ANTIOXIDANT AND ANGIOTENSIN 1 CONVERTING ENZYME INHIBITORY FUNCTIONS FROM CHICKEN COLLAGEN HYDROLYSATES

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Abstract- Chicken collagen was explored for its possible multifunctional physiological tendencies including ACE inhibitory and antioxidant activities. Flavourzyme, Neutrase and Alcalase were employed for hydrolysis of chicken collagen at varying predetermined times with optimal conditions for corresponding enzymes. The hydrolysate produced from Flavourzyme showed the highest antioxidant activity as measured by ORAC-FL assay (20942 µmol TE/100g) followed by Neutrase (19207 µmol TE/100) and Alcalase (14352 µmol TE/100). Further purification of the hydrolysates by size exclusion chromatography showed that lower molecular weight fractions (between 170 – 700 Dalton) have highest antioxidant capacity which is about 5 fold higher than the initial activity of untreated collagen (52787 and 44093 µmol TE/100 for Flavourzyme and Neutrase fractions respectively). The ACE inhibitory activity of collagen hydrolysates also appeared to be higher with low molecular weight fractions (between 1200–450 Dalton) having IC_{50} value of about 47.2 and 59.7 µg/ml for Flavourzyme and Neutrase respectively. The present study suggests collagen as an effective candidate for both ACE inhibitory and antioxidant activity which can be employed in functional food/soup formulations.

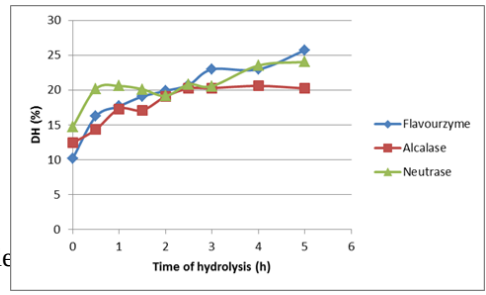
Key Words – functional foods, enzyme, molecular weight fractions, peptides.

I. INTRODUCTION

The worldwide increase in chronic lifestyle related diseases (CLRD) has demanded more concerted efforts from all relevant fields including the scientific community to discover cheap, yet effective remedies for this precarious human health status. Oxidation is an important process in the physiology of all living organisms as oxidative metabolisms are important for cells survival. However, its side effect is the production of free radicals. Although human bodies possess several mechanisms to eradicate or control these oxidation products, these mechanisms may be overpowered either due to excess of free radical production or inability to adequately eradicate them, resulting in attack to the closest molecules causing destructive and lethal cellular effect by oxidizing lipids, proteins, DNA and enzymes in the body [1], the process which can lead to several human diseases including cancer, diabetes, stroke, arteriosclerosis, Alzheimer's, heart diseases among others[2,3]. Several mechanisms have been proposed showing the link between oxidative stress and hypertension [3], although controversies exist on their relationships.

Erdmann *et al* [4] have shown however that consumption of antioxidant laden food products appears to provide further benefits to the endogenous defense mechanisms with fighting oxidative stress. Explorations of peptides generated from various protein sources with high antioxidant activity may also be a step in the right direction. The vast and cheap nature of collagen in various animal byproducts has increased its exploration interest for nutritional and pharmaceutical applications, reducing both industrial cost in waste disposal when these byproducts are put into use and also government spending when the hydrolysate are incorporated in diets of the consumers. Aside this, chicken soups have widely been acclaimed as medicinal due to its healing power [4] and peptides fractions may be parts of the functional parts of this commonly consumed delicacy. Hence, this study aims to identify possible bioactive peptides fractions from chicken collagen hydrolysates with either antioxidant or/and ACE inhibitory function, the application of which can be useful in chicken soup preparation.

II MATERIALS AND METHODS



Avian collagen (AC) (partially hydrolyzed, with ~ 97 % protein content) from Ingredis Distribución Ingredientes, Reus, Spain was used and three different enzymes were employed in this experiment for hydrolysis: Flavourzyme®, Neutrase® and Alcalase®, all purchased from Novozyme A/S, Bagsvaerd, Denmark. Degree of hydrolysis (DH) was carried out by adapting the TNBS (2, 4, 6-trinitrobenzenesulfonic acid) method of Jens, [5] and Spellman *et al*., [6] to a 96-well microplate reader method. Size Exclusion Chromatography (SEC) was used to classify the derived hydrolysates based on molecular weights using the regression line of the generated standard curve plotted using Aprotinin (6512), Ribonuclease A (13700), Cobalamin (1355), and leu- Enkephalin (556). Oxygen radical absorbance capacity (ORAC-FL) assay was based on the method of Franka & Dell, [7] with slight modifications and ACE (Angiotensin-converting enzyme) inhibiting activity was according to Sentandreu & Toldrá, [8]. All reagents were of analytical grades and StatgraphicsPro version 5.1 was used for the data processing. All analyses were done in triplicates with results expressed as mean ± SD.

II. RESULTS AND DISCUSSION

Figure 1 shows the DH of the three enzyme hydrolysates measured by TNBS analysis. Flavourzyme appeared to produce the highest DH up to about 26%, followed by Neutrase (24%) and then Alcalase (20.6%). The first few minutes of hydrolysis seem to be very important in whole hydrolysis process. In all cases, up to 50% of the whole DH achieved seemed to occur in the first 1 hour of hydrolysis. This result is in accordance with that of Alemán *et al*. [9] who found that the hydrolysis rate of marine gelatin were fast in the initial stage of hydrolysis and then gradually decreased until reaching a stationary phase.

Fig 1: Degree of hydrolysis with time progression

All fractions obtained from the three enzymes employed in this study showed consistent increase in antioxidant activity as hydrolysis progresses after which a maximum activity was attained at some point and a subsequent decline appeared with time (Fig. 2). Flavourzyme hydrolysate appeared to be the most effective, attaining a maximum value of about 21000 µmol of Trolox Equivalence/100g of protein at 3 h. Neutrase and Alcalase hydrolysates had their highest ORAC value between 1.5 to 2 h of hydrolysis (19207 and 14400 µmol TE/100g respectively). These respective ORAC values are relatively high compared to values from previous studies and USDA database for the Oxygen Radical Absorbance Capacity of selected foods (www.oracvalues.com). Wei & Shiow [10] earlier reported values between 188 and 2230 µmol TE/100g for some selected medicinal herbs, much lower compared to that of our study. The values between 235 and 9218 µmol TE/100g were also reported in the same study for some culinary herbs, all of which were much below that of the hydrolysates in our present study.

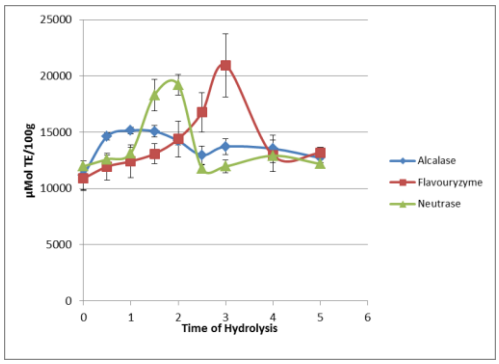


Fig 2: ORAC values for the 3 enzymes hydrolysate

Some researchers have found relationship between DH and antioxidant capacity [9]. Although DH does not seem to always have a direct relationship with the antioxidant and ACE activity as observed in this present study, an extent of relationship still exists between ORAC-FL antioxidant value and Flavourzyme hydrolysed substrate where gradual increase in DH leads to a corresponding increase in ORAC values. Moreover, this relationship seems to occur in two linear phases with the first phase (0 – 1 h) having a linear relationship of

about 98.6 % and the second phase (1.5–3 h) about 99.4 % relationship between DH and ORAC value. Maximum antioxidant capacity was attained at about 23 % DH and subsequent increase in DH leads to its decline. Similar trend was also noticed in the other enzyme hydrolysates. The ACE inhibitory activities of the three hydrolysates considered in this study do not improve significantly with hydrolysis time until later during the hydrolysis and the results do not seem to vary significantly from enzyme to enzyme (Table 1).

Table 1: ACE inhibitory activities ($IC_{50;}$ µg/ml) of enzyme hydrolysate with time progression

Time (h)	Flavourzyme	Alcalase	Neutrase
0	1110±3^{a}	1134±35^{a}	1030±14^{bc}
0.5	1091±62^{ab}	1085±15^{ab}	1037±36^{bc}
1	1062±92^{ab}	1030±10^{ab}	1067±70^{c}
1.5	996±43^{ab}	1031±12^{ab}	1081±90^{c}
2	986±112^{ab}	1096±61^{ab}	1048±12^{bc}
2.5	972±92^{ab}	1076±38^{ab}	1003±64^{b}
3	1008±76^{ab}	1047±11^{ab}	959±55^{a}
4	975±56^{ab}	1018±20^{bc}	949±44^{a}
5	961±71^{b}	995±18^{c}	1046±14^{bc}

$^{a-c}$ results with the same letter in the same column are not significant at $P < 0.05$

Moreover, the raw material employed in this experiment seems to possess some level of ACE inhibiting activity (see time 0) and this value improves gradually but not significantly with time for most of the enzyme. However this cannot be consistently implied as there are reductions in activity at some points during hydrolysis which may be due to the enzyme attack on some active peptide sequences responsible for the ACE inhibition. It has to be explored if the rather slow increase in the inhibitory activities of the hydrolysates may be improved by increasing the enzyme to substrate ratio or other parameters.

Degree of hydrolysis appears to affect ACE inhibitory activity in some of our enzyme treatments. Overall, the linear relationship between degree of hydrolysis and ACE activity in Flavourzyme is about 77.4 % (result not shown). A strong linear relationship was noticed within the first few periods (120 min) of hydrolysis with Alcalase (99.2 %) although this relationship was lost at time point from 2 h. All these results suggest that ACE inhibition activity of avian collagen peptides should be due not only to its molecular weight but also to the amino acid composition and sequence.

Two different hydrolysates (Flavourzyme, 3h and Neutrase, 2 h) were selected for further separation into different molecular weight fractions and subsequently, antioxidant and ACE inhibitory functions of the fractions were assayed. These two fractions were selected based on their highest antioxidant activity measured by ORAC-FL among all the enzyme treatments. With size exclusion chromatography, eleven fractions were identified and collected. The highest activity were observed particularly with fractions of 170-267 Da in Flavourzyme hydrolysates and these fractions appeared to contribute largely to the antioxidant capacity observed in the hydrolysate, attaining about 5 fold increase (47048–52787 µmol TE/100g) in antioxidant capacity compared to the initial activity of the crude hydrolysate at zero time (Fig 3). Fairly similar trend was also observed in Neutrase fractions where the highest activity was distributed between peptide fractions of low molecular weight between 347-798 Da (41353 – 44093 µmol TE/ 100g) resulting in about 4 fold increase in antioxidant activity compared with initial time (Fig 3). Alemán *et al*. [11] have emphasized the fact that biological activities of peptides are to a large extent affected by their molecular weight distribution which in turn may be influenced by processing conditions. The same authors [11] have also stated that majority of the antioxidant peptides derived from food origin have molecular weight ranging between 500 and 1500. These results are confirmed to an extent in the present study as even much lower fractions were found to possess higher antioxidant activity. The high radical scavenging activities of these low molecular fractions could be due to the amino acid composition, structure and hydrophobicity of the resulting peptides.

For the ACE inhibitory activity, in both selected samples, it is obvious that the fractions with molecular weight of between 450-1200 Dalton are responsible for the ACE inhibitory activities (fraction 591-1074 in Flavourzyme and 589-1277 in Neutrase) attaining the maximum activity of about 47.2 and 59.7 µg/ml in Flavourzyme and Neutrase respectively (Fig 3). These values are

about 20 fold improvement compared to the values of the selected crude hydrolysate fraction. Rui-Zeng *et al.*, [12] have reported that small sized peptides are better candidate than longer ones in

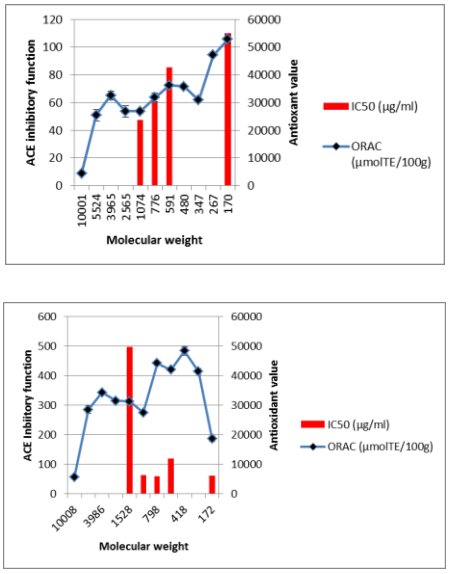


Fig 3: ACE and Antioxidant activity of SEC classified fractions for (a) Flavorzyme & (b) Neutrase hydrolysates

playing physiological roles *in vivo* as they are less susceptible to gastrointestinal hydrolysis. Considering the molecular weight of these active fractions, it can be said that either dipeptides or tripeptides are mostly involved in the acclaimed ACE inhibitory and antioxidant activities.

III. CONCLUSION

Our study confirms the fact that chicken collagen hydrolysates possess both antioxidant and ACE inhibitory activity which can be mostly implicated on low molecular weight fractions. Degree of hydrolysis also influences peptides activities however, to a varying extent. Incorporating the knowledge in this study in production of chicken soups may help in deriving a tremendous health benefit from this commonly consumed delicacy unconsciously improving the health of consumers and reducing government spending on CLRDs and other related diseases.

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