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Purification and identification of antioxidant and ACE-inhibitory peptide from *Saccharomyces cerevisiae* protein hydrolysate

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ABSTRACT

Yeast protein hydrolysate may be considered as a good source of bioactive peptides. Yeast hydrolysate was prepared by two different physical-enzymatic and autolysis treatments to identify the most active angiotensin I-converting enzyme (ACE) inhibitory and antioxidant peptides. The most active hydrolysate was obtained after sonication-trypsin hydrolysis. The hydrolysate was subjected to fractionation by ultrafiltration. Fraction with molecular weight of <3 kDa exhibited the highest activity. Reverse phase high performance liquid chromatography (RP-HPLC) resolved this fraction into five fractions, one of which (fraction F3) with amino acid sequence of Tyr-Gly-Lys-Pro-Val-Ala-Val-Pro-Ala-Arg (MW:1057.45 Da) exhibited ACE inhibitory (IC_{50} = 0.42 ± 0.02 mg/ml) and antioxidant activities (26.25 ± 0.13 μM TE/μg protein). Taken together, the results of this study show that *S. cerevisiae* proteins contain specific peptides in their sequences which can be released by enzymatic hydrolysis. These peptides have excellent bioactive properties that can potentially replace the antioxidant and antihypertensive agents with chemical origin.

1. Introduction

Oxidation is an important contributor to many deteriorative phenomena, such as food spoilage and progress of many human diseases, including cardiovascular disease, diabetes, inflammatory disease and ageing. Therefore, protection against free radicals induced oxidation is a key factor in preventing diseases and deterioration of foods. The use of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG), in food products is under strict regulation owing to their potential health hazards and having toxic effects. Therefore, the usage of natural and safe antioxidants as an alternative to synthetic

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ones is gaining interest (Mohamed, Issoufou, & Zhou, 2012; Rao et al., 2012).

Hypertension is one of the risk factors for cardiovascular diseases. It is well established that the renin–angiotensin system plays an important role in the regulation of blood pressure. The angiotensin converting enzyme (ACE or kinase II; peptidyl- dipeptidase A, EC 3.4.15.1) catalyses hydrolysis of the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II, an octapeptide (Coates, 2003). Recently, ACE inhibitors are widely used in hypertension treatments (Kanauchi, Igarashi, Ogata, Mitsuyama, & Andoh, 2005).

There has been some evidence that proteins are potentially excellent sources of antioxidants and antihypertensive peptides, and enzymatic hydrolysis is an effective means to release them from the protein molecules (Xia, Bamdad, Gänzle, & Chen, 2012).

The combination of ACE inhibition and antioxidant activity in one product could be very useful for the control of cardiovascular diseases (Vercruysse, Smagghe, Beckers, & Camp, 2009). Some of the protein hydrolysates, such as flax seed protein (Udenigwe & Aluko, 2010), rapeseed (Yu et al., 2013), camel milk casein (Salami et al., 2011), protein isolate from pumpkin oil cake (Vaštag, Popović, Popović, Krimer, & Peričin, 2011), hen egg white lysozyme (Rao et al., 2012), and fermented camel and bovine milk (Moslehishad et al., 2013), have been reported to show both ACE inhibitory and antioxidant activities.

Many studies have reported the great benefits of yeast hydrolysate as a food supplement because of the enormous availability of the yeast cell and its high-quality protein. Also, it has been found that yeast protein has high proportion of hydrophobic and basic residue, hence can be considered as a source of ACE inhibitor and antioxidant peptides (de la Hoz et al., 2014).

The aim of this research was the isolation and identification of a peptide with antioxidant and ACE inhibitory activities obtained by physical-enzymatic (sonication-trypsin and chymotrypsin hydrolysis) and autolysis of *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Materials

S. cerevisiae (PTCC 5269) was obtained from Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST). Chymotrypsin (EC 3.4.21.1; activity 55 unit/mg solid), trypsin (EC 3.4.21.4; activity 1300 unit /mg solid), ortho-phthalaldehyde (OPA), furanacrylolyl tripeptide (FAPGG), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2- carboxylic acid (trolox), trifluoroacetic acid (TFA), and acetonitrile were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany).Ultrafiltration membranes with 10, 5 and 3 kDa cut-off were purchased from Sartorius Stedim (Biotech Company, Goettingen, Germany).

2.2. Culture conditions

S. cerevisiae was grown aerobically in yeast mold (YM) culture [glucose (1%), yeast hydrolysate (0.3%), malt extract (0.3%), pepton (0.5%)]. The temperature was controlled at 28 °C in a shaking incubator (IRC-1-U) at 150 rpm. Cells were harvested in the 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.

2.3. Preparation of yeast protein hydrolysates

2.3.1. Autolysis treatment

The yeast pellet was resuspended in distilled water (2.5% dry yeast cell/water) and incubated at 52 °C, pH 5 (adjusted by adding 0.1 M NaOH) with agitation at 120 rpm for 96 h (Běehalová & Beran, 1986). Ethyl acetate was added to a final concentration of 1.5% (v/v) as autolysis inducer (Conway, Gaudreau, & Champagne, 2001). After 96 h, samples were heated at 85 °C for 15 min to terminate enzyme activity of yeast cell (Tanguler & Erten, 2008). Cell debris was removed by centrifugation at 11,500 × g for 10 min.

2.3.2. Physical-enzymatic (sonication-trypsin and chymotrypsin hydrolysis) treatment

To obtain protein extract, 50 ml of 2.5% dry yeast cells in 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 cell debris and particles were removed by centrifugation at 11,500 × g for 10 min. The intrinsic yeast cell enzymes in the supernatant were inactivated by heating at 85 °C for 15 min.

To prepare hydrolysate, a solution of extracted protein (4 mg/ml) in 50 mM phosphate buffer, pH 7.8 was subjected to enzymatic hydrolysis using trypsin and chymotrypsin. The ratio of protein substrate to each enzyme was 10:1 (w/w). Each enzyme was dissolved separately in the same buffer. After 5 h incubation at 37 °C, the enzymatic hydrolysis was stopped by heating in water at 85 °C for 15 min. One sample that contained only intrinsic yeast cell enzymes and protein and peptides from yeast source was also considered as control sample.

2.4. Purification of peptide from yeast protein hydrolysate

To purify antioxidant and ACE-inhibitory peptides, the hydrolysates derived from two physical-enzymatic and autolysis treatments were passed through ultrafiltration membranes with a cut-off of 10, 5 and 3 kDa. The filtrates were then freeze-dried and stored at –20 °C. The resulting filtrate with the highest antioxidant and ACE-inhibitory activities was fractionated using reverse phase-high performance liquid chromatography (RP-HPLC) on an analytical C_{18} column (Perfect sil target, ODS-3, 250 × 4.6 mm, 5 μm, 100 Å) with a linear gradient system from eluent A (0.1% TFA in distilled water) to eluent B (0.1% TFA in acetonitrile) at a flow rate of 0.5 ml/min for 45 min. The chromatographic column was conditioned with 100% of eluent A, after which 15 μl of the peptide solution was applied onto the C_{18} column. Absorbed peptide was eluted using the following procedure: 0–15 min, 90% eluent A; 15–30 min, 90–10% eluent A; 30–32 min, 10–90% eluent A; 32–45 min, 90% eluent A. The absorbance of the elution peaks was monitored at 215 nm using a UV detector. Major peaks were collected, and lyophilised for

further analysis. Fraction demonstrating the highest antioxidant and ACE inhibitory activities was re-chromatographed on the same condition except for reducing the flow rate to confirm the purity of the peak.

2.5. Protein assay

Protein concentration in supernatants was determined by Hartree (1972) modified Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). Bovine serum albumin (BSA) was used as a protein standard.

2.6. Evaluation of the degree of hydrolysis (DH)

The extent of hydrolysis was studied using o-phthaldialdehyde (OPA) spectrophotometric assay, which has been previously described by Church, Swaisgood, Porter, and Catignani (1983) and Salami et al. (2011). A fresh OPA solution was prepared daily as follows: 25 ml of 100 mM sodium tetrahydroborate, 2.5 ml of 20% SDS (w/v), 40 mg of OPA (dissolved in 1 ml methanol) and 100 μl of β- mercaptoethanol were adjusted to a final volume of 50 ml with distilled water. To assay proteolysis with yeast protein as substrate an aliquot (usually 10–50 μl 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 UV-visible spectrophotometer. Free amino groups were calculated from a standard curve constructed by using L-leucine (0–4 mg/ml). DH was calculated using the following formula:

$$\mathrm{DH} = \frac{L1 - L0}{Lmax - L0}$$

where L1 is the amount of free amino groups released after hydrolysis, L0 is the amount of free amino groups in the original yeast hydrolysate and Lmax is the total amount of free amino groups in the original yeast hydrolysate obtained after acid hydrolysis (6 M HCl at 120 °C for 24 h).

2.7. Measurement of antioxidant activity

Antioxidant activity was measured based on 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) and 2, 2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate) (ABTS) radical-scavenging activity.

2.7.1. DPPH radical-scavenging activity test

The experiment previously described by Son and Lewis (2002) was used to evaluate the antioxidant activity. A 1 ml peptide solution (or ethanol as control) was added to 1 ml DPPH solution in ethanol (0.002%). The mixture was shaken for 10 sec and incubated for 30 min in the darkness at room temperature. The absorbance of resulting solution was measured at 517 nm and radical-scavenging activity (%) calculated as follows:

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

The trolox standard curve was used to determine trolox equivalent antioxidant capacity (TEAC).

2.7.2. ABTS radical-scavenging activity test

The ABTS radical-scavenging activity was determined by the decolourisation assay according to the method described by Re et al. (1999). Briefly, ABTS radical was generated by a mixed solution of the 7 mM ABTS solution and 2.45 mM potassium persulphate. After 12–16 h reaction in the dark, the solution was diluted with 5 mM phosphate buffer (pH 7.4) to the absorbance level of 0.70 ± 0.02 at 734 nm. Sample (25 μl) was added to 1 ml of reagent and incubated at 25 °C. Scavenging of the ABTS radical was measured by an absorbance decrease at 734 nm using a spectrophotometer after 6 min of initial mixing. The extent of radical-scavenging activity was calculated as follows:

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

The trolox standard curve generated with 0–25 μM of trolox was used to express trolox equivalent antioxidant capacity (TEAC).

2.8. Determination of ACE-inhibitory activity

The rabbit lung powder was prepared following the procedure of Lossow, Migiorini, Brot, and Chaikoff (1964) with modifications. Rabbit lungs purchased from the Pasteur Institute of Iran (IPI) were used as starting material. The lung tissue was diced and homogenised in one volume of 10 mM ice-cold potassium phosphate buffer (pH 8.3). Then 10 volumes of cold acetone were added. The homogenate was centrifuged at 3000 × g for 30 min. The sediment was washed with 15 volumes of cold acetone and centrifuged at 3000 × g for 30 min and stored at 4 °C. Rabbit lung extract as the source of ACE was prepared by dissolving one gram of lung acetone powder in 10 ml 50 mM, pH 8.3 tris-HCl and 5% (v/v) glycerol. The mixture was centrifuged at 14,000 × g for 20 min at 4 °C after overnight storage at 4 °C. ACE-inhibitory activity was measured by spectrophotometric assay (Vermeirssen, Van Camp, & Verstraete, 2002). Briefly, 75 μl of 5 mM FAPGG dissolved in 50 mM tris-HCl buffer, pH 8.3 containing 400 mM NaCl and 25 μl peptide fractions or water) was added to each well of ELISA plate and pre-incubated for 20 min at 37 °C. Then 10 μl of ACE extract was added to the mixture and the absorbance was measured at 340 nm using an ELISA reader Expert 96 (Power wave xS2, BioTek, Winooski, VT, USA) for periods of 30 min at 37 °C. The slope of the decrease in absorbance of samples (ΔA sample) and control (ΔA control) as a function of time was calculated, and the ACE % inhibitory activity was calculated as follows:

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

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

2.9. Determination of amino acid sequence of the most active peptide

In order to determine the sequence of the peptide having the highest ACE inhibitory and antioxidant activities, the sample

was analysed with MALDI/TOF/TOF mass spectrometer using a 5800 Proteomics Analyzer [Applied Biosystems at Proteomics International Pty Ltd., Nedlands, Western Australia]. MS/MS spectra was analysed using PEAKS Studio Version 4.5 SP2 [Bioinformatics Solutions] and manual interpretation.

2.10. Statistical analysis

All experiments were performed in triplicate (n = 3). Data are presented as mean value ± standard deviations (SD). 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.

3. Results

3.1. Antioxidant and ACE inhibitory activities of yeast protein hydrolysates

According to the results reported earlier (Mirzaei, Mirdamadi, Ehsani, Aminlari, & Hoseini, 2015), autolysis and enzymatic hydrolysis were completed after 96 and 5 h, respectively. Finally DH values of, respectively, 48.75, 18.51, and 17.87% were measured by the OPA assay for autolysis, trypsin, and chymotrypsin hydrolysis.

The DPPH and ABTS radical-scavenging and ACE-inhibitory activities of the end products of autolysis and physical-enzymatic hydrolysis are presented in Table 1. Antioxidant activity is expressed as TEAC, and ACE inhibitory activity is determined as IC_{50} value based on inhibiting ACE activity.

Different yeast protein hydrolysates showed the IC_{50} values between 0.8 and 2.24 mg/ml in ACE- inhibition assay. These results are comparable to those reported by others. For example, IC_{50} values were seen in the range from 0.0069 to 7 mg/ml in a variety of other food protein enzymatic hydrolysates, including *phaseolus lunatus* and *phaseolus vulgaris* seeds (Torruco-Uco, Chel-Guerrero, Martínez-Ayala, Dávila-Ortíz, & Betancur-Ancona, 2009), egg protein (Majumder & Wu, 2010), flax seed (Udenigwe & Aluko, 2010), cowpea (Segura-Campos, Chel-Guerrero, & Betancur-Ancona, 2011), pumpkin oil cake (Vaštag et al., 2011), gelatin (Herregods et al., 2011), and *jatropha curcas* flour (Marrufo-Estrada, Segura-Campos, Chel-Guerrero, & Betancur-Ancona, 2013). The DPPH and ABTS radical-scavenging activities of yeast protein hydrolysates were measured to be between 52.23–179.24 μM TE/mg protein and 1932.5–4653.36 μM TE/mg protein, respectively (Table 1). Nalinanon, Benjakul, Kishimura, and Shahidi (2011) reported DPPH and ABTS radical-scavenging activities of 6210 and 151,000 μM TE/mg protein, respectively, for hydrolysate from *threadfin bream* muscle, and Vaštag et al. (2011) reported ABTS radical-scavenging activity of 7590 and 3270 μM TE/mg protein for enzymatic hydrolysates from pumpkin oil cake protein isolate prepared by alcalase and flavourzyme, respectively.

The results indicated that the trypsin hydrolysate with DH = 18.51% has the highest DPPH (179.24 ± 4.8 μM TE/mg protein), ABTS (4653.36 ± 50 μM TE/mg protein) radical-scavenging, and ACE-inhibitory (IC_{50} = 0.84 ± 0.01 mg/ml) activities.

3.2. The size-dependent antioxidant and ACE inhibitory activities of yeast hydrolysates

In this study, the reducing ability of whole protein hydrolysates and their peptide fractions towards DPPH was calculated, and the results are presented in Fig. 1.

Also, the antioxidant activity of whole yeast protein hydrolysates and peptide fractions was calculated measuring TEAC value, based on the consumption of coloured ABTS radicals (Fig. 2).

Fractions with molecular weight of less than 3 kDa in control and physical-enzymatic treatments showed higher DPPH and ABTS radical-scavenging activities. In autolysis samples (48.75% DH) peptide fraction with MW = 5–10 kDa showed higher DPPH and ABTS radical-scavenging activities compared to lower molecular weight peptides (Figs. 1 and 2).

The results of considering the size-dependent ACE-inhibitory activity of whole yeast protein hydrolysates and peptide fractions are presented in Table 2.

Peptide fraction (MW < 3 kDa) of physical-trypsin hydrolysate exhibited the strongest DPPH (489.12 ± 0.001 μM TE/mg protein), ABTS (7718.30 ± 57 μM TE/mg protein) radical-scavenging (Figs. 1 and 2), and ACE inhibitory (IC_{50} = 0.32 mg/ml) activities (Table 2). Also peptide fraction (MW = 3–5 kDa) exhibited good ACE inhibitory activity with an IC_{50} values of 0.296 mg/ml.

Considering both the inhibitory effect on ACE and antioxidant activities, peptide fraction (MW < 3 kDa) obtained by physical-trypsin hydrolysis was selected for further purification process.

Table 1 – ACE inhibitory and antioxidant activities of yeast protein hydrolysates.

Samples	DH (%)	IC_{50} (mg/ml)	DPPH radical-scavenging activity (μM TE/mg protein)	ABTS radical-scavenging activity (μM TE/mg protein)
Control	10.59^{a}	2.24 ± 0.2^{d}	116.28 ± 0.8^{b}	1932.50 ± 4.7^{a}
Sonication-trypsin hydrolysis	18.51^{b}	0.84 ± 0.01^{a}	179.24 ± 4.8^{d}	4653.36 ± 5^{d}
Sonication-chymotrypsin hydrolysis	17.87^{b}	1.71 ± 0.1^{b}	160.73 ± 11.17^{c}	3323.40 ± 40^{c}
Autolysis	48.75^{c}	2.18 ± 0.12^{c}	52.23 ± 4.61^{a}	2211.59 ± 69^{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 significantly different at P < 0.05.

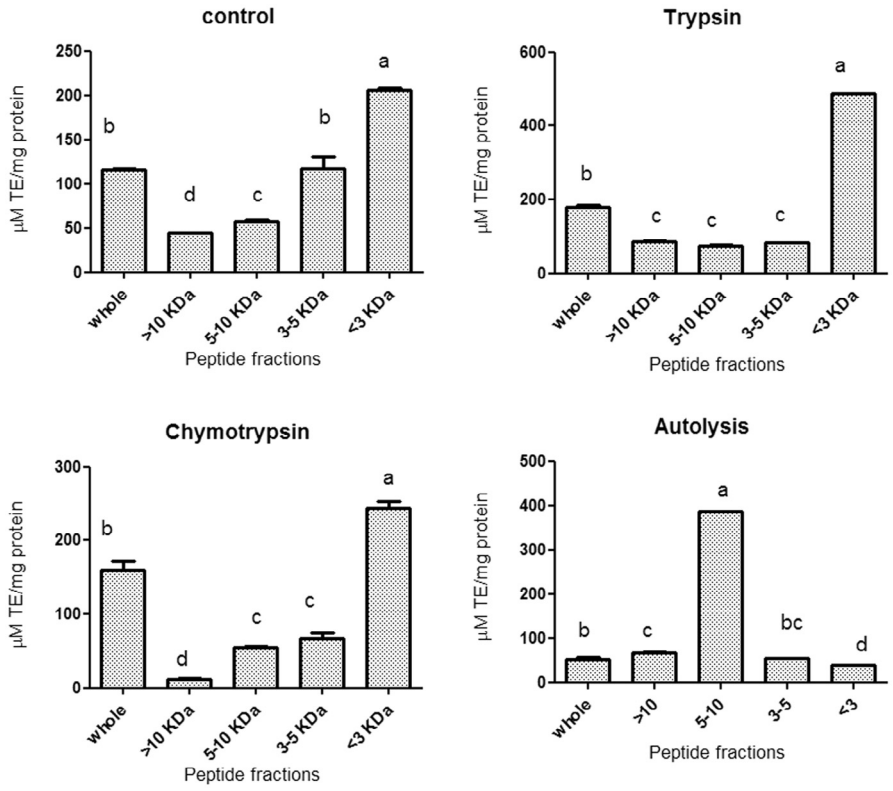

Fig. 1 – The size-dependent DPPH radical-scavenging activity of whole yeast protein hydrolysates (control, physical-trypsin hydrolysate, physical-chymotrypsin hydrolysate and autolysate) and its peptide fractions. The data are expressed as trolox equivalent antioxidant capacity (µM TE/mg protein). The results are mean values of experiments carried out in triplicate. Values with different letters are statistically different at $P < 0.05$.

Table 2 – The size-dependent ACE inhibitory activity of whole yeast protein hydrolysates and their peptide fractions.

Sample	Whole	5–10 kDa	3–5 kDa	<3 kDa
Control	2.24 ± 0.2^{Cd}	3.79 ± 0.03^{Dc}	1.203 ± 0.037^{Ab}	1.54 ± 0.02^{Bc}
Sonication-trypsin hydrolysis	0.84 ± 0.01^{Ba}	2.63 ± 0.015^{Cb}	0.296 ± 0.04^{Aa}	0.32 ± 0.011^{Aa}
Sonication-chymotrypsin hydrolysis	1.71 ± 0.1^{Cb}	1.62 ± 0.02^{Ba}	2.42 ± 0.02^{Dc}	0.79 ± 0.09^{Ab}
Autolysis	2.18 ± 0.12^{Bc}	1.66 ± 0.051^{Aa}	3.05 ± 0.07^{Dd}	2.7 ± 0.05^{Cd}

IC_{50} is the concentration (mg/ml) of an ACE inhibitor needed to inhibit 50% of ACE activity. Values with different capital letters in each row and different small letters in each column are statistically different at $P < 0.05$.

3.3. *Purification of antioxidant and ACE-inhibitory peptide by reverse phase-HPLC*

The trypsin-hydrolysate peptide fraction with MW < 3 kDa showing the highest antioxidant and ACE inhibitory activities was fractionated by RP-HPLC, and the major peaks were selected for assaying ABTS radical-scavenging and ACE-inhibitory activities. The fractions were numbered sequentially (F1–F5).

Major fractions were collected, lyophilised and assayed for ABTS radical-scavenging (Fig. 3B) and ACE-inhibitory (Fig. 3C) activities. As it was mentioned previously, the ABTS radical-scavenging activity of parent purified peptide (sonication-trypsin hydrolysate, MW < 3 kDa) was measured as 7.718 µM TE/µg protein, and it was in the range of 6.22–26.25 µM TE/µg protein for purified peptide fractions. Among all of the fractions collected, the ABTS radical-scavenging activity was the highest in fraction F3 (26.25 ± 0.13 µM TE/µg protein), followed by fractions F5, F4, F1, and F2. The antioxidant activity increased by 3 folds after purification step, indicating 3 times improvement in purity.

The ACE-inhibitory activity of different peptide fractions was expressed as IC_{50} value (Fig. 3C). The IC_{50} value was corresponded to 0.32 mg/ml for parent peptide fraction (MW < 3 kDa) and in the range of 0.29–0.73 mg/ml for purified peptide fractions. The IC_{50} value could not be measured for fraction F1. Among all of the fractions collected, the ACE inhibitory activity was the highest in fractions F5 ($IC_{50} = 0.29 \pm 0.001$ mg/ml) and F3 ($IC_{50} = 0.42 \pm 0.02$ mg/ml), followed by F4 and F2. The measured IC_{50} values showed no significant difference ($P > 0.05$) in fraction F5 compared to parent peptide, and other fractions showed significantly ($P < 0.05$) higher values of IC_{50}. The fraction

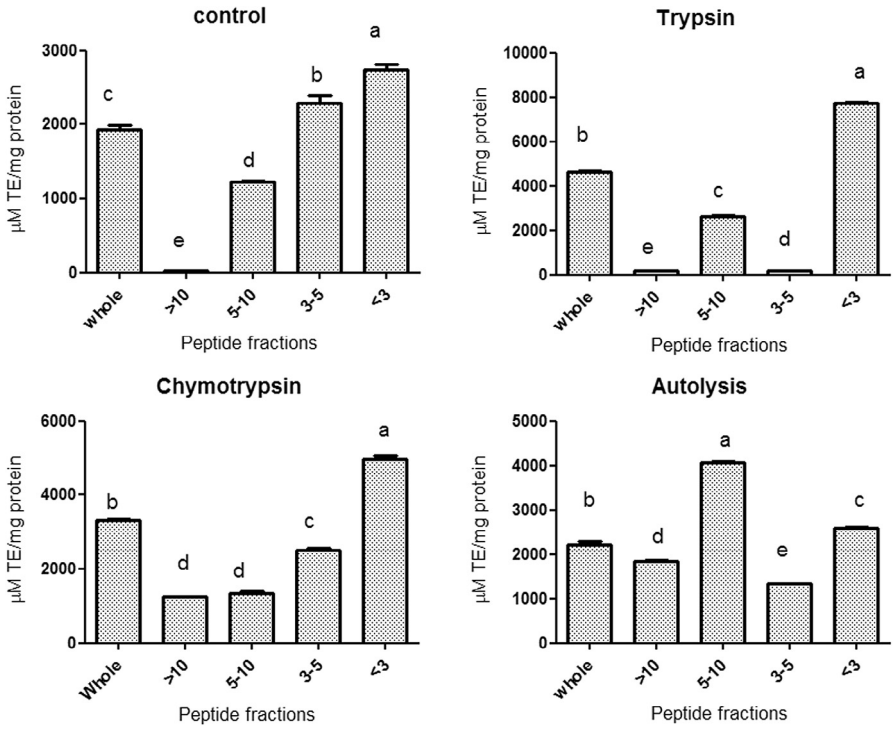

Fig. 2 – The size-dependent ABTS radical-scavenging activity of whole yeast protein hydrolysates (control, physical-trypsin hydrolysate, physical-chymotrypsin hydrolysate and autolysate) and its peptide fractions. The data are expressed as trolox equivalent antioxidant capacity (µM/mg protein). The results are mean values of experiments carried out in triplicate. Values with different letters in each column are statistically different at $P < 0.05$.

F3 purified from the first step of RP-HPLC with ABTS radical-scavenging and ACE-inhibitory activity was applied in the second step RP-HPLC in the same situation except for reducing the flow rate. The chromatogram showed a major peak, suggesting the fraction F3 had a purity satisfactory for amino acid sequencing.

3.4. *Peptide identification*

The molecular mass and the sequence of the active peptide (fraction F3) were determined by MALDI-TOF-TOF mass spectrometer. The peptide sequence was provided by Proteomics International Pty Ltd and found to be Tyr-Gly-Lys-Pro-Val-Ala-Val-Pro-Ala-Arg with a molecular weight of 1057.45 Da. Due to preparing of hydrolysate by trypsin, the resulting sequence has been terminated by Arg residue. It consists of 70% hydrophobic amino acid residues (Fig. 4).

4. Discussion

The results of this study show that the prepared hydrolysate, especially trypsin hydrolysates, possibly contains peptides with significant DPPH and ABTS radical-scavenging as well as ACE inhibitory activities. These properties are probably related to the total content of hydrophobic amino acids in the trypsin hydrolysate as compared to other hydrolysates. Hydrophobic amino acid residues with aromatic or branched side chains at each of the C-terminal tripeptide position are common features among potent antioxidant and ACE inhibitors (Memarpoor-Yazdi, Mahaki, & Zare-Zardini, 2013; Ruiz, Ramos, & Recio, 2004).

Protein hydrolysates showed both DPPH and ABTS radical-scavenging activities. The results so obtained suggest that the hydrolysates contained amino acids or peptides that were hydrogen donors and could react with free radicals to convert them to more stable products (Martysiak-Zurowska & Wenta, 2012; Prior, Wu, & Schaich, 2005; Thiansilakul, Benjakul, & Shahidi, 2007).

As it can be found in Table 1, Figs. 1 and 2, the TEAC values determined by the ABTS method were significantly ($P < 0.05$) higher than those measured by DPPH assay. This difference can be due to differences of free radicals solubility and diffusivity in the reaction medium. The same results were previously reported by Nalinanon et al. (2011), Martysiak-Zurowska and Wenta (2012), and Tang et al. (2010).

According to our results, the peptide fractions with molecular weight <3 kDa and 5–10 kDa obtained by physical-enzymatic and autolysis treatments, respectively, exhibited higher antioxidant and ACE-inhibitory activities compared to the whole hydrolysates, but other peptide fractions showed mostly the same or lower antioxidant and ACE-inhibitory activities. These results suggest that the overall antioxidant and ACE inhibitory activities of physical-enzymatic yeast hydrolysate and autolysate are the result of a concerted action of fractions with MW <3 kDa and 5–10 kDa, respectively.

The physical-enzymatic peptide fraction (MW < 3 kDa) exhibits the highest antioxidant and ACE-inhibitory activities. The

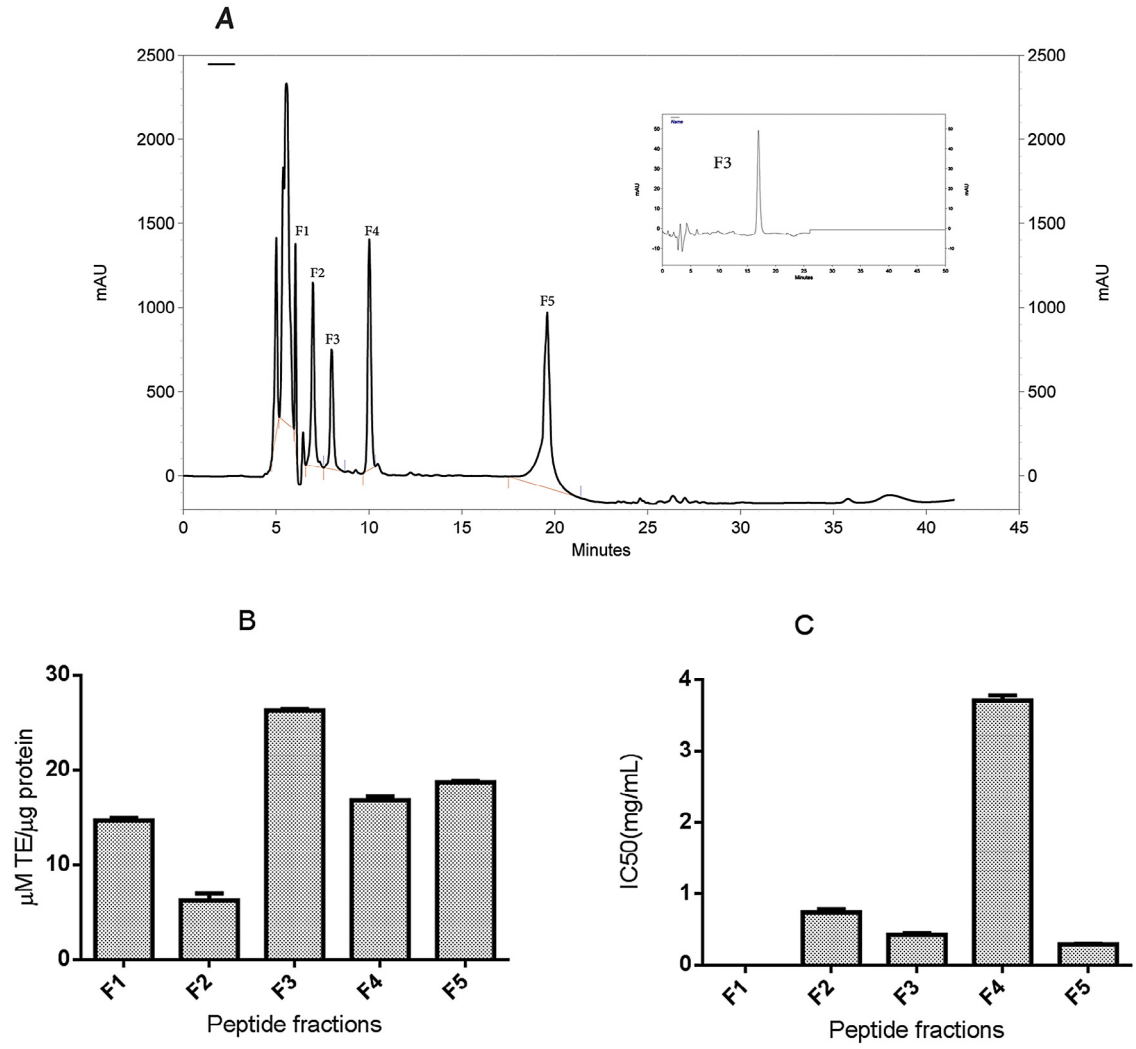

Fig. 3 – (A) RP-HPLC chromatogram of the prepared yeast protein hydrolysate (physical-trypsin hydrolysis) with MW < 3 kDa. The insets confirm the purification of F3 fraction using the second run of C_{18} RP-HPLC. (B) ABTS radical-scavenging activity of purified peptide fractions. The data are expressed as trolox equivalent antioxidant capacity (μM/μg protein). (C) ACE inhibitory activity of purified peptide fractions. 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.

majority of previous research has shown that the antioxidant and ACE inhibitory activities of hydrolysates depend on their molecular weight distribution, and short peptides are the most efficient antioxidants and ACE inhibitors because of their greater accessibility to the oxidant/antioxidant test system and better binding to the ACE active site than that of macropeptides and proteins (Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005; Wang, Li, Chi, Zhang, & Luo, 2012).

Furthermore, when the peptides of < 3 kDa range were subjected to RP-HPLC, the fraction F3 with intermediate hydrophobicity (intermediate elution time) was identified to be highly potent ABTS radical-scavenging and ACE-inhibitory peptide.

Structure–activity correlations among different peptide inhibitors of ACE indicated that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate. It has been suggested that peptides that contain hydrophobic amino acids at these positions are potent inhibitors (He et al., 2012; Li, Le, Shi, & Shrestha, 2004). The existence of Ala and Pro at the C-terminal tripeptide positions (He et al., 2012) and the presence of hydrophobic amino acids (Pro, Val, Ala) at the internal position of peptide F3 (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; Meisel, Walsh, Murray, & FitzGerald, 2006) seem to positively influence the peptide ACE inhibitory activity. The purified peptide contains Tyr at N-terminal position. Although Tyr residues were present at the C-terminus of many reported potent ACE inhibitors (Fahmi et al., 2004; Hernandez-Ledesma, del Mar Contreras, & Recio, 2011), the relationship between the N-terminal of peptide structure and ACE inhibitory activity is not completely clear (Saiga et al., 2003). Also, the positive charge on the side chain of Arg residue at the C-terminal may be a requirement for potent ACE-inhibition of the purified peptide and suggests a possible interaction between the inhibitor and ionic binding site of ACE (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980; Ondetti, Rubin, & Cushman, 1977; Shahidi & Zhong, 2008).

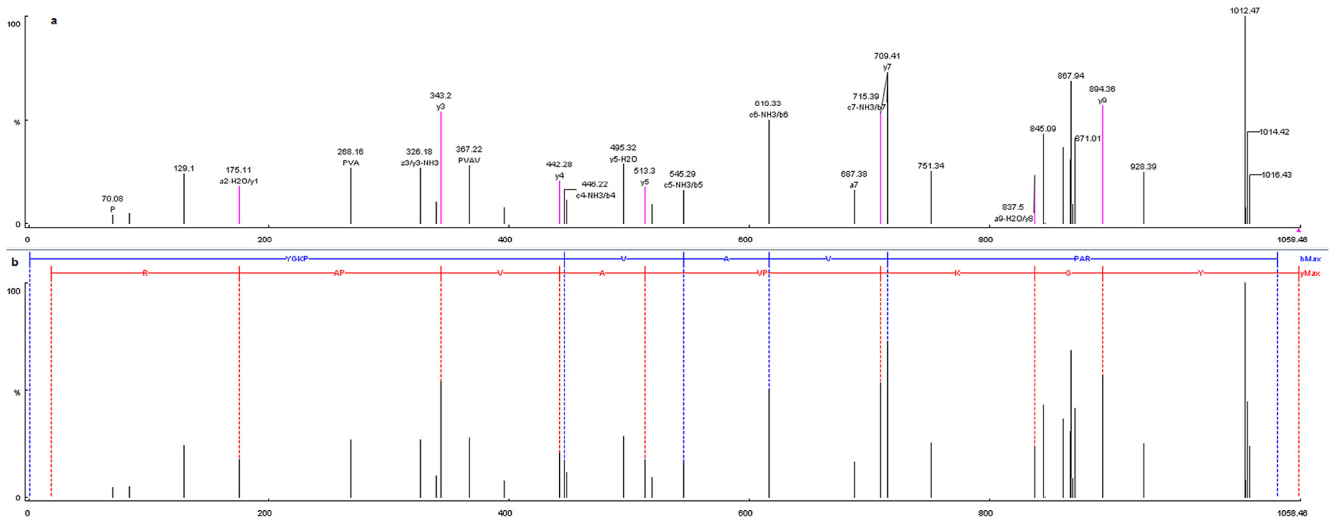

Fig. 4 – Identification of the molecular mass and amino acid sequence of the F3 peptide using MALDL-TOF-TOF mass spectrometer. (A) MS/MS spectra of the F3 peptide, and (B) the interpretation of the obtained spectra.

The presence of hydrophobic amino acid in the peptide sequences has been postulated to play an important role in the antioxidant activity (Jiang et al., 2014; Sarmadi & Ismail, 2010). There is a relation between the presence of aromatic/imino amino acids and antioxidant activity of peptide (Peña-Ramos, Xiong, & Arteaga, 2004). Pro residue plays an important role in antioxidant activity of purified peptide (Samaranayaka & Li-Chan, 2011).

The radical-scavenging activity of antioxidant peptide may be due to the hydrogen donor activity of the hydroxyl group of Tyr as an aromatic amino acid (Shahidi & Zhong, 2008). Gly residue may contribute significantly to antioxidant activity since the single hydrogen atom in the side chain of Gly serves as protons-donating and neutralises active free radical species (Chen, Chi, Zhao, & Xu, 2012) .

5. Conclusion

In conclusion, this study confirmed that the yeast protein hydrolysate of *S. cerevisiae* and its purified peptides can show multifunctional bioactivities and may be beneficial as food additives and pharmaceutical agents. However, it is important to study the techno-functional properties of active peptides fractions and develop model foods that contain these peptides and retain their activity in different targeted food matrices. Molecular studies are needed to assess the mechanisms by which bioactive peptides exert their activities. It is also important to consider a relationship between *in vitro* and *in vivo* activity of peptides since they are subject to degradation and modification in the intestine, vascular system and liver. Also, synergistic or antagonistic effects with other antioxidant and/or trace metal present in food and biological systems should be taken into consideration.

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