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Evaluation of *In-Vitro* Angiotensin Converting Enzyme-I Inhibitory Activity of Duck Egg Protein Hydrolysates and their Fractions

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ABSTRACT

The present study was conducted to extract ACE-I inhibitory peptides from duck egg using proteases from different sources viz. plant (papain and ficin), animal (trypsin) bacterial (Alcalase). Initially, the whole duck egg liquid was defatted with multiple washing with ethanol. The partially denatured whole duck egg proteins were subjected to hydrolysis using preoptimized conditions (enzyme substrate ratio, pH, temperature, incubation time) w.r.t. each enzyme. Four treatments viz duck egg hydrolysed with alcalase (DEA), duck egg hydrolysed with ficin (DEF), duck egg hydrolysed with papain (DEP) and duck egg hydrolysed with trypsin (DET) were prepared. The collected respective hydrolysates were fractionated using ultrafiltration to obtain different fractions on the basis of molecular weight (kilo dalton*) viz.* whole: DEPH, >10 kDa: DEPH-1, 5-10 kDa: DEPH-2, 1-5 kDa: DEPH-3 and <1 kDa: DEPH-4. The whole hydrolysates and their fractions were evaluated for their ACE-I inhibitory activity under *in-vitro* condition. Amongst all the hydrolysates and their fractions, the ACE-I inhibitory activity of DEPH were significantly ($p<0.05$) higher than their respective fractions and DEPH of DEP exhibited the highest activity. However, all the fractions displayed varied ($p<0.05$) ACE-I inhibitory activity with each other. Results suggested that the duck egg protein hydrolysates and their fractions have strong antihypertensive activity which can be exploited to develop nutraceuticals or functional foods.

Keywords: ACE-I inhibitory peptides, , Duck eggs, Ultrafiltration, Enzymatic hydrolysis

According to WHO (2019), raised blood pressure/ hypertension is estimated to cause 7.5 million deaths, accounts for 12.8% of the total deaths worldwide and appraised for 57 million disability adjusted life years (DALYS) or 3.7% of total DALYS. Angiotensin converting enzyme-I (ACE-I) (EC 3.4.15.1) is the key enzyme in the regulation of blood pressure and electrolyte homeostasis of the body (Balti *et al*., 2015). ACE is a dipeptidyl carboxy peptidase and its inhibition is largely related to two types of blood pressure systems, the RAS (renin–angiotensin system) and NOS (nitric oxide system) (Lee and Hur, 2017). ACE inhibitors can exert antihypertensive effects by decreasing the formation of Angiotensin II and degradation of bradykinin/vasoldilator. ACE inhibitors effectively prevent heart failure, independent of blood pressure values and considered better than calcium channel blockers commonly used to regulate heart muscle movement (Sur *et al*., 2011).

Protein chains remain encrypted for different functions, till they are broken down into short chain 2-25 amino acids bioactive peptides (Kitts and Weiler, 2003; Korhonen and Pihlanto 2006). The functionalities of the peptides are directed by their inherent amino acid composition and sequence (Meisel and Fitzgerald, 2003). ACE inhibitory peptides have been found in enzymatic hydrolysates of many foodstuffs, such as casein (Kumar *et al*., 2016), whey (Pan *et al*., 2012), eggs (Zhipeng *et al*., 2011; Eckert *et al*., 2014), tuna backbone protein, porcine muscle, shark meat, clam, soy protein (Lo and Li-Chan 2005) and many other food proteins (Balti *et al*., 2015).

The food derived ACE inhibitory peptides are less potent than the synthetic drugs such as captopril and enalapril, but could be effective in maintaining blood pressure at a healthy level (Eckert *et al*., 2014). ACE inhibitory peptides are mostly short chain with 2 to 12 amino acids residues

mostly carrying polar amino acid residues such as proline (Milan *et al*., 2014).

Various functional and biological activities of egg proteins make them potential precursor for the production of bioactive peptides (Zambrowicz *et al*., 2015; Liu *et al*., 2017). Enzymatic hydrolysis is recognized as the effective technique to develop bioactive peptides from egg proteins (Liao *et al*., 2018). The high quality of duck egg proteins attributed to high biological value and essential amino acids makes it a potent candidate for the development of multifunctional bioactive peptides. The membrane technology especially ultrafiletration with molecular weight cut offs (MWCOs) membrane is considered as convenient and rapid method to segregate as retentate unhydrolyzed proteins, proteases and large protein segments from protein hydrolysates whereas, known range of molecular weight peptides as permeate. (Liu *et al*., 2017). Milan *et al*., 2014 documented spectrophotometric and high performance liquid chromatography (HPLC) as common methods for evaluation of ACE inhibitory activity of bioactive peptides under *in vitro* conditions. The scanty literature is available on the duck egg bioactive peptides with ACE inhibitory activities. Therefore, the present study was designed to extract and evaluate the ACE inhibitory activity of duck egg derived protein hydrolysates and their fractions under *in-vitro* conditions.

MATERIALS AND METHODS

Sources of materials

Duck eggs

Fresh duck eggs (10 no.) of Khaki Campbell (weighing around 50-60 g) were procured from farmers in and around Ludhiana. The duck eggs having protein percentage more than 18% (on weight basis) were selected for the study. The eggs were broken manually, egg liquid was blended and packed in LDPE pouches and stored in frozen condition (-18±1˚C) for further use.

Chemicals and reagents

Enzyme Papain (E.C. 3.4.22.2, activity ≥10 units/mg protein), Ficin (E.C. 3.4.22.3, activity ≥200 units/g protein) and Trypsin (E.C. 3.4.21.4, activity >250 USP units/mg protein), were procured from Hi-media Laboratories Private Limited, Mumbai India, and MP Biomedicals, France, respectively and Alcalase (EC3.4.21.62, activity > 5 units/g protein) and ACE (EC 3.4.15.1 >2.0 units/mg protein) were procured from Sigma-Aldrich Chemical Co., USA. Analytical grade chemicals were procured from reputed firms like SRL, Fisher Scientific, LobaChemie, MP Bio-Medicals, Hi-Media and Sigma-Aldrich etc.

Enzymatic hydrolysis of duck eggs (DE)

The enzymatic hydrolysis of duck egg proteins were carried out following the method described by Kumar *et al.* (2016). Four different enzymes (Alcalase, Ficin, Papain and Trypsin) were used for the hydrolysis of duck egg proteins. The egg liquid was defatted by repeated (4-5) washing with 95% ethanol. The defatted egg liquid was hydrolyzed by incubating in a stirred water bath maintained at constant temperature (55˚C for Alcalase and Papain; 50˚C for Ficin and 37˚C for Trypsin) for 6 h (Table 1). Each hydrolyzed samples were immediately heated to 85˚C for 15 minutes in water bath to terminate any further enzymatic activity. The samples was then cooled and centrifuged in a refrigerated centrifuge (Thermo Scientific, Fiberlite F21-8x50y Rotor, USA) at 11200 g for 25 min. The supernatant was collected and stored at -20˚C in sterilized polycarbonated sample bottle for further use.

Fractionation of duck egg protein hydrolysates

The duck egg protein hydrolysates were ultra-filtered sequentially in Millipore 8400 ultrafiltration unit (Amicon, Millipore, USA) using cellulose membranes (Diameter 76 mm, Amicon Bioseparations, USA with different molecular weight (MW) limits (Fig. 1). Briefly, the hydrolysates were first ultra-filtered through a membrane with 10 kDa nominal molecular weight limit (NMWL) under 40 psi pressure of nitrogen gas. The process yielded two fractions i.e. retentate (>10 kDa) and permeate (<10 kDa). Further, ultrafiltration of permeate was done through a 5 kDa NMWL membrane so as to obtain the second retentate i.e. (5-10 kDa) and permeate (<5 kDa). Subsequently, the second permeate was ultra-filtered through a 1 kDa NMWL membrane to yield the third retentate (1-5 kDa) and permeate (<1 kDa). All the retentates and permeates were collected and stored at -20ºC until further use.

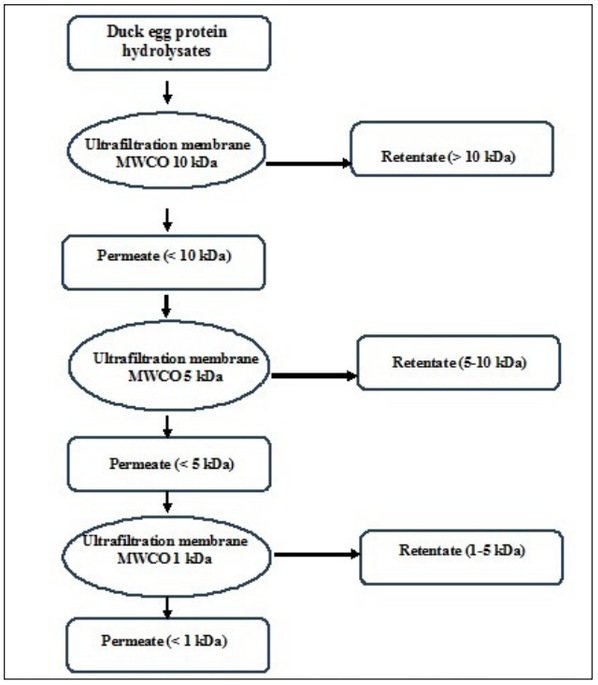

Fig. 1: Flow chart of sequential ultra-filtration protocol of duck egg protein hydrolysates using cellulose membranes with different molecular weight cut off (MWCO) limits

Table 1: Optimum conditions for enzymatic hydrolysis of duck egg protein (DE)

Enzyme	E:S ratio	pH	Buffer	Temperature (°C)
Alcalase	1:100	8.0	Phosphate buffer	55°C
Ficin	1:100	5.5	Phosphate buffer	50
Papain	1:100	6.5	Phosphate buffer	55
Trypsin	1:100	8.0	Phosphate buffer	37

Determination of ACE-inhibitory activity

ACE (EC 3.4.15.1) inhibitory activity was determined *in-vitro* using spectrophotometric method of Cushman and Cheung (1971) modified by Miguel *et al*. (2004). Briefly, 20 µL of the hydrolysate solution was mixed with 110 µL Hippuryl-His-Leu (HHL) substrate solution (5 mM HHL in 0.1 M borate buffer containing 0.3 M sodium chloride, pH 8.3). The reaction was initiated by the addition of 20 µL (4 mU) of ACE solution and then incubated at 37°C for 30 min. The enzymatic reaction was terminated by adding 40 µL of 1 M HCl. The hippuric acid formed was extracted using 1 ml of ethyl acetate by centrifugation at 3000g for 10 min. An aliquot of 750 µL of the upper organic layer was collected and transferred into a test tube; dried out completely under 600 mmHg vacuum at 50 ± 2°C and re-dissolved in 1ml of distilled water. The absorbance was measured spectrophotometrically at λ = 228 nm. All samples were tested in 3 replications. Double distilled water was used in place of hydrolysate solution as a positive control. Blank was prepared by adding only substrate and water. The results were calculated and expressed as the peptide concentration required to inhibit 50% of the original ACE activity.

$$\% \text{ ACE} = [(\text{Control- Sample}) / (\text{Control-Blank})] \times 100$$

STATISTICAL ANALYSIS

The experiments was repeated three times and all the parameters were analyzed in triplicate (n=9). The data were analyzed using SPSS software (Version 20.0 for Windows; IBM SPSS Inc, Chicago, 111, USA) and expressed as mean with standard error. Two way analysis of variance (ANOVA) was done by comparing the means using Duncan's multiple range test at 95% confidence level (Steel and Torrie, 1981).

RESULTS AND DISCUSSION

Enzymatic hydrolysis and fractionation

The hydrolysates of duck egg proteins extracted at 6 h incubation time using different enzymes *viz*. Trypsin (DET), Papain (DEP), Ficin (DEF) and Alcalase (DEA) with DH of 23.06±0.18%, 20.85±0.12%, 15.42±0.09% and 14.24±0.08%, respectively were fractionated using ultrafiltration to obtain different fractions viz. whole: DEPH, >10 kDa: DEPH-1, 5-10 kDa: DEPH-2, 1-5 kDa: DEPH-3 and <1 kDa: DEPH-4.

ACE inhibitory activity of duck egg protein hydrolysates and its fractions

Angiotensin-converting enzyme (ACE) plays a central role in blood pressure regulation and inhibition of ACE is a major target in the prevention of hypertension. All the hydrolysates and their fractions showed ACE inhibitory activity and statistically analyzed data are presented in Table 2.

Table 2: ACE-I inhibitory activity of duck egg protein hydrolysates and its fractions (Mean ± SE)

Fractions	ACE inhibition (%)			
	DEA	DEF	DEP	DET
DEPH	37.65±0.03^{aE}	57.43±0.03^{cD}	57.60±0.03^{cE}	54.11±0.02^{bD}
DEPH-1	27.40±0.03^{aA}	45.92±0.04^{dC}	36.37±0.03^{cA}	35.37±0.04^{bA}
DEPH-2	36.90±0.02^{bD}	35.37±0.02^{aAB}	37.28±0.03^{bB}	45.36±0.06^{cB}
DEPH-3	28.86±0.02^{aB}	36.53±0.03^{cB}	41.11±0.03^{dC}	34.97±0.06^{bA}
DEPH-4	31.61±0.02^{aC}	34.57±0.03^{bA}	51.60±0.04^{dD}	48.54±0.02^{cC}

Mean ± SE values bearing different superscripts row-wise (small alphabets) and column wise (capital alphabets) and differ significantly ($p<0.05$) n=9

Whole hydrolysate: DEPH, >10 kDa: DEPH-1, 5-10 kDa: DEPH-2, 1-5 kDa: DEPH-3 and <1 kDa: DEPH-4; DEA: Duck egg protein hydrolyzed with Alcalase; DEF: Duck egg protein hydrolyzed with Ficin; DEP: Duck egg protein hydrolyzed with Papain; DET: Duck egg protein hydrolyzed with Trypsin.

Amongst the fractions of DET, the DEPH exhibited significantly ($p<0.05$) higher ACE inhibitory activity (54.11±0.02) followed by DEPH-4, DEPH-2, DEPH-1 and DEPH-3. For the group DEP, significantly ($p<0.05$) higher inhibition was recorded for DEPH (57.60±0.03) followed by DEPH-4, DEPH-2, DEPH-3 and DEPH-1. In the group DEF, the highest inhibitory activity was exhibited by DEPH (57.43±0.03) followed by DEPH-1, DEPH-3, DEPH-2 and DEPH-4. The ACE inhibitory activity shown by DEA was lower compared to the other enzyme groups, however, within this group the highest was recorded for DEPH (37.65±0.03) followed by DEPH-2, DEPH-4, DEPH-3 and DEPH-1. Asoodeh *et al*. (2012) also reported ACE inhibitory activity of hen egg white lysozyme hydrolysate prepared using trypsin, papain and a combination of two enzymes. In all the groups, the whole hydrolysate exhibited significantly ($p<0.05$) higher ACE inhibitory activity as compared to the fractions. On comparison of results, it was observed that papain treated DE hydrolysates exhibited highest ACE inhibitory activity than other enzyme treated groups. The difference in the ACE-inhibition among hydrolysates and their fractions could be related to the differences in amino acid compositions and their sequences, as well as the peptide sizes determined by the specificity of the enzyme to substrate (Milan *et al*., 2014). Khueychai *et al*. (2018) also reported that ostrich egg white hydrolysate of 8 h showed ACE inhibitory activities from 10.05% to 42.29% in a concentration dependent manner. However, Mullaly *et al*. (1997) reported that there was no correlation between DH and ACE inhibition.

The value recorded for all the fractions of different groups significantly ($p<0.05$) vary with each other. In the group DEP and DET, a significantly ($p<0.05$) higher activity was exhibited by DEPH-4 fractions whereas in group DEF and DEA, no particular trend was exhibited by the different fractions and the highest ACE inhibitory activity was exhibited by the DEPH-2 and DEPH-1 fractions of DEA and DEF, respectively. A significant correlation between peptide molecular weight and ACE inhibitory activity has been reported by Li *et al*. (2016) who observed that less than 3 kDa peptides of razor clam hydrolysates exhibited higher ACE inhibitory activity than that of larger peptides. Lee and Hur (2017) suggested that the greater ACE inhibition in lower molecular weight peptides may be due to their higher absorbency in the body.

In this study, the 6 h hydrolysates of duck egg proteins using different enzymes were fractionated and evaluated for its ACE inhibitory activity under *in-vitro* condition. All the whole hydrolysates and their fractions produced by different enzymes exhibited ACE inhibitory activity. The whole hydrolysate of papain exhibited significantly higher activity as compared to the other enzyme groups. Moreover the fractions with the lower weight molecular size peptide i.e. DEPH-4(<1 kDa) exhibited higher activity as compared to the higher molecular weight fractions in DEP and DET. Our findings are in line with Miguel *et al*. (2004) who reported that peptides with lower molecular weight possess better ACE inhibitory activity. In contrast to our findings, Miguel *et al*.(2007) reported longer chain ACE inhibitory peptides of egg white hydrolysates prepared using pepsin. However, the ACE inhibitory

activity was in the range of 27.40 and 57.60±0.03%. The current study suggested that, protein hydrolysates from duck egg have the potential as suitable candidate as natural and multifunctional compound to control hypertension and other related diseases.

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