2% SDS, 40 mg of OPA (dissolved in 1 mL methanol) and 100 μL of β- mercaptoethanol were mixed and adjusted to a final volume of 50 mL with distilled water. To assay proteolysis with yeast protein as substrate, 10-50 μL aliquots containing 5-100 μg protein was added directly to 1 mL of OPA reagent. The solution was mixed briefly and incubated for 2 min at room temperature. Subsequently the absorbance was read at 340 nm using a spectrophotometer (Jenway, Model 6315). L-leucine (0-4 mg/mL) was used to construct a standard curve and free amino groups were calculated using standard curve. The DH value was calculated using following formula:

$$DH = \frac{L1 - L0}{L\,max - L0}$$

L1: The number of free amino groups released after hydrolysis.
L0: The numbers of free amino groups in the original yeast extract.
L max: The total numbers of free amino groups in the original yeast extract obtained after acid hydrolysis (6N HCl at 120 ºC for 24 h).

**Measurement of antioxidant activity**

The antioxidant activity was measured based on DPPH and ABTS$^+$ radical-scavenging activity according to the methods described by Son and Lewis [21]  and Re et al. [22], respectively, as explained in our last paper [14]. Radical-scavenging activity (%) calculated as follows:

$$Radical\text{-}scavenging\ activity\ (\%) = \frac{A\ control - A\ sample}{A control} * 100$$

The trolox standard curves showed a linear concentration response in the range of  0-30 μM and 1.5-25 μM of trolox for respectively, DPPH and ABTS assays and were used to determine trolox equivalent  antioxidant capacity (TEAC).

**Determination of ACE-inhibitory activity**

The rabbit lung powder was prepared following the procedure of Lossow et al. [23] with modifications [14] and  ACE-inhibitory activity was measured by the spectrophotometric assay

[24] . Briefly, furanacrylolyl tripeptide (75 µL, 5 mM) dissolved in 50 mM tris-HCl buffer (pH 8.3) containing 400 mM NaCl and Peptide fractions or water (25 µL) were added to each well of Elisa plate and pre-incubated for 20 min at 37 ºC. ACE extract (10 µL) was added to the mixture and the absorbance was measured at 340 nm using an ELISA reader Expert 96 (Power wave xS2, Bioteck, America) for periods of 30 min at 37ºC. The slope of the decrease in absorbance of samples (ΔA sample) and control (ΔA control) as a function of time were calculated and ACE-inhibitory activity (%) was determined as follows [24]:

$$\text{ACE-inhibitory activity (\%)} = (1 - \frac{\Delta A\ sample}{\Delta A\ control}) * 100$$

The 50% inhibitory concentration ($IC_{50}$) was determined from a plot of percentage of ACE-inhibitory activity against peptide concentration.

**Antibacterial activity**

Antibacterial activity of yeast protein hydrolysates and peptide fractions were performed against *Bacillus cereus* (PTCC 1015), *Listeria monocytogenes* (PTCC 1306), *Staphilococcus aureus* (PTCC 1112), *Escherichia coli* (PTCC 1330), and *Micrococcus luteus* (PTCC 1110), by the well diffusion method described by Hou, Li [25] with some modification.

An aliquot of bacteria with a titer of $10^6$ CFU/mL was mixed with 20 mL Tryptocase soy broth (TSB) medium containing 0.8% agarose and was poured in a plate. Holes were then created in the medium using a punch. The peptide sample (50 µL) was loaded into the wells and the plates were incubated at 37ºC for 24 h. An aliquot of 5 µL nisin (2000 IU/mL) was used as positive control and water as negative control. For calculating antibacterial activity (%), each bacterium was first grown separately in BHI medium at 37ºC for 24 h. Approximately, 100 µL of culture was transferred to new culture and incubated for 3 h at 37ºC. To a 96-well microplate, 10 µL of bacterial suspension and 15 µL of BHI medium were poured. Stock serial dilution of 5.6-26.6 mg/mL peptides were prepared and 50 µL of peptide stocks were added in each well. The microplate was incubated at 37ºC for 24 h. Later, the absorbance at 600 nm for each well in zero time and after 24 h (ΔA) was read with a microplate reader, with the results then being compared to the control sample. The antimicrobial activity was calculated as follows:

$$\text{Antibacterial activity(\%)} = 100 \times \frac{\Delta A\ negative\ control - \Delta A\ sample}{\Delta A\ negative\ control}$$

The MIC values were determined as the lowest concentration of peptides at which no visible growth was observed. For the determination of Minimum Bactericidal Concentration (MBC), a portion of liquid from each sample that exhibited no visible growth were cultured in the plate and then incubating 37ºC for 24 hr. The lowest concentration that revealed no bacterial growth was taken as MBC [7, 26, 27].

**Statistical analysis**

In this study the results are the average of triplicate analysis conducted on one application of the treatments. Data are presented as mean value ± standard deviations. The significance between mean values was determined using independent-samples T test and one-way ANOVA. Statistical analysis was performed in SPSS software, version 20. P value < 0.05 was considered significant.

## RESULTS AND DISCUSSION
### Antioxidant, ACE inhibitory and antimicrobial activities of yeast peptide fractions
In this study, *K. marxianus* protein hydrolysates was used as a possible source of ACE inhibitory, antioxidant and antibacterial peptides. The yeast protein hydrolysates were prepared by two different enzymatic (sonication-trypsin and chymotrypsin hydrolysis) and autolysis treatments. Antioxidant activity is expressed as TEAC and ACE inhibitory activity is determined as $IC_{50}$ value based on inhibiting ACE activity.

### Antioxidant activity
The DPPH and $ABTS^{+}$ radical-scavenging activity of the control sample, trypsin and chymotrypsin hydrolysate, and autolysate samples is shown in figure 1.
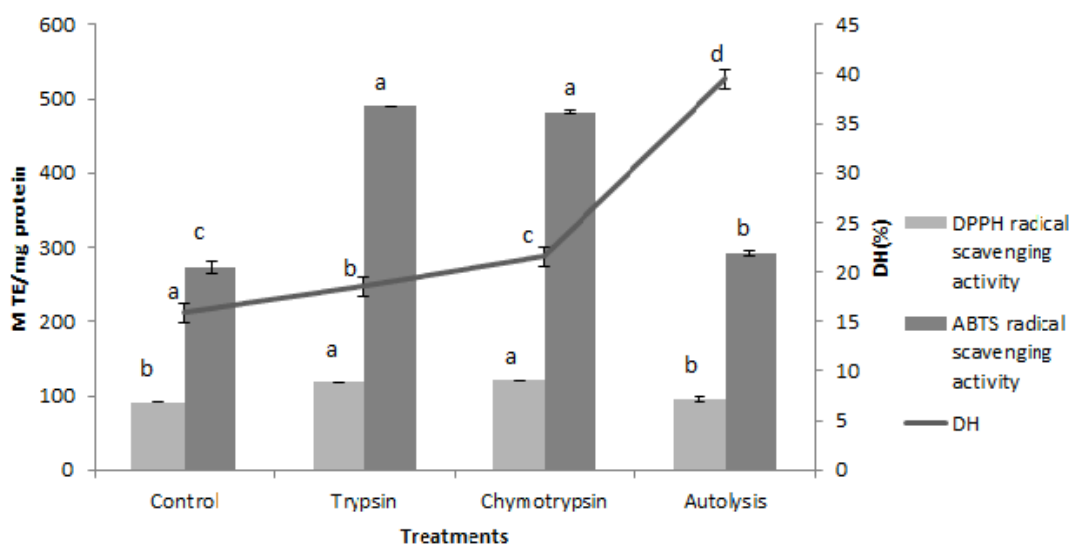


**Figure 1.** Antioxidant activity of yeast protein hydrolysates. The data of DPPH and ABTS radical-scavenging activities are expressed as trolox equivalent antioxidant capacity (μM TE/mg protein). The data of DH were measured using OPA assay and are expressed as percent values. The results are mean values of experiments carried out in triplicate. Values with different letters for each assay are statistically different at P<0.05.

As it can be found, trypsin (18.52% DH) and chymotrypsin (21.59% DH) hydrolysis causes significant (P<0.05) increase in DPPH and $ABTS^{+}$ radical-scavenging activity of protein hydrolysates compare to control sample (P<0.05). It can be explained by a better exposure of the antioxidant amino acid residues due to peptide bonds cleavage. Tyr, Trp, Met, Lys and His, and aromatic amino acids improve the radical scavenging properties of amino acid residues and the SH group in cysteine has a crucial antioxidant action due to its direct interaction with radicals [28]. Additionally, it has been reported that during hydrolysis, the protein chains unfold and results in the development of hydrophobicity and so antioxidant activity of protein hydrolysates, since the balance between hydrophilic and hydrophobic forces of peptides has a crucial influence on the solubility of protein hydrolysate and its antioxidant activity [28-32] .

The differences between trypsin and chymotrypsin were not significant (P>0.05) in DPPH and ABTS assay (figure 1). As it was previously reported by Pihlanto [31], the antioxidant activity of protein hydrolysates is influenced by characteristic amino acid sequences of derived peptides, depending on the protease specificity. Protease specificity affect amount, size, composition and amino acid sequence of producing peptides which in turn influences the antioxidant activity of the hydrolysates [33-35]. These observations suggest that trypsin and chymotrypsin digestion resulted in hydrolysis of inactive peptides within the protein and release of shorter peptides with radical-scavenging activity. Probably, hydrophobic amino acid side chain groups are exposed by tryptic and chymotryptic digestion, which might increase their ability to scavenge free radicals as it was previously reported by You, Zhao [36], and Memarpoor-Yazdi, Mahaki [37].

According to the results of this research (figure 1), the autolysate sample (39.51% DH) showed the lowest DPPH (94.96±3 μm TE/mg protein) and ABTS$^+$ (292.23±3.4 μm TE/mg protein) radical-scavenging activity. Our results indicated that antioxidant activity does not necessarily increase with DH. Jun, Park [38] also reported that the lowest DH of pepsin hydrolyzed yellow fin sole frame exhibited the highest antioxidant activity than those hydrolyzed by alcalase and trypsin. Wiriyaphan et al. Wiriyaphan, Chitsomboon [39] also reported that threadfin bream surimi by products hydrolysate prepared by pepsin (5% DH) and *Virgibacillus* SP. SK33 proteinase (13% DH) showed higher ABTS$^+$ radical-scavenging activity than the hydrolysate prepared by alcalase (27% DH) and trypsin (11% DH). Wang, Li [32] also reported the same results. Deeper hydrolysis, may change the conformation of producing peptides, with enhancing levels of free amino acid carboxyl groups resulting in enhanced solubility [28, 40]. Additionally, it can contribute to the production of too much amino acids during extensive proteolysis. Peptides are potentially better antioxidants than amino acids, due to unique chemical and physical properties related to their amino acids sequences [38, 39]. Therefore, too many free amino acids produced as a result of extensive proteolysis may reduce the antioxidant activity of protein hydrolysate.

As can be seen in figure 1, TEAC levels determined by the ABTS radical cation decolorization method were significantly (P<0.05) higher than those measured by DPPH assay. It can be explained by more reactivity of ABTS$^+$ radical toward most antioxidants, as it was previously reported by Martysiak-Zurowska and Wenta [41] for human milk. They also reported the lower sensitivity of DPPH assay when compared to ABTS radical cation decolorization assay and our results confirm it.

**ACE inhibitory activity**
In the present study, *in vitro* ACE-inhibitory activity of the final products of autolysis and physical-enzymatic treatments was also investigated. The ACE-inhibitory activity is expressed as IC$_{50}$ value based on inhibiting ACE-activity.

Results (figure 2) revealed that the ACE-inhibitory activity of the yeast protein hydrolysate was influenced by the type of enzymatic treatment. Trypsin (15.91% DH, IC$_{50}$= 0.49±0.009 mg/mL) and chymotrypsin ( 21.59% DH, IC$_{50}$=0.51±0.01 mg/ml) were the most effective. These results indicated that enzymes specificity plays an important role in the preparation of bioactive peptides and tryptic and chymotryptic digestion resulted in more ACE-inhibitory

activity of protein hydrolysate. As it can be seen from the results, the higher DH value obtained during autolysis caused no significant (P>0.05) increase in ACE-inhibitory activity of protein hydrolysate. It may be due to the type of active enzymes during autolysis and extent of proteolysis. As it was previously reported by Chen et al. [42] extensive proteolysis may lead to disruption of peptides that were previously generated and so decreasing ACE-inhibitory activity.
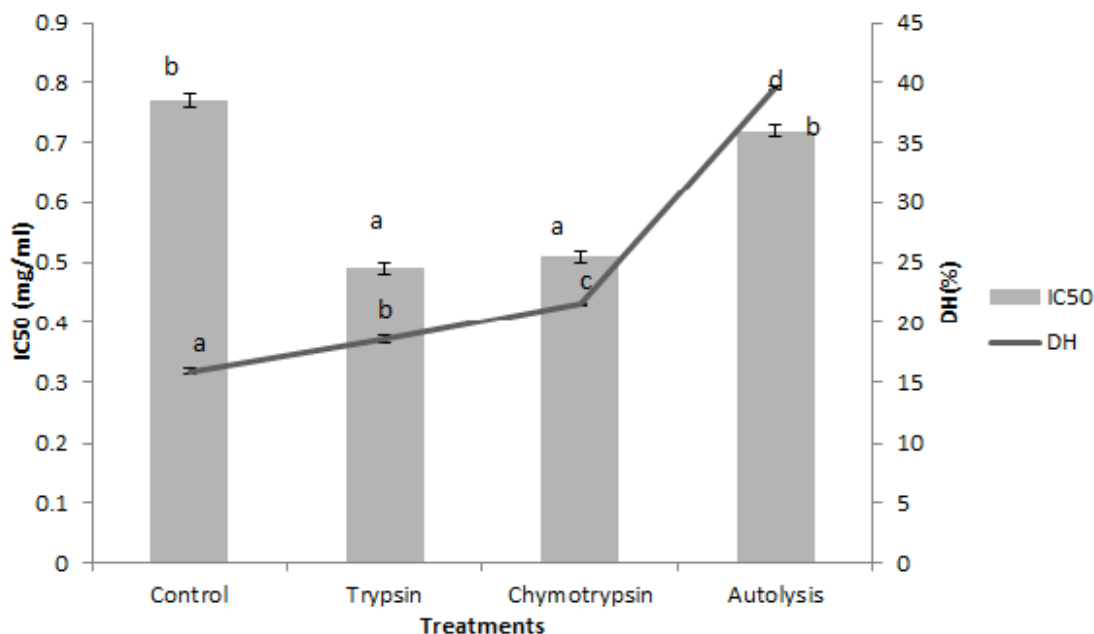


**Figure 2**. ACE-inhibitory activity of yeast protein hydrolysates. $IC_{50}$ is the concentration (mg/mL) of an ACE inhibitor needed to inhibit 50% of ACE activity. The results are mean values of experiments carried out in triplicate. Values with different letters in each column are statistically different at P<0.05.

Although the structure-activity relationship of ACE-inhibitory peptides from food proteins is not fully studied, it is known that the binding of inhibitory peptides to ACE is strongly influenced by the C-terminal sequence and hydrophobic amino acids e.g., Proline, are more active if present at each of the three C-terminal sequence. Additionally, the presence of the positive charge of Lys and Arg as the C-terminal residue may contribute to the inhibitory activity [43]. Based on López-Fandiño, Otte [43], enzymes with specificity towards the carboxylic side of aromatic or other hydrophobic amino acid residues or the basic amino acids may be useful for producing ACE-inhibitory peptides. These observations suggest that trypsin and chymotrypsin hydrolysis were the most effective for releasing ACE inhibitory peptides from parent protein. Salami, Moosavi-Movahedi [44] also reported the increasing in ACE inhibitory activities of camel whole casein and camel β-CN after trypsin and chymotrypsin hydrolysis.

**Antibacterial activity**

Preliminary assay for antimicrobial activity was carried out using crude hydrolysate and autolysate with protein concentration of 4 mg/mL. They exhibited no antibacterial activity in well diffusion assay against the tested strains. It can be explained by low peptide concentration

as it was previously reported by Sibel Akalın [45]. Thus, the antibacterial activity was examined after partial purification by ultrafiltration technique.

**Antioxidant, ACE inhibitory and antimicrobial activities of yeast peptide fractions**

The ultrafiltration was used to perform peptide separation according to their molecular weight. Trypsin and chymotrypsin hydrolysates and autolysate were fractionated using membranes with molecular weight cut-off of 3, 5, and 10 kDa. The DPPH and ABTS radical scavenging activities of yeast protein hydrolysates and autolysate and their UF-fractions are presented in figure 3 and 4.

Our results (figure 3, 4, 5) confirm that peptide size has substantial influence on the antioxidant and ACE inhibitory activities of yeast protein hydrolysates and their peptide fractions. Onuh, Girgih [46] also reported that the antioxidant activity of hydrolysates were closely associated with the MW distributions and it is reported that a common feature of ACE-inhibitory peptides is their relatively restricted size [43]. The chymotrypsin 3-5 kDa (301.6±22.81 µM TE/mg protein) and trypsin< 3 kDa (280.16±39.16) permeate peptide fractions showed the highest DPPH radical scavenging activity (figure 3). The trypsin <3 kDa permeate peptide fraction showed the highest ABTS radical scavenging (1691.1±48.68 µM TE/mg protein) (figure 4) and ACE inhibitory (IC50=0.03± 0.001 mg/mL) (figure 5) activities.
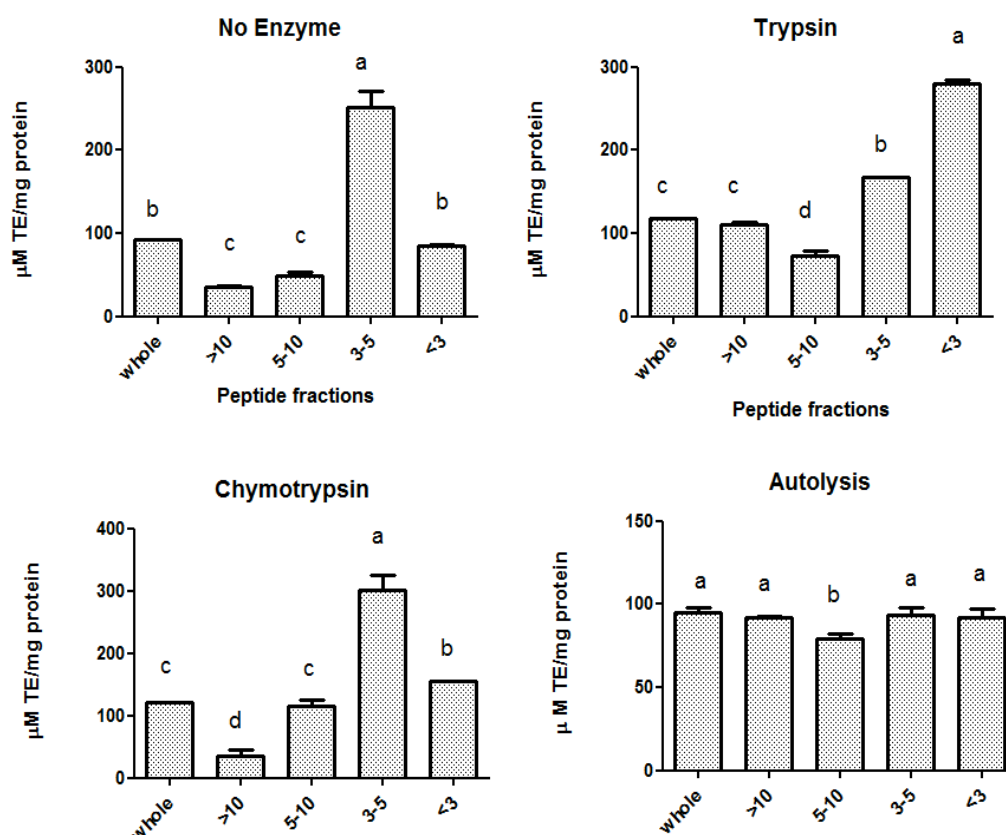


**Figure 3.** DPPH radical-scavenging activity of whole yeast protein hydrolysates (physical-trypsin hydrolysate, physical-chymotrypsin hydrolysate) and their peptide fractions. The data are expressed as trolox equivalent antioxidant capacity (µM TE/mg protein). The results are mean values of experiments carried out in triplicate. Data marked with asterisk are not statistically different at P<0.05.
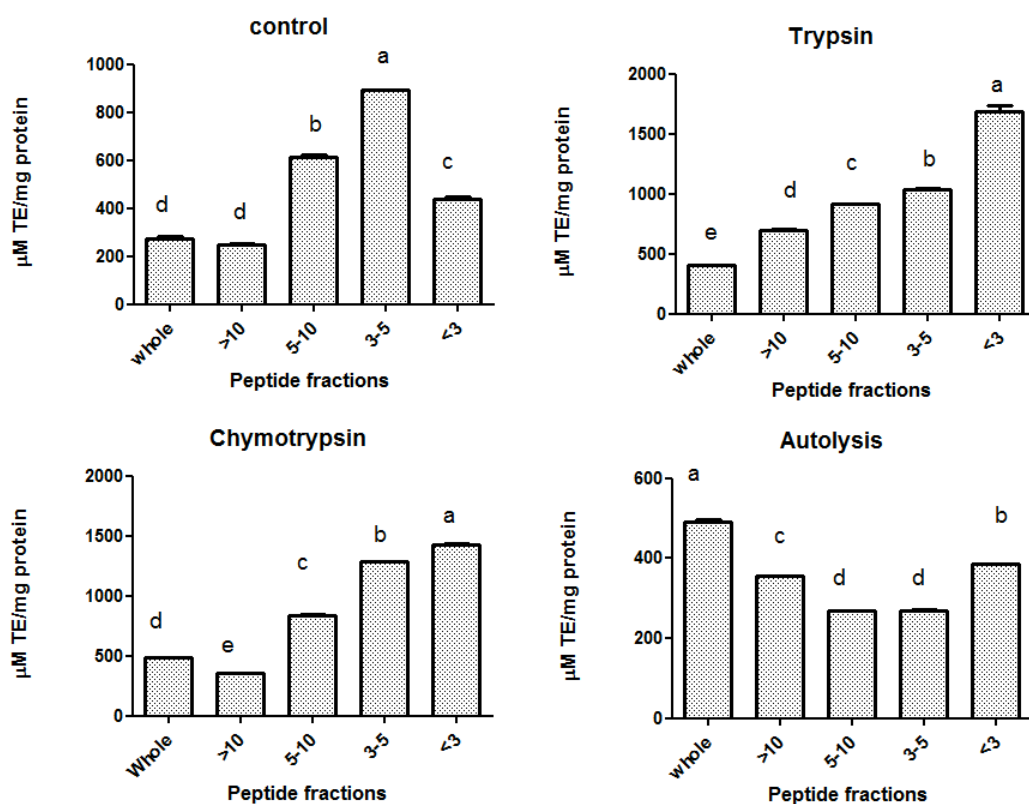
**Figure 4.** ABTS radical-scavenging activity of whole yeast protein hydrolysates (physical-trypsin hydrolysate, physical-chymotrypsin hydrolysate) and their peptide fractions. The data are expressed as trolox equivalent antioxidant capacity (µM TE/mg protein). The results are mean values of experiments carried out in triplicate. Data marked with asterisk are not statistically different at P<0.05.



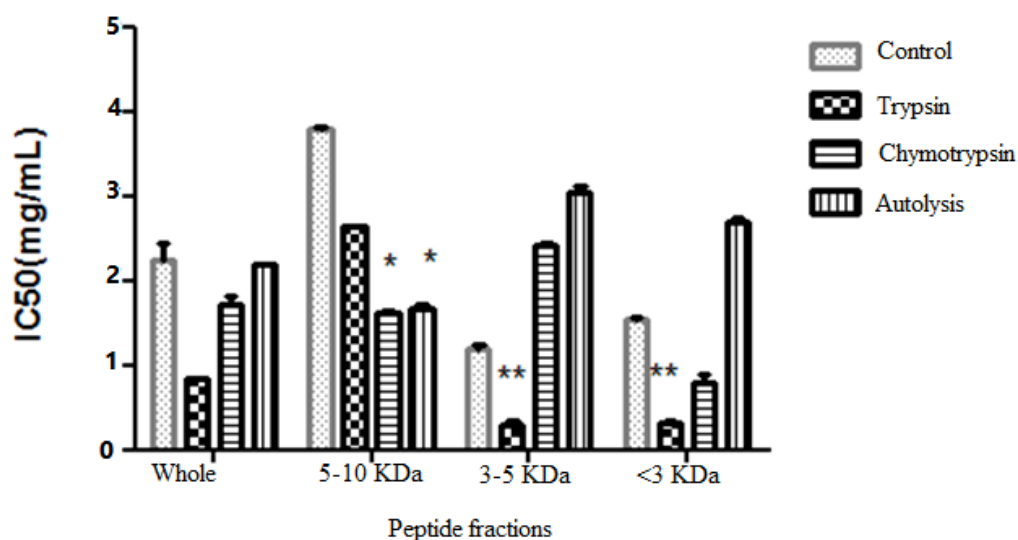**Figure 5.** The size dependent ACE–inhibitory activity of  yeast protein hydrolysates and autolysate and their peptide fractions. IC50 is the concentration (mg/mL) of an ACE inhibitor needed to inhibit 50% of ACE activity. Data marked with asterisk are not significantly different (P>0.05).

Most the antioxidant and ACE inhibitory peptides are less than 3 kDa and 3-5 kDa and have a significantly higher activity than their parent hydrolysates. This agrees with many reports where the lower molecular weight (LMW) peptides had greater antioxidant and ACE inhibitory activities than those of the larger peptides [47] due to more effective interaction with radicals intervening in the oxidizing process [8]. As it was previously reported, this is the case for barley hordein protein fractions [48], hemp hydrolysates [49] and canola meal protein hydrolysate [50]. Mullally, Meisel [51] also found the highest ACE inhibitory peptides in the 3kDa permeates, compared with the 1 and 10 kDa permeates and with the original tryptic digest of whey protein.
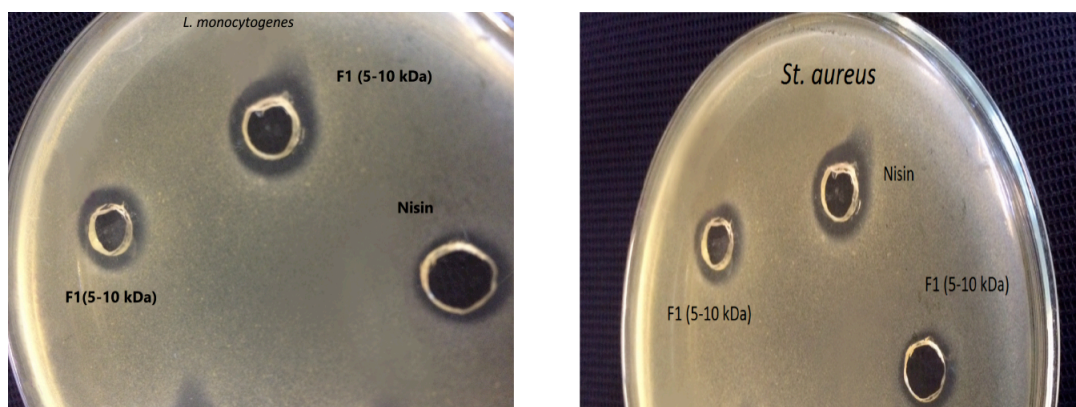


**Figure 6.** Inhibition zone of peptide fraction (5-10 kDa) in two plate containing *L. monocytogenes* and *St. aureus* in well diffusion assay.

The fraction (MW=5-10 kD) obtained after autolysis of *K. marxianus* showed antibacterial activity against *St. aureus* and *Lis. monocytogenes* in well diffusion screening (Figure 6). Peptide length is an important factor for the interaction of bioactive peptides and phospholipid membranes. Peptides need to be able to span the thickness of the membranes to stabilize the pore and exhibit antibacterial activity. In addition, by decreasing peptide lengths, the tendency of peptides to form ordered secondary structure will be reduced [52] and these can explain the higher efficiency of peptide fraction with MW=5-10 kDa.

Growth inhibitory (%) activity of peptide fraction was calculated as it was described in material and method section. The results are presented in table 1.

**Table 1.** ACE inhibitory and antioxidant activity of yeast protein hydrolysates

| Samples | DH(%) | IC50 (mg/ml) | DPPH radical scavenging activity (µM TE/mg protein) | ABTS radical scavenging activity (µM TE/mg protein) |
|---|---|---|---|---|
| Control | 15.91[a] | 0.77±0.01[b] | 91.85±0[b] | 273.04±8.63[c] |
| Physical-trypsin hydrolysis | 18.52[b] | 0.49±0.009[a] | 118.53±0[a] | 489.53±0[a] |
| Physical-chymotrypsin hydrolysis | 21.59[c] | 0.51±0.01[a] | 121.11±0[a] | 482.03±2.34[a] |
| Autolysis | 39.51[d] | 0.72±0.01[b] | 94.96±3[b] | 292.23±3.4[b] |

IC$_{50}$ is the concentration (mg/ml) of an ACE inhibitor needed to inhibit 50% of ACE activity. The data of DPPH and ABTS radical-scavenging activities are expressed as trolox equivalent antioxidant capacity (µM TE/mg protein). The results are mean values of experiments carried out in triplicate. Values with different letters in each column are statistically different at $P<0.05$.

This study shows that autolysis of *K. marxianus* can release peptides (MW=5-10 kDa) with antibacterial activities with MIC value of 13.3 mg/mL against *St. aureus* and *Lis. Monocytogenes.* While other peptide fractions showed no activity against tested strains. The antibiotic activity of peptide fraction at 21.3 mg/mL protein concentration was proven to be lethal for *St. aureus* as they were not capable of resuming growth on agar plate after 24 h incubation at 37 ºC.

**CONCLUSION**
Altogether, the results of this study confirmed that the yeast protein hydrolysates of *K. marxianus* with antioxidant, ACE-inhibitory and antimicrobial activity can be provided through enzymatic hydrolysis and autolysis treatments of yeast cell and peptides size has an important role in their biological activities. They may be beneficial as food additives a functional ingredient, pharmaceutical agents and diet nutrients.

**Competing Interests:** The authors have no financial interests or conflicts of interest.

**Author's Contributions:** All authors contributed sufficiently to this work.

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