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Identification of potent antioxidant bioactive peptides from the soluble proteins of chicken egg yolk

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ABSTRACT

BACKGROUND: Eggs are an excellent nutrient-dense food containing proteins, fats, carbohydrates, minerals, and vitamins. While many proteins are present in egg yolk, there arefew studies on their health benefits.

OBJECTIVE: The aim of this study was to explore the antioxidant peptides derived from the soluble protein fractions of egg yolk. Lipids and lipoproteins were removed with activated carbon and centrifugation after water dilution at acidic pH.

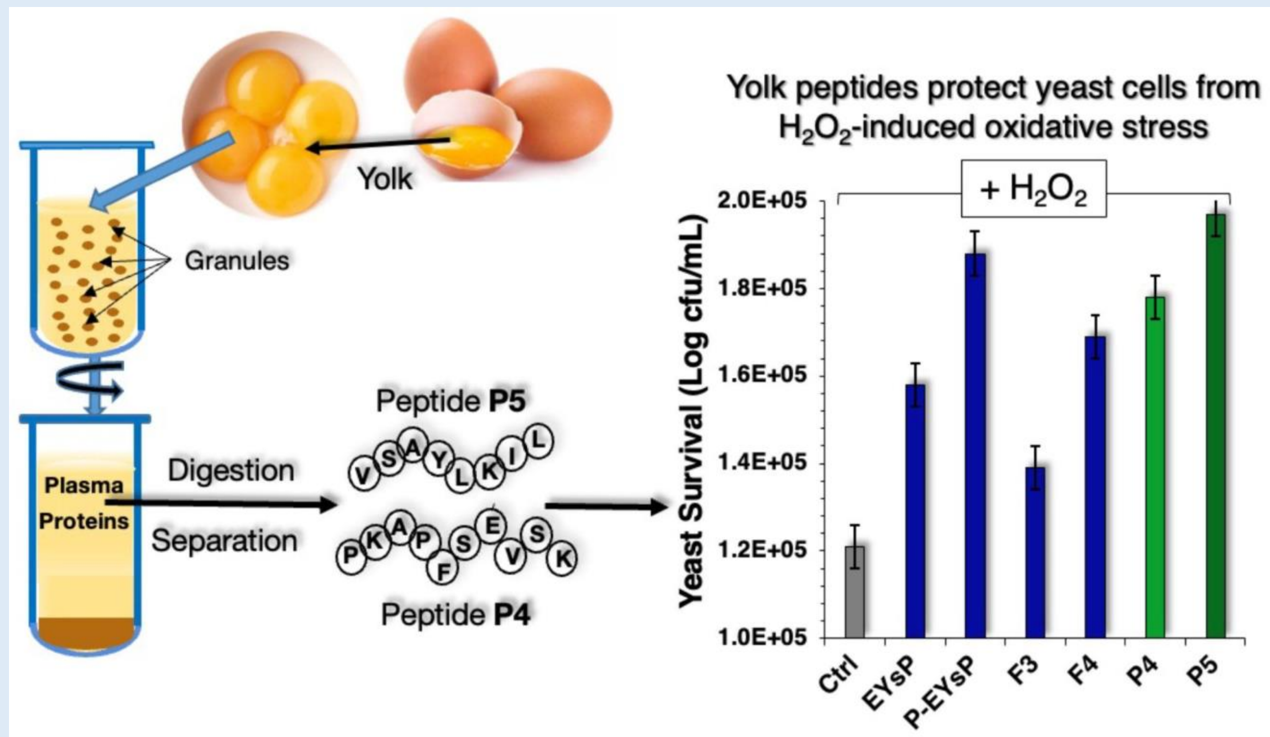
MATERIALS AND METHODS: Compared with 3.6 grams of protein in egg white, egg yolk contains 2.7 grams of protein in a single large egg. The egg yolk soluble protein (EYsP) was digested with pepsin for 2h at pH 3.0 (P-EYsP), followed by digestion with trypsin (P-EYsP-T), α-chymotrypsin (P-EYsP-α), or both enzymes (P-EYsP-T/α). The most active digest was fractionated using sephacryl S-100 gel filtration, followed by reversed phase-HPLC of the most active fraction. The antioxidant activities were evaluated using a superoxide anion-generating system of xanthine oxidase, DPPH-scavenging assay, and yeast cells as an oxidative-stress tolerance cellular model.

RESULTS: The intact proteins (EYsP) showed antioxidant activity, but pepsin hydrolysate (P-EYsP) exhibited greater superoxide-scavenging activities than EYsP, while P-EYsP-T, P-EYsP-α and P-EYsP-T/α lacked activities. The P-EYsP and its subsequent proteases (P-EYsP-T, P-EYsP-α and P-EYsP-T/α) exhibited significant DPPH reduction, but P-EYsP-T, P-EYsP-α and P-EYsP-T/α exhibited the strongest DPPH reduction activities. MALDI-TOF-MS analysis revealed five major

antioxidant peptides, two derived from yolk glycoprotein 40 (859 Da, 883Da), two from lipovitelline (901Da, 945 Da), and one from livetin (1089 Da). The peptides exhibited potent superoxide anion as well as DPPH scavenging activities and markedly enhanced the tolerance of yeast cells against peroxide-induced oxidative stress.

CONCLUSION: The results show that these bioactive peptides hold a fascinating opportunity for their potential as nutraceuticals in prevention and combating oxidative stress-associated diseases.

KEYWORDS: Egg yolk proteins; bioactive peptides; antioxidant; superoxide-scavenging; DPPH-reduction; yeast tolerance



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INTRODUCTION

Oxidative stress associated with the elevated level of reactive oxygen species (ROS) contributes to the pathogenesis of many diseases, including inflammatory diseases, rheumatoid arthritis, diabetes, hypertension, and carcinogenesis [1]. Oxidative stress occurs when excess reactive oxygen species (ROS) exceeds the capacity of the endogenous antioxidant systems. When ROS production becomes overwhelming, compensatory mechanisms are inadequate and pathophysiological consequences ensue. Multiple enzymatic systems, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase (XOD), nitric oxide synthases (NOS), and myeloperoxidase (MPO), are involved in the excess production of ROS in pathogenesis such as superoxide ($O_2^{\cdot-}$) and its derivatives. Antioxidant peptides have been exploited to treat or prevent several human pathologies in which oxidative stress seems to be the trigger. The scavenging of $O_2^{\cdot-}$ using nature-derived peptides is hence of huge therapeutical and nutraceutical demand. Numerous studies have focused on developing antioxidant peptides with $O_2^{\cdot-}$ scavenging capacities from

food proteins as natural sources of bioactive peptides [2,3].

Chicken eggs have health benefits due to the contents of biologically active proteins that help preven a number of human diseases [4]. There have been a relatively large number of studies investigating the health benefits of chicken egg white proteins and their derived bioactive peptides [4,5]. Yet, there have been relatively fewer studies investigating the antioxidant peptides derived from egg yolk. Egg yolk contains non-soluble proteins such as low-density lipoproteins (LDLs), high-density lipoproteins (HDLs), and soluble proteins, including some lipovitellin fractions, livetins, and phosvitins. The non-soluble proteins aggregate, making up what is called granules, which account for about 50% of yolk proteins, suspended in a yellow fluid called plasma. Plasma fluid contains soluble proteins [6,7]. Although soluble proteins are responsible for most of the physical properties of yolk, surprisingly, they have not been studied reliably [8]. Apolipoprotein B, apovitellenin-1, vitellogenins, serum albumin, immunoglobulins, and some egg white protein such as riboflavin binding protein (also called ovoflavoprotein) are the most abundant proteins of egg yolk [9]. Yolk also contains proteases, protease inhibitors, biotin-binding proteins, and antioxidative enzymes (superoxide dismutase and glutathione peroxidase) [10]. Vitellogenin, a serum lipoprotein, is proteolytically cleaved by cathepsin D, a pepsin-like protease, into two lipovitellins (heavy and light chains), phosvitin, and the yolk glycoprotein of 40 kDa (YGP40) after transportation into oocyte [11].

The egg yolk proteins, accounting for 17% of egg yolk, are an inexpensive natural and safe source of bioactive proteins. The interesting features of egg yolk proteins are suggested to show various medicinal activities [12]. It has been reported that hen egg proteins exhibit antioxidant activities, which play a role in human health [13–15] and in food systems to increase the shelf life of products [16]. The sequences of these proteins may encode bioactive peptides, which could be released through proteolysis in the gastrointestinal tract or food fermentation. Bioactive peptides of egg yolk are expected to find wide application in food nutraceuticals or therapy. In recent years, research on bioactive peptides is intensifying, particularly, on antioxidant peptides and their applications in health foods and cosmetics [3]. There is significantly growing evidence that oxidative stress, increase of reactive oxygen species (ROS), is involved in the pathogenesis of a large number of chronic diseases in humans [17]. Thus, the antioxidants' bioactive peptides from natural proteins have significant potential as nutraceuticals and as alternatives to synthetic antioxidants in food formulations.

The biological function of yolk Is to provide the lipids, proteins, and minor substances needed by the growing embryo. Some of the lipids in yolk are chemically reactive and are liable to oxidation once the egg has been laid. Yolks would therefore be expected to have specific antioxidant proteins or peptides to prevent oxidative damage that might affect their use by the embryo. These proteins and peptides are not fully understood. The egg yolk proteins, accounting for 17% of egg yolk, are an inexpensive natural, and safe source of bioactive proteins. The interesting features of egg yolk proteins are suggested to show various medicinal activities [12]. It has been reported that hen egg proteins exhibit antioxidant activities, which play a role in human health [13–15] and in food systems [16]. These studies were performed on the whole yolk proteins obtained by ultrafiltration [18], or by-product of delipidated egg yolk by organic solvents [19], or just proteomic-based research on the composition of egg yolk proteins [20], or individual proteins such phosvitin [21].

For food and pharmaceutical industries, egg yolks are valuable sources for the extraction of lipids and phospholipids. Lipids and phospholipids are localized in the yolk granules associated with apoproteins in the form of insoluble lipoproteins. The lipids extraction process using organic solvents and heating impairs the structures of yolk proteins and, subsequently, their biological activities. On the other hand, yolk plasma contains water-soluble proteins, livetins, and some lipovitellenin fractions. Hence, the native forms of water-soluble proteins could be of added value as sources of antioxidant bio-active peptides if the plasma, which contains proteins [6], was isolated from the yolk before the protein-denaturing lipid extraction process. The sequences of these water-soluble proteins may encode bioactive peptides, which could be released through proteolysis in the gastrointestinal tract. These bioactive peptides are expected to find wide application in the nutraceuticals or pharmaceutical industries. Particularly, the interest in antioxidant bioactive peptides is intensifying for their applications in health foods and cosmetics [3]. Especially, there is growing evidence that oxidative stress, an increase of reactive oxygen species (ROS), is involved in the pathogenesis of a large number of chronic diseases in humans [17].

Therefore, this study aimed to explore antioxidant peptides of the fractionated plasma water-soluble proteins of egg yolk obtained through peptic and gastrointestinal tract proteolytic simulation.

MATERIALS AND METHODS

Materials: Fresh white eggs were obtained from Keiran farm, Kagoshima. Pepsin (porcine), trypsin, α-chymotrypsin, xanthine, xanthine oxidase, nitro-blue tetrazolium and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were from Sigma-Aldrich (Tokyo, Japan). Peptides calibration standard and α-cyano-4-hydroxy-cinnamic acid (α-HCCA) were from Bruker Daltonik (Bremen, Germany). Sephacryl S-100 was a product of Amersham-Pharmacia Biotech (Tokyo, Japan). TSK gel ODS-120T column (TOSOH, Tokyo, Japan). All other reagents were of analytical grade.

Isolation and proteolysis of proteins: Freshly laid eggs were maintained for 2h at room temperature (20˚C) before breaking to separate the egg yolk from albumen using a stainless steel egg separator. As shown in Scheme 1, the yolk spheres were separated from egg white and washed with distilled water over filter paper (Whatman No. 5A) before the rupture of the vitelline membrane to release its contents (Egg Yolk). Egg yolk contents were diluted 10X with distilled water,then adjusted to pH 5.0 with 0.5N HCl and subjected to centrifugation at 4800 ×g for 20 min at 10˚C. The supernatant was passed through a column of activated carbon to remove the remaining lipoproteins. The supernatant was then filtered through a paper filter and freeze-dried, referred to as egg yolk soluble protein (EYsP). The isolation of the peptides is based on an *in vitro* digestion process, which stimulates the *in vivo* digestion of the proteins into the intestine. Portions of EYsP were adjusted to pH 7.5 and then treated with trypsin (EYsP-T), α-chymotrypsin (EYsP-α), or both enzymes (EYsP-T/α) to give 100:1 (w/w) protein to the enzyme at 37˚C for 2h. For the gastrointestinal tract digestion simulation, the remaining portion was adjusted to pH 3.0 and mixed with pepsin to give 100:1 (w/w) protein to the enzyme. After incubation at 37˚C for 2 h, pepsin was inactivated by heating at 85˚C for 5 min. The reactions were centrifuged at 3000 ×g for 10 min, and the supernatants were adjusted to pH 7.5 and then treated with trypsin (P-EYsP-T), α-chymotrypsin (P-EYsP-α), or both enzymes (P-EYsP-T/α) as above mentioned. The hydrolysates were analyzed by reducing SDS-PAGE (4-15% acrylamide gels) according to standard protocols

[22] and tested for the ability to scavenge superoxide or DPPH radicals. The most active hydrolysate was fractionated on Sephacryl-S100 (80 x 1.5 cm) attached to Biologic LP system (Bio-Rad), and elution of peptides was achieved at 25˚C with 25 mM pyridine-acetate buffer（pH 5.5）and monitored at 280 nm. The active fraction was then purified by reversed-phase HPLC “RP-HPLC” on C18 column (7.5 x 250 mm) with a linear gradient of 1-40% acetonitrile over 180 min, and elution was monitored at 214 nm [23]. Collected peptides were dried and re-suspended in distilled water at the desired concentration.

Antioxidant activity assay: Antioxidant activity was evaluated by measuring superoxide ($O_2^{\cdot-}$)-scavenging capacity and the reduction of 2,2-Diphenyl- 1-picrylhydrazyl (DPPH). The superoxide scavenging capacity is expressed as the degree of nitro-blue tetrazolium reduction by superoxide, measured spectrophotometrically at 562 nm [23,24]. A reaction mixture (100 µL) reaction mixture containing nitro-blue tetrazolium (40 µM), xanthine (5 mU), and test proteins or peptides (100 µg/mL) in Na-phosphate buffer (pH 8.0) in a 96-well plate was mixed with 100 µL of 5mM xanthine oxidase (XOD). The flux of superoxide anion was monitored at 562 nm (37˚C) kinetically for 20 min using an infinite 200 microplate reader (Tecan Trading, Switzerland). Control (Ctrl) contained water instead of test samples. The results were expressed as the rate of absorbance change, by subtracting the reading at 0 time from the subsequent readings. Results are representative of two experiments with three wells per sample.

The DPPH-scavenging capacity was assessed by monitoring the fading of purple color kinetically for 20 min at 562 nm (37˚C) of DPPH solution (1.4 mM) containing 100 µg/mL proteins or peptides, using an infinite 200 microplate reader. Control (Ctrl) contained water instead of test samples. The results were expressed as the rate of absorbance change by subtracting the reading at 0 time from the subsequent readings. Also, the DPPH-scavenging capacity was examined in a dry-state assay by spraying DPPH solution over spots of proteins or peptides onto a thin layer chromatography (TLC) sheet [25]. Samples of proteins or peptides (6 µL) were spotted onto the TLC sheet. Upon drying, the sheets were sprayed with DPPH (2 mM ethanolic solution). Within the purple background, white spots will appear when spots contain DPPH-reducing sample.

The yeast model for oxidative stress: As a system for modeling mitochondrial disease, yeast cells were employed [25,26]. Saccharomyces cerevisiae (YNN27) cells grown in yeast extract peptone dextrose (YEPD) broth, suspended at 0.1 absorbance of 600 nm, were mixed with peptides (200 µg/mL) and then incubated at 28˚C for 1 h. The oxidizing agent, hydrogen peroxide (H_2O_2), was added at 2 mM, and the incubation was extended for 24h. A portion of the serially diluted suspensions (10 µL) in 2 mM H_2O_2, was spotted on YEPD agar plates containing H_2O_2 (2 mM). The colony forming units (CFU) of survival yeast cells was estimated after incubation of the plates for 72h at 28˚C. Data was expressed as log CFU/mL.

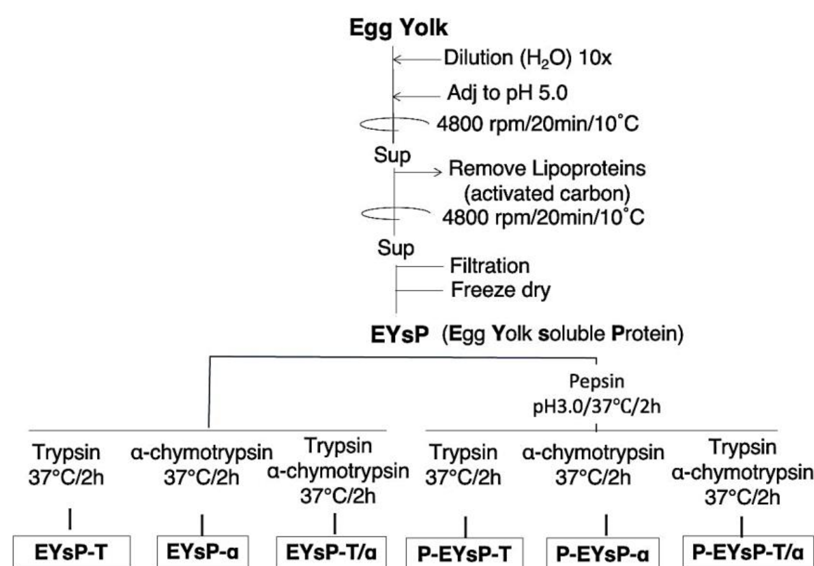
MALDI-TOF peptides identification: Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) was employed to identify peptides in the RP-HPLC peaks. Peptide solution (1 µL) was mixed with an equal volume of saturated α-HCCA matrix solution, then spotted onto a steel target plate and air-dried. Autoflex Speed mass spectrometer (Bruker Daltonik GmbH, Germany) was used for analysis in positive reflector mode within the mass range of 1000 -

3200 Da. Upon calibration using peptides calibration standard 700-4000 Da (Bruker Daltonik GmbH, Germany), the peptides were subjected to TOF/ MS analysis of the major precursor ions in each peak. By using flexControl tools, de novo routine and MASCOT, as well as SEQUEST database were employed to identify peptide sequences. Mass spectrum was generated and viewed by using mMass 5.5 software [27].

Statistical analysis: Experiments were carried out in three wells per sample in triplicate measurements, and mean values were used for the statistical analysis. The analysis of one-way ANOVA was used to explore the difference between controls and samples using Excel's data analysis ToolPak[28,29]. Data shows mean values, and error bars show standard deviations.

RESULTS

Isolation and proteolysis of egg yolk proteins: The egg yolk soluble proteins (EYsP) were separated from the lipids and lipoproteins of egg yolk by acidification and passing through activated carbon (Scheme 1). EYsP was hydrolyzed with trypsin (EYsP-T), α-chymotrypsin (EYsP-α) or double digested with both enzymes before (EYsP-T/α) and after hydrolysis for 2 h by pepsin (P-EYsP-T, P-EYsP-α, P-EYsP-T/α) followed by dialysis and lyophilization. The hydrolysates were analyzed on reduced SDS-polyacrylamide gel electrophoresis (Fig. 1). The non-proteolyzed egg yolk soluble proteins (EYsP, none) showed four major bands and three minor ones. The major bands were apovitellenin Va (122 kDa), α-livetin (73 kDa), β-phosvitin (59 kDa), and YGP40 (42 kDa), where the minor bands were apovitellin 5-6 (78 kDa), and two forms of β-livetin (35 and 31 kDa) based on the literatures [8]. Digestion with trypsin (EYsP-T) produced minor proteolysis, while α-chymotrypsin (EYsP-α) hydrolyzed all proteins into bands with molecular masses ranging from 31 kDa to less than 8 kDa. Double digestion with α-chymotrypsin and trypsin (EYsP-T/α) showed a similar pattern as with α-chymotrypsin alone. However, pepsin alone (P-EYsP, P alone) completely hydrolyzed all protein bands into small peptides with molecular masses of 6 kDa or less. But digestion with α-chymotrypsin or trypsin or both proteases after pepsin treatment (P-EYsP-T/α) did not change the electrophoretic pattern of P alone.



Scheme 1. Outline of the isolation of egg yolk soluble proteins (EYsP) as well as the gastrointestinal simulated hydrolysis of the fraction.

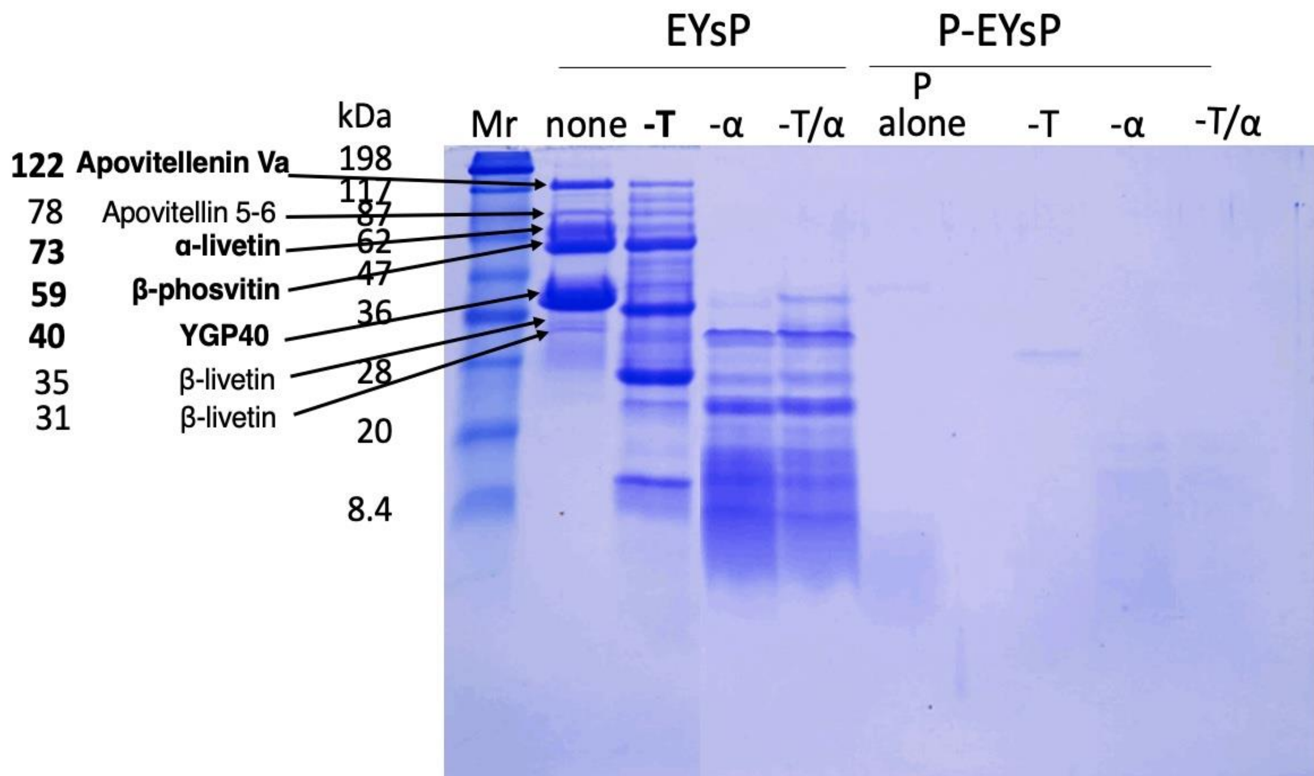


Figure 1. Electrophoretic patterns of EYsP and its peptic digest (P-EYsP) before (none) and after digestion with trypsin (-T), α-chymotrypsin (-α), or both enzymes (-T/α) on 15% polyacrylamide gels of reducing SDS-PAGE. Mr, molecular weight marker.

Antioxidant activity of EYsP hydrolysates: The intact proteins and the hydrolysates were assessed for their abilities to scavenge superoxide anion and the chemical radical DPPH (Figure 2). The intact proteins (EysP) exhibited remarkable superoxide-scavenging capacity, while EysP-T, EysP-α and EysP-T/α lacked any superoxide-scavenging capacity compared to the control reaction (Figure 2A). The results of DPPH reduction (Figure 2A, upper) did not parallel that of the superoxide-scavenging activity, whereas EYsP-α and EYsP-T/α digests showed DPPH reduction with EYsP-T/α being the strongest reduction, as it produced more intense white spots (Figure 2A). The pepsin hydrolysate (P-EYsP) exhibited greater superoxide-scavenging activity than its intact proteins (EYsP), while P-EYsP-T, P-EYsP-α and P-EYsP-T/α, wich again lacked superoxide-scavenging activities (Figure 2B). All hydrolysates of pepsin (P-EYsP) and their subsequent proteases (P-EYsP-T, P-EYsP-α and P-EYsP-T/α) exhibited significant DPPH reduction (Figure 2B, upper). But P-EYsP-T, P-EYsP-α and P-EYsP-T/α exhibited stronger DPPH reduction than pepsin alone, P-EYsP (Figure 2B, upper). The results demonstrated that EYsP proteins remarkably scavenge oxygen superoxide, but their peptic hydrolysate, as well as the subsequent trypsin or α-chymotrypsin hydrolysates, possess ability to reduce the DPPH radical.

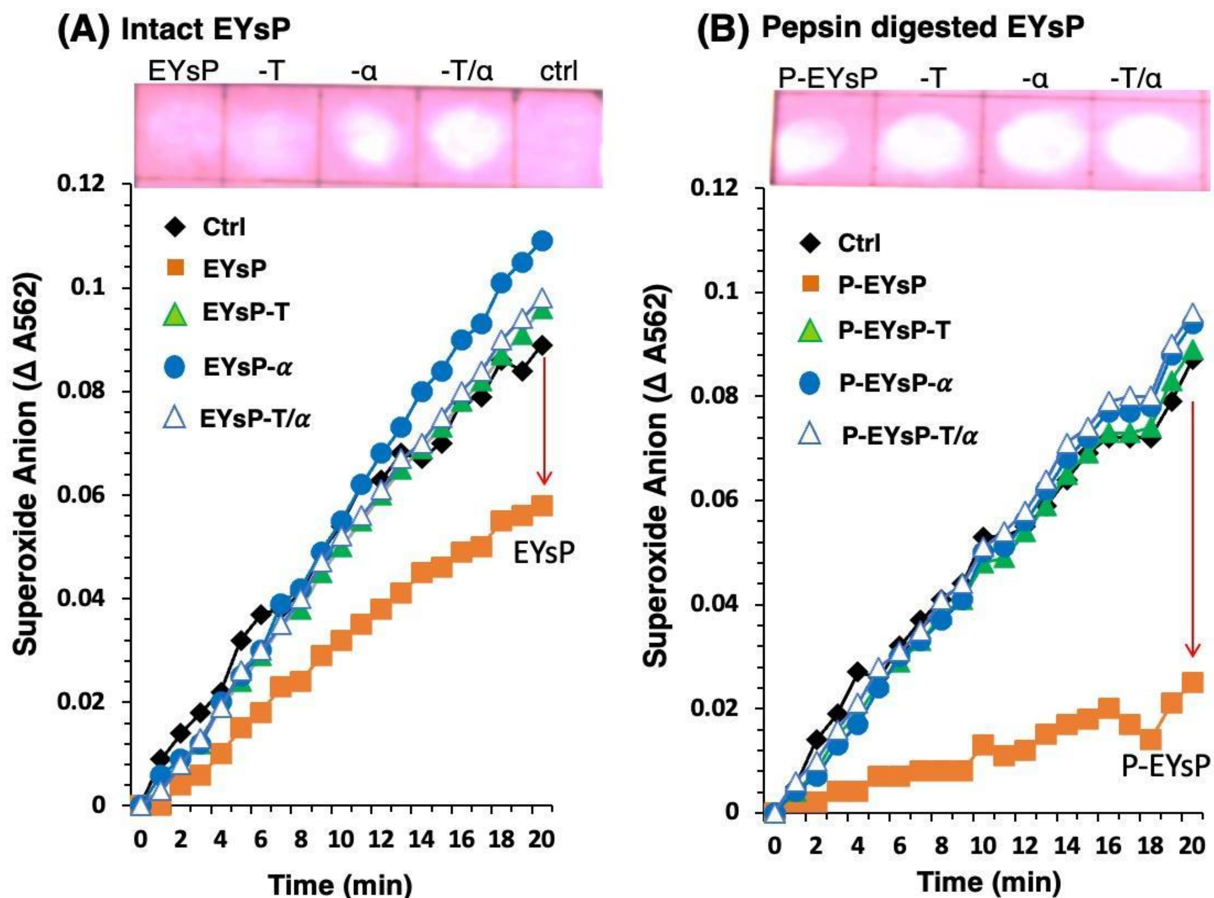


Figure 2. Superoxide-scavenging activities of EYsP (A) and P-EYsP (B) upon digestion with trypsin (-T), α-chymotrypsin (-α) or both enzymes (-T/α). The superoxide ($O_2^{\cdot-}$)-scavenging activity was measured in X/XOD/NBT reduction assay at 100 μg/mL protein. The rate of $O_2^{\cdot-}$ accumulation measured in real-time kinetics presented as the rate of absorbance change at 562 nm due to NBT reduction at 37˚C for 20 min. DPPH reduction of the fractions are shown above the panels.

Fractionation of the antioxidant peptides: Peptides in the most potent antioxidant peptic hydrolysate (P-EYsP) were separated into four fractions, designated F1-F4, using Sephacryl S-100 size-exclusion column (Figure 3A). The antioxidant activities of the fractions were examined by measuring the superoxide-scavenging activity (Figure 3B) and DPPH-reducing capacity (Figure 3C). The slow-eluting fractions F3 and F4 exhibited strong superoxide-scavenging activities while fast-eluting fractions F1 and F2 showed moderate and weak activities, respectively (Figure 3B). The fraction F4 showed potent DPPH-reducing capacity, and F3 showed moderate capacity in a time-dependent manner, while F1 and F2 had weak capacities (Figure 3C). All fractions F1-F4 showed DPPH reducing capacity in a dry-state assay, but F3 and F4 exhibited equally the highest DPPH reducing capacities (Figure 3C, upper TLC sheet), thus parallel that in the time-dependent assay (Figure 3C).

The most potent antioxidant peptic hydrolysate (P-EYsP) was further separated, using RP-HPLC with C18 column, into five peptide peaks, designated P1-P5 (Figure 4A). The superoxide scavenging activities of the peptide peaks were tested at a concentration of 40 μg/mL (Figure 4B). All peptide peaks showed stronger scavenging activity than the total hydrolysate (P-EYsP), indicating that peptic hydrolysis produces potent superoxide-scavenging peptides from EYsP. However, slow-eluting peptides P4 and P5, showed the strongest scavenging activities, while fast-eluting peptide peaks (P1, P2 and P3) exhibited moderate activities. Although all peptide peaks

exhibited DPPH-reducing capacities.P5, P2 and P1 showed stronger DPPH-reducing capacities than the total hydrolysate (P-EYsP) or P3 and P4 (Figure 4C). The results demonstrate that hydrophobic peptides (P5) are the most potent antioxidants with the ability to equally scavenge superoxide as well as DPPH radical. The results also indicate that the antioxidant peptides from EYsP exert their superoxide scavenging and DPPH-reducing activities through different mechanisms of action.

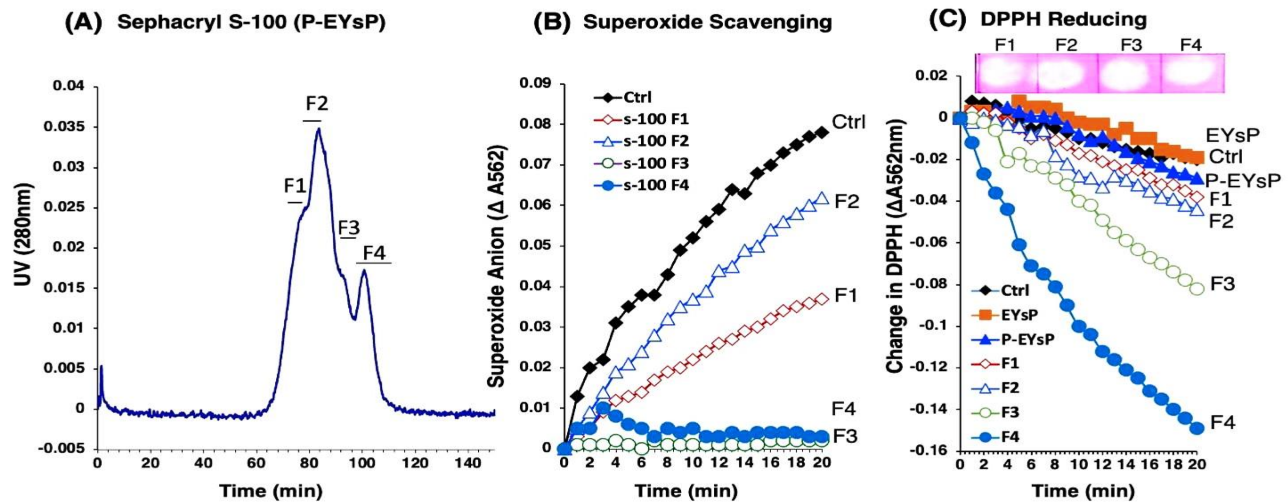


Figure 3. Size-exclusion chromatography pattern, on Sephacryl S-100 column, of the P-EYsP (A) and superoxide-scavenging (B) and DPPH-reducing capacities (C) of the peptide peaks. (A) Elution was monitored at 280 nm representing four peptide fractions (F1 ~ F4). (B) The accumulation rate of superoxide anions was measured in real-time kinetics assay and presented as the rate of absorbance change at 562 nm due to NBT reduction. (C) DPPH-reduction was assessed by monitoring the fading of the purple color kinetically for 20 min at 562 nm of DPPH solution. DPPH reduction in the dry-state on TLC-blot assay of the fractions on a silica gel TLC plate sprayed with DPPH solution is shown above the panels.

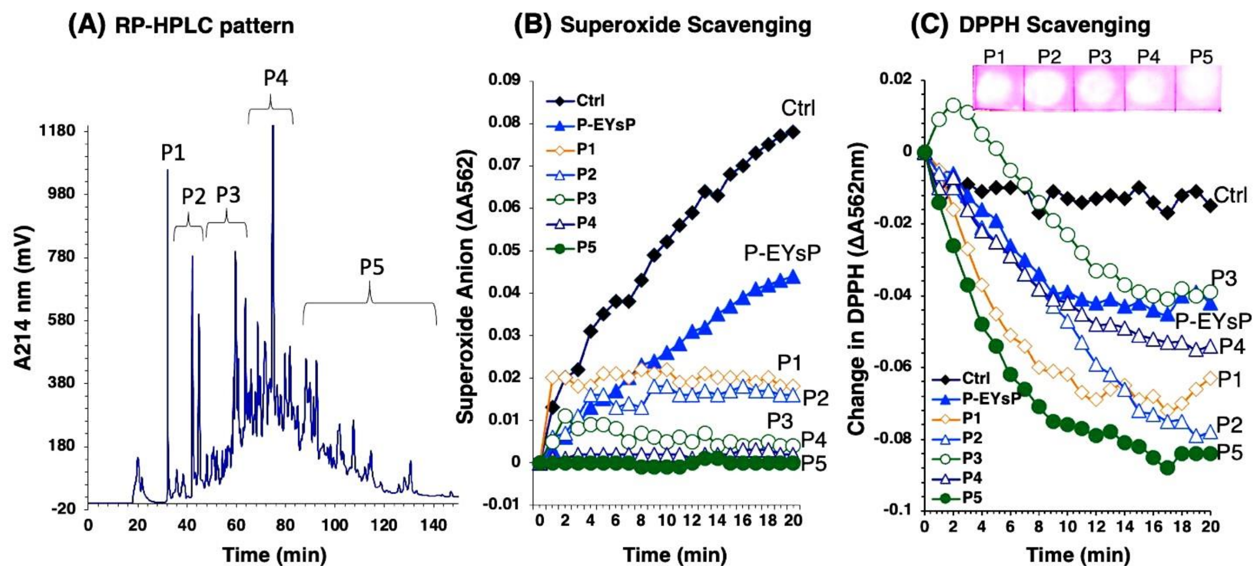


Figure 4. Reversed-phase HPLC pattern, on C18 column, of the P-EYsP (A), superoxide-scavenging (B), and DPPH-reducing capacities (C) of the peptide peaks. (A) Elution was monitored at 215 nm, and five peptide peaks (P1 ~ P5) were collected. The superoxide-scavenging (B) and DPPH-reducing (C) capacities are presented as in Figure 3.

Effect of peptides on the yeast cells tolerance against oxidative stress: The ability of the most active antioxidant peptides to enhance the tolerance of yeast cells against oxidative stress was examined. The yeast Saccharomyces cerevisiae cells were incubated with peptides for 24hr then exposed to the oxidative H_2O_2 (2 mM) for a further 24 hr. The mixture was spotted on agar plates containing 2 mM of H_2O_2 (Figure 5). The EYsP as well as its peptic hydrolysate (P-EYsP) exhibited a remarkable increase in yeast survival (log CFU/mL), with P-EYsP being more potent. The active antioxidant peptide fractions from Sephacryl S-100 (F3 and F4) showed higher survival than the control cells (Ctrl), whereas F4 was more efficient than F3. However, the active antioxidant peptide peaks from RP-HPLC (P4 and P5) were much more efficient than those from Sephacryl S-100, whereas peptides of P5 were the most potent in enhancing the tolerance of yeast cells against oxidative stress. Previously, it has been suggested that antioxidanttreatment forces a metabolic switch toward reduced ROS levels and enhanced survival in yeast mitochondria [30,31]. The results indicate that antioxidant peptides released from EYsP by pepsin may act as modulators of the redox state in the live cells and thus enhance tolerance against oxidative stress.

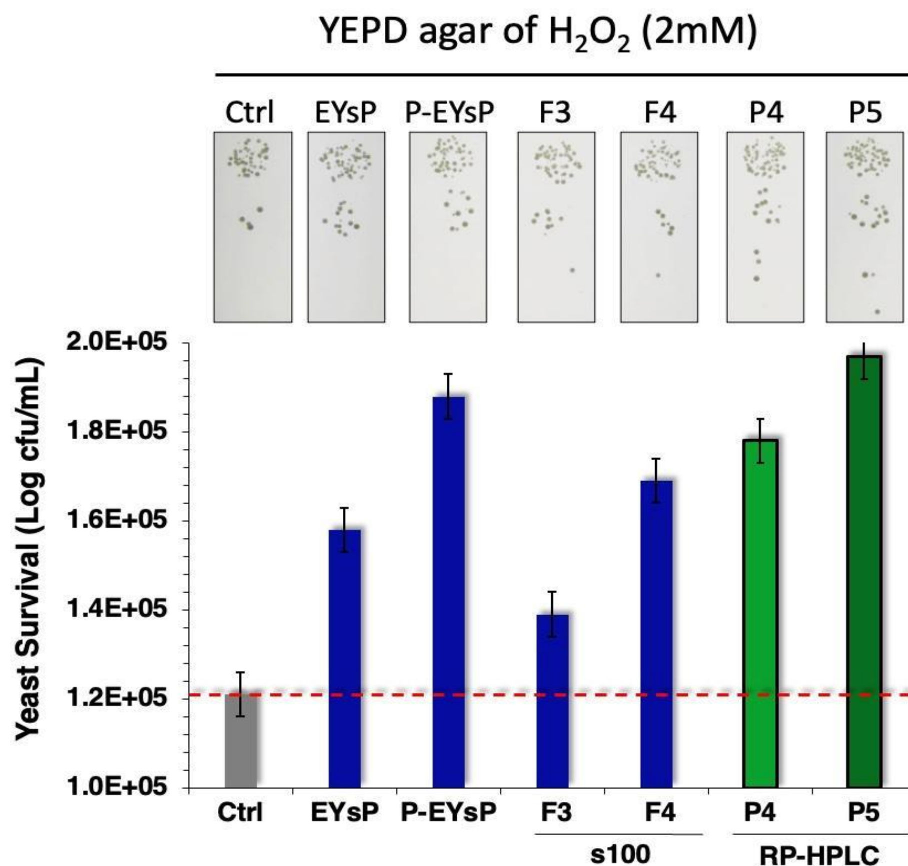


Figure 5. Yeast cells' tolerance against H_2O_2-induced oxidative stress in the presence of Sephacryl S-100 fractions (F3 and F4) and RP-HPLC peptide peaks (P4 and P5) of P-EYsP. Yeast cells were grown to exponential phase in YEPD broth, then treated with peptide peaks in YEPD broth containing H_2O_2 (2 mM) and shaken for 48 h at 28 °C. Cells were then serially diluted in YEPD broth containing H_2O_2 (2 mM) and spotted onto YEPD agar plates containing 2 mM H_2O_2. Plates were incubated at 28 °C for 72 h (upper image), then colonies were counted to estimate yeast survival. Data are represented as log CFU/mL.

Identification of the active peptide: The peptides in P5 of RP-HPLC were found to be the most potent antioxidant that equally scavenges superoxide and DPPH radical (Figure 4) and is able to enhance the yeast tolerance against oxidative stress (Figure 5). Five peptides, in the active peak P5 of RP-HPLC, with masses ranging from 859.28 to 1089.66 m/z (Figure 6 and Table 1) were identified using MALDI-TOF-TOF analysis. The major peptides were two that originated from either lipovitellin or yolk glycoprotein-40 (YGP40) and one peptide from livetin-2. The main peptides with the highest intensity were ones with masses of 901.06 m/z originating from the soluble lipovitellin [32], VSAYLKIL, and one with masses of 883.22 m/z originating from YGP40, LHRSFVK (Table 1).

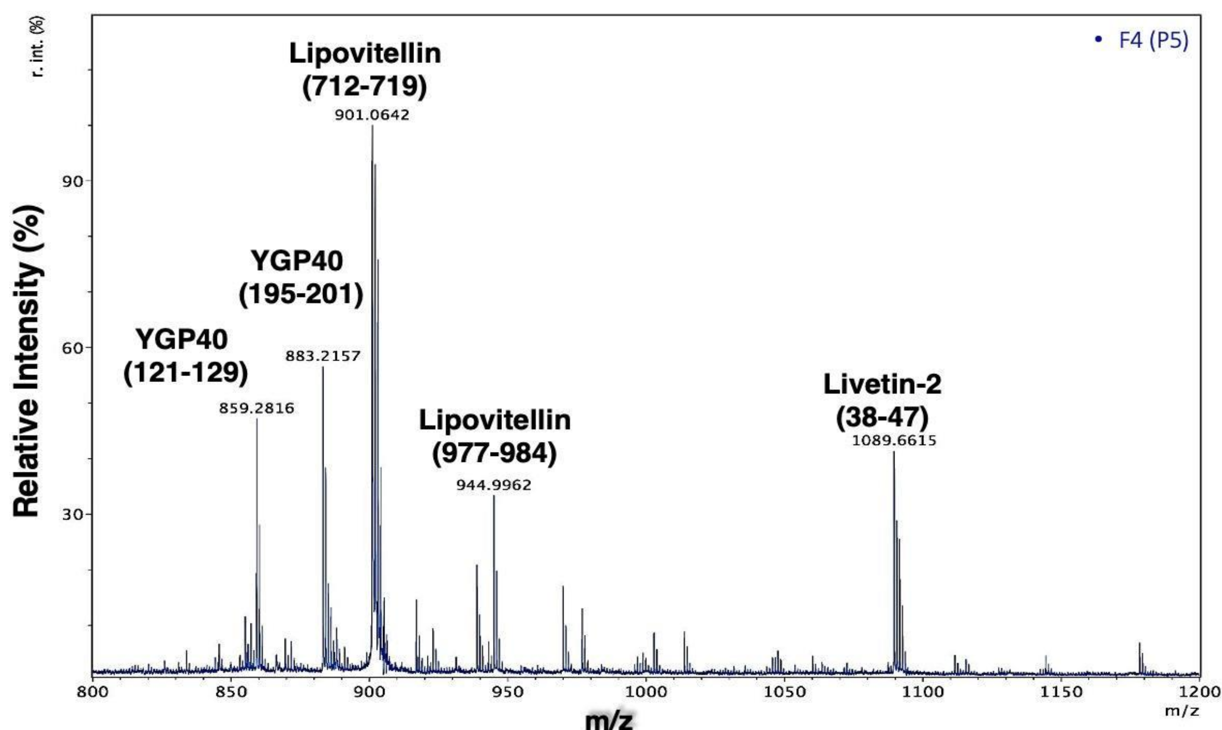


Figure 6. MALDI-TOF mass spectra of peptide peak 5 (P5) from RP-HPLC. Details about peptide sequences are shown in Table 1, whereas the origin of the peptides within their parent proteins is provided. The sequence of the peptides was obtained by de novo sequencing protocol of the fragments as assigned to protein BNBI database.

Table 1. Peptides identified by MALDI-TOF/MS in the RP-HPLC active peak P5.

TOF-MS (Da)	Calculated mass (Da)	Sequence*	Relative Intensity (%)	Protein Identity (fragment)
859.28	864.43	AAPGHGIDK	47.22	YGP40 (f 121-129)
883.22	884.52	LHRSFVK	56.91	YGP40 (f 195-201)
901.06	905.56	VSAYLKIL	100.00	Lipovitellin-1, 2 (f 712-719)
945.00	951.44	KMHHIGCQ	33.08	Lipovitellin-1, 2 (f 977-984)
1089.66	1089.26	PKAPFSEVSK	41.79	Livetin-2 (f 38-47)

*Peptide sequences were identified through the interpretation of the ion series in MALDI-TOF/MS.
The major peptide is shown in bold letters. YGP40, Yolk plasma GlycoProtein 40.

DISCUSSION

Oxidative stress is associated with many chronic diseases, including rheumatoid arthritis, cardiovascular diseases, chronic inflammation, diabetics, Alzheimer, and cancers [33]. Antioxidants act to scavenge radicals for prevention of the oxidative stress and associated diseases. The radical scavenging effect of small molecules represents a limitation in antioxidant defense because radicals react with almost all small molecules. One promising way to achieve an antioxidant role is to inhibit the generation of oxidants such as H_2O_2 or eliminate its signaling [34]. Hence it is highly rewarding to search for macromolecules as antioxidants with low toxicity that possess nutritional and natural physiological functions in biological systems.

Egg is a natural source of bioactive proteins and peptides [35,36]. Although bioactive peptides from egg white proteins have been intensively described, the egg yolk proteins acting as precursors of bioactive peptides are still less characterized. Egg yolk contains many vital proteins with important biological roles in embryonic process [4,12,36]. Egg yolk is composed of granules suspended in an aqueous phase called plasma. The proteins of egg yolk are 68% low-density lipoproteins (lipovitellins), 12% high-density lipoproteins (HLD), 12% livetins, and 7% phosvitin. The aqueous phase, plasma, contains 85% lipovitellins besides 15% livetins and 40 kDa glycoprotein (YGP40), which are lipid-free proteins. Livetins and YGP40, as well as number of lipovitellin forms, are water-soluble proteins. Surprisingly, the aqueous phase has not been studied intensively, although it is responsible for most of the physical properties of yolk. The components of yolk are difficult to isolate, however they can be fractionated into granules (pellet) and plasma (supernatant) by dilution and centrifugation [37]. This study is focused on the soluble protein fraction, which could be obtained from egg yolk by dilution, acidification, and centrifugation, as shown in Scheme 1. The soluble protein fraction showed a distinct electrophoretic protein pattern in the range of 31-122 kDa (Figure 1). The isolated protein fraction shows the presence of 7 bands, whereas 4 are major protein bands with high intensity, YGP40 (42 kDa), β-phosvitin (59 kDa), α-livetin (73 kDa), and apovitellenin Va (122 kDa) and less intense 3 bands, two β-livetin forms (31 and 35 kDa) and apovitellin 5-6 (78 kDa)[8]. The results confirm that these 7 proteins correspond to the soluble fraction of egg yolk proteins.

The soluble egg yolk proteins EYsP exhibited strong superoxide and DPPH scavenging activities, and pepsin digestion greatly increased these activities (Figure 2). Fractionation of the peptic hydrolysate through size-exclusion chromatography and RP-HPLC further produced several peptide fractions with various antioxidant activities. Among the fractions, peak number 5 (P5) from RP-HPLC possessed the strongest radical scavenging activity (Figure 3 and 4) compared to other fractions, which were enriched in peptides with molecular masses ranging from 864 to 1089 Da (Figure 6). In addition, P5 slowly eluted from the C18 column in RP-HPLC exerted higher protection of yeast cells against oxidative stress induced by H_2O_2 (Figure 5). The antioxidant activity of a peptide is related to its amino acid sequence and composition [23–25]. MALDI-TOF/MS analysis identified five peptides in P5 of RP-HPLC (Figure 6). The peptides were two from lipovitellin, two from YGP40, and one from livetin domain 2. Among the five peptides, one peptide (VSAYLKIL) originated. Lipovitellin was the most dominant at 100% intensity (Table 1). It has been demonstrated that the amino acids Tyr, Lys, Leu, Ile, Val, His, Pro, and Ser in peptide sequence are responsible for antioxidant properties [38]. These amino acids have the ability to donate electrons, serving as hydrogen donors, to inactivate the radical. The dominant peptide

(VSAYLKIL) from lipovitellin is composed of a group of these amino acids, particularly possessing hydrophobic residues (Val and Leu) at both N- and C-terminals. Although lipovitellin is a constituent of egg yolk granules, it is not surprising that the main peptide (VSAYLKIL) is derived from lipovitellin because a class of lipovitellin is unusually found to be soluble lipoprotein in yolk [32].

Vitellogenin is an egg yolk precursor that makes up lipoproteins and phosphoproteins containing four domains, lipovitellin-1, lipovitellin-2, phosvitin, and a peptide of about 40 kDa named YGP40. These domains are produced after intra-oocytic proteolytic processing by cathepsin D, an endogenous aspartic protease [39]. Upon limited proteolysis of lipovitellin by cathepsin D, the YGP40 dissociates from the lipovitellin-phosvitin complex in the oocyte and is released into the yolk plasma during the localization of the complex into the yolk granule (see supplementary material, Figure S1). Interestingly, cathepsin D is an aspartyl protease of the pepsin family, also detected in whole yolk prepared from growing oocytes [40]. This study revealed that proteolysis of EYsP with pepsin at pH 3.0, an aspartyl protease, generated peptides with the strongest scavenging abilities for superoxide anions and DPPH. The results highlight, for the first time, the importance of cathepsin D in triggering the biological roles of egg yolk proteins. Furthermore, the newly found peptides in this study which originated from YGP40 and livetin, were not reported so far in the previous study.

Despite the health benefits and preventive effects of bioactive peptides against oxidative stress-associated diseases, there are growing concerns about their bioavailability and intestinal absorption. The peptides must be absorbed into blood circulation through the intestinal barrier to exert their physiological effects. In this study, pepsin completely hydrolyzed all proteins of EYsP into small peptides with molecular masses of less than 6 kDa. Upon oral administration, peptide absorption is unequally distributed throughout the digestive tract. In general, the proximal portions of the small intestine, the duodenum and jejunum, are the major site for small peptide absorption [41]. This intestinal uptake process involves a proton-driven transmembrane protein called peptide transporter 1, PepT1[3,42]. The activity of PepT1 is mostly located in the proximal small intestine, the duodenum and jejunum [42]. Accordingly, the absorbance of a large portion of the pepsin derived peptides (P-EYsP) of EYsP is expected to take place in the proximal small intestine, duodenum, before exposure to trypsin or α-chymotrypsin whose actions abolish the antioxidant activities as demonstrated in Figure 2. However, the *in vitro* assays performed in this study cannot accurately predict the bioavailability and antioxidant performance of these peptides *in vivo*. Therefore, intestinal absorption and antioxidant activities *in vivo*, in animals and humans of the pepsin hydrolysate P-EYsP and its derived antioxidant peptides would merit further investigation to explore in depth their health benefits as bioactive peptides.

CONCLUSIONS

Our data highlights that aqueous phase proteins of hen egg yolk represent a promising source of bioactive peptides with potent antioxidant activities. Although peptic hydrolysis of the soluble egg yolk proteins produced a larger number of peptides with variable antioxidant potencies, slow-eluting peptide fractions (hydrophobic peptides) from RP-HPLC exhibited higher superoxide scavenging potencies. Our isolation procedure proved to produce peptides with over 8 times higher superoxide-scavenging activity or DPPH-reducing capacity. Using the yeast cells model, the biological activity of the peptides as antioxidants was validated. The active peptides were derived from lipovitellin (VSAYLKIL

and KMHHIGCQ), YGP40 (LHRSFVK and AAPGHGIDK), and livetin-2 (PKAPFSEVSK), with VSAYLKIL peptide being the dominate peptide in the most antioxidant fraction. It has been demonstrated, for the first time, that livetin and YGP40 possess potent antioxidant peptides. Further clinical studies of these peptides focusing on human intestinal cell absorbance and in vivo biological activity will be highly rewarding. These findings suggested that soluble proteins of egg yolk contain potent bioactive peptides that could potentially contribute to advancing Functional foods applications and therapeutics with the aim of ameliorating oxidative stress and related diseases. Supplementary Materials: Figure S1: Amino acid sequences of three proteins in egg yolk from which antioxidant peptides originated.

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Abbreviations:

EYsP: egg yolk soluble protein

P-EYsP: EYsP digested with pepsin.

P-EYsP-T: P-EYsP digested with trypsin.

P-EYsP-α: P-EYsP digested with α-chymotrypsin.

P-EYsP-T/α: P-EYsP digested with both trypsin and α-chymotrypsin.

YGP40: yolk plasma glycoprotein-40

MALDI-TOF-MS: Matrix-assisted laser desorption ionization time of flight mass spectrometry

RP-HPLC: reversed-phase high performance liquid chromatography.

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