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# Multifunctional peptides from egg white lysozyme

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ABSTRACT

Hen's egg white lysozyme (HEWL) is one of the major egg white proteins with well demonstrated antimicrobial activity. Bioactive peptides other than antimicrobial peptides from HEWL have not been reported; therefore, the purpose of the study was to explore new bioactivities of lysozyme-derived bioactive peptides. HEWL was hydrolysed with Alcalase and fractionated by cation-exchange chromatography. The Alcalase HEWL hydrolysate and its fractions were analyzed for inhibitory activities against calmodulin-dependent phosphodiesterase (CaMPDE) and antioxidant activities using oxygen radical absorbance capacity-fluorescein (ORAC-FL), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\cdot+}$) and 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) radical scavenging methods. The fractionated peptides had higher CaMPDE inhibition activity, ORAC-FL value and $ABTS^{\cdot+}$ scavenging activity than those of the hydrolysate. Peptide sequences in the most overall active fractions were characterized by LC–MS/MS. Our results showed that HEWL hydrolysate and its peptide fractions may serve as useful ingredients in the formulation of functional foods and nutraceuticals.



## 1. Introduction

Lysozyme is a basic protein consisting of 129 amino acids and has a molecular weight of 14.3 kDa and an isoelectric point of 10.7. Hen's egg is the richest source of lysozyme, accounting for 3.5% of total egg white proteins. As a well-known antimicrobial protein, hen's egg white lysozyme (HEWL) has been commercialized for applications as a natural preservative to control bacteria in meat products such as sausage, salami, pork, beef, or turkey (Hughey, Wilger, & Johnson, 1989), to prevent the growth of *Clostridium tyrobutyricum* in cheese making (Proctor & Cunningham, 1988), or to control lactic acid bacteria such as *Lactobacillus* spp. in wine and beer production (Daeschel, Bruslind, & Clawson, 1999). It can also be used in other pharmaceutical applications. It is estimated that over 100 tons of lysozyme is used each year for these purposes (Tenuovo, 2002).

Interestingly, recent studies showed that lysozyme denatured by heating shows improved antimicrobial activity (Ibrahim et al., 1996); it has been clearly demonstrated that the polymerization of lysozyme during heating treatment is the reason for its broader spectrum of antimicrobial activity. Moreover, peptides released from lysozyme's primary sequence by protease digestion displayed bacteriostatic activity independent of its enzymatic activity (Ibrahim, Thomas, & Pellegrini, 2001; Mine, Ma, & Lauriau, 2004). Lysozyme peptide preparation, produced from the protein lysozyme by partial enzymatic hydrolysis using pepsin, is commercially available as a natural food preservative (Abdou, Higashiguchi, Abouelenin, Kim, & Ibrahim, 2007). It seems that the bactericidal potency of lysozyme is not only due to its muramidase activity, which amounts to only about 11% of the lytic activity of the whole lysozyme protein, but is also due to its cationic and hydrophobic properties (Pellegrini et al., 1997). Lysozyme-derived peptides other than antimicrobial peptides have not been reported in literature.

Food protein-derived bioactive peptides are believed to have various bioactivities. Peptides with antihypertensive activity and/or antioxidant have been extensively studied for prevention of high blood pressure, a well-defined risk factor for cardiovascular disease (CVD). CVD is the leading cause of death in developed countries. Moreover, the onset and development of CVD is also related to oxidative stress in cells caused by excessive production of free radicals and causally involved in the atherosclerotic process (Moosmann & Behl, 2002; Touyz, 2004).

Cyclic nucleotide phosphodiesterase (PDE) is a key enzyme that controls the contraction and relaxation of vascular smooth muscle and cardiac myocytes via second messengers ($Ca^{2+}$ and cAMP), which are assigned for signal transduction (Omori & Kotera, 2007). The $Ca^{2+}$-binding protein calmodulin (CaM) is a multifunctional protein that may exert an influence on the enzyme activity and different cellular processes (Klee, Crouch, & Richman, 1980). Disturbance in the component of the signaling pathway could lead to disease progression such as development of cancer or hypertension related to

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$Ca^{2+}$-induced changes in membrane functions (Lal, Raju, & Sharma, 1998). CaM-dependent cyclic nucleotide phosphodiesterase (CaMPDE) can be activated by simultaneous presence of both $Ca^{2+}$ and CaM which are present in physiological concentrations; however, antagonists of $Ca^{2+}$ and CaM may act as a CaMPDE inhibitor thus reducing disease progression (Kakkar, Raju, & Sharma, 1999). Since CaM is negatively charged at physiological pH, it was indicated that peptides containing positively charged amino acids is an important structural feature of CaMPDE inhibitors (Itano, Itano, & Penniston, 1980; Weiss, Prozialeck, & Wallace, 1982). Previous structure and activity studies of bioactive peptides also indicated the importance of positively charged amino acids in determining the potency of peptides as CaMPDE inhibitors and antioxidants (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998; Li & Aluko, 2005). Lysozyme has an isoelectric point of 10.7 and it contains a high content of positively charged amino acids; therefore, lysozyme may be a great substrate for production of bioactive peptides with multifunctional activities indicated above. Moreover, many researchers have reported that peptides and protein hydrolysates from various food sources have significant antioxidant activity (Zhu, Chen, Tang, & Xiong, 2008). Also HEWL has been shown to suppress reactive oxygen species (ROS) generation and protect against acute and chronic oxidant injury (Liu et al., 2006). Since there are potential health risks associated with the use of synthetic antioxidants (BHA and BHT) in foods, natural antioxidants such as ascorbate, catechin, tocopherols, peptides and phenolic compounds, are extensively studied as alternatives to the synthetic ones. Therefore, peptidic antioxidants also could be attractive to use in the food industry and pharmaceutical applications.

The objectives of the study were to determine if lysozyme-derived peptides have in vitro inhibitory activities against CaMPDE in addition to antioxidant activity, and to elucidate the major peptide sequences in the active fractions.

## 2. Materials and methods

### 2.1. Materials

HEWL was obtained from Neova™ Technologies (Abbotsford, BC, Canada). Fluorescein and Trolox® were obtained from Acros Organics (Morris Plains, NJ, USA). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH), Alcalase (EC 3.4.21.14; from *Bacillus licheniformis*), aprotinin, glycine, $(glycine)_3$, potassium persulfate, Vitamin $B_{12}$, were obtained from Sigma–Aldrich® (Oakville, ON, Canada).

### 2.2. Preparation of HEWL hydrolysate

HEWL was prepared into 5% (w/v) slurry in water. After heating the sample slurry at 80 °C for 10 min, the temperature was adjusted to 50 °C by putting the sample into an ice bucket; the slurry was then adjusted to pH 7.5 by adding 1 M NaOH. The pH of the slurry was maintained constant during digestion using a Titrando® instrument (Metrohm, Herisan, Switzerland) and the temperature of the slurry was maintained constant by using jacketed beaker with circulating water bath. Alcalase (0.5%, w/w, on the basis of protein content of slurry) was added and the slurry was incubated for 3 h; the hydrolysis was terminated by holding the slurry at 95 °C for 10 min. After centrifugation at 10,000*g* for 25 min, a portion of the supernatant was collected and freeze-dried while the remaining portion of the supernatant was passed through a 3 kDa molecular weight (MW) cut-off membrane to prepare the ultrafiltration permeate of lysozyme hydrolysate (UPLH).

### 2.3. Ion-exchange chromatography

Ion-exchange chromatography was carried out on a AKTAexplorer 10XT system (GE Healthcare, Uppsala, Sweden) using a cation-exchange column (16 × 100 mm, HiPrep 16/10 SP FF, GE Healthcare). The freeze-dried UPLH was dissolved in 0.01 M ammonium acetate buffer (100 mg/ml), filtered through 0.2 μm membrane disk and loaded onto the above column using an injection volume of 4 ml. Elution was achieved by a linear gradient of 0.5 M ammonium carbonate in ammonium acetate buffer at a flow rate of 5 ml/min and monitored at 280 nm. The chromatogram was then obtained by using UNICORN 5.11 software (GE Healthcare). Each fraction was pooled and freeze-dried for further use.

### 2.4. Size exclusion chromatography

Molecular weight (MW) distribution of samples was analyzed by a Superdex peptide 10/300 GL column coupled with an AKTAexplorer 10XT system (GE Healthcare, Uppsala, Sweden). Each sample was dissolved in 30% aqueous acetonitrile containing 0.1% trifloroacetic acid (TFA), at a concentration of 0.5 mg/ml. A 100 μl aliquot was injected onto the column and eluted with the aqueous acetonitrile solution; fractions were monitored at 215 nm. Peptide molecular masses were determined by reference to a calibration curve created by running molecular mass markers on the Superdex peptide 10/300 GL column under identical running conditions to samples.

### 2.5. Oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay

ORAC-FL assay was performed according to Ou, Hampsch-Woodill, and Prior (2001) as modified by Dávalos, Miguel, Bartolomé, and López-Fandiño (2004). Briefly, the final assay mixture (150 μl) contained fluorescein (200 nM), and an antioxidant [Trolox (2–16 μM) as a standard or sample (at different concentrations)] in 75 mM phosphate buffer (pH 7.4) was transferred into a 96-well microplates (96F untreated, Nunc, Denmark) and incubated at 37 °C for 15 min. After mixing with 50 μl of AAPH (80 mM), the plate was automatically shaken before the first reading, and the fluorescence was recorded at 1 min intervals for 100 min. All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. Fluorescence measurements were normalized to the curve of the blank (no antioxidant). From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as followed:

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=100} f_i/f_0$$

where $f_0$ is the initial fluorescence reading at 0 min and $f_i$ is the fluorescence reading at time *i*. The net AUC of the sample was calculated by subtracting the AUC of blank. Regression equations obtained from net value of Trolox was used to calculate the ORAC value for each assay. Final ORAC values were expressed as micromoles of Trolox equivalent (TE) per mg of protein determined by the modified Lowry Protein Assay Kit (Pierce, USA) using bovine serum albumin as standard.

### 2.6. Radical-scavenging activity (RSA)

#### 2.6.1. Measurement of ABTS RSA

The analysis was performed using an $ABTS^{\cdot+}$ decolorization assay (Strube, Haenen, Berg, & Bast, 1997). To generate the ABTS radical cation ($ABTS^{\cdot+}$), the ABTS stock solution (7 mM) and potassium persulfate (2.45 mM) in phosphate buffer saline (PBS, pH 7.4) were allowed to stand for 16 h and the absorbance was 0.7 at 734 nm. RSA of samples was measured by mixing 100 μl of the samples