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Evaluation of Anti-Inflammatory and Antihypertensive Activities of Bioactive Peptides Derived from Trypsin Treated Cow Milk Casein

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Abstract

Cow milk proteins are considered as the rich source of bioactive peptides which has a great influence in the various biological processes. The present study was aimed to evaluate anti-inflammatory (stabilizing of Erythrocyte Membrane Integrity) and antihypertensive (Angiotensin Converting Enzyme inhibition) bioactive peptides derived from cow milk protein casein after treatment with trypsin. Casein was prepared and treated with trypsin & extent of hydrolysis was analyzed. Bioactive peptides thus generated were evaluated for their said activities. Maximum anti-inflammatory effect was shown after 4 hours of enzymatic treatment; while maximum antihypertensive effect was shown after 3 hours of treatment. The results obtained hence proved that bioactive peptides released during tryptic digestion at different incubation times show significant effect on metabolic activities providing the need for further investigation for their targeted use as human nutritional supplements for specific medical conditions.

Keywords: Angiotensin Converting Enzyme (ACE), Antihypertensive, Anti-Inflammatory, Bioactive Peptides, Erythrocyte Membrane Integrity, Trypsin

Introduction

Milk is an excellent source of well-balanced nutrients for young mammals and adult. Milk exhibits range of biological activities that influence digestion, metabolism, growth & development of organs and resistance to disease. In addition to some fully active components, many bioactivities of milk proteins are latent, requiring an enzymatic proteolysis for release of bioactive peptides (Meisel, 1998; Clare & Swaisgood, 2000). Though significant data has been generated on the various effects of bioactive peptides derived from milk of different species but still there are some shortfalls as there is not much information available regarding anti-inflammatory and antihypertensive bioactive peptides derived from cow casein and need further investigation including study of effect of enzymatic activity at different incubations. The present work was designed with the aim to investigate the presence of anti-inflammatory and antihypertensive activities of bioactive peptides derived from cow milk casein upon treatment with enzyme trypsin.

Milk is an important source of nutrition for both neonates and adults as milk contains components which provide nutritive elements, immunological protection and bioactive substances (Schanbacher *et al.,* 1997). Cow milk is very much rich in protein having a wide range of potential health benefits and functional properties. About 80% of milk protein is a phosphoprotein Casein (Swaisgood, 1973, Jonness & Holt, 1987). Casein is considered as the most important source of bioactive peptides (Clare & Swaisgood, 2000; Korhonen & Pihlanto-Leppala, 2003). Bioactive peptides are protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts & Weiler, 2003). In milk bioactive peptides are latent until released and activated (e.g. during digestion or fermentation). Once activated these peptides are potential modulators of many biological regulatory processes thus exhibiting variety of biological effects (Haque *et al.,* 2009).

In the modern society, hypertension is the one of the major health risks involved in the development of cardiovascular diseases and stroke. Nutritional supplements are of major area of interest for modern researchers as it has significant role in the prevention of hypertension. In human body, an enzyme ACE (Angiotensin Converting Enzyme) is responsible of increase in blood pressure by converting angiotensin-I to a potent vasoconstrictor angiotensin-II (Fitzgerald & Meisel, 2000), and by degrading bradykinin, a vasodialatory peptide, and enkephalins. Therefore, researchers are trying to develop foods and nutritional supplements with potent ACE inhibitiory effects as ACE inhibition leads to a hypotensive effect.

Inflammation is a complex biological response of vascular tissues to harmful stimuli like pathogens or damaged cells (Denko, 1992). It is a defensive mechanism of body to remove injurious stimuli and to repair damaged tissue, but if run unchecked, it can lead to onset of diseases like rheumatoid arthritis, atherosclerosis etc. (Henson & Murphy, 1989). Thus, inhibition of overproduction of inflammatory mediators and pro-inflammatory cytokines may prevent or suppress inflammatory diseases (Kim *et al.,* 2003). A study by Mao *et al.* (2011) demonstrates that yak casein hydrolysate can substantially decrease NO production, as well as pro-inflammatory cytokines IL-6, TNF-a and IL-1b secretion in LPS-stimulated murine peritoneal macrophages.

Materials and Methods

Trypsin, ACE and HHL are from Sigma-Aldrich; while all the other chemicals used were of analytical grade.

Preparation of Casein

Cow milk sample were collected from indigenous cow in the locality of Suddhowala, Dehradun, India. Casein was prepared from milk using isoelectric-precipitation method. 500ml milk, immediately after collection, was defatted by centrifuging twice at 5000gfor 20 min at 4°C. The filtrate was diluted with equal volume of double distilled water (DDW); pH adjusted to 4 .6 with 1 N HCl and the mixture was stirred for 20 min. The precipitate so formed was separated by filtration through four layers of cheese-cloth, washed, solubilized in distilled water at pH 7.0 (equal to initial volume of milk) with 1N NaOH, reprecipitated and washed 3-4 times with distilled water. The wet casein after thorough washing with distilled water was dried at room temperature for 28 hours to obtain a dry powder.

Preparation of Sodium Caseinate

Dried casein was solubilised in distilled water (3gm/50 ml) by continuous stirring with the help of magnetic stirrer

and simultaneous addition of 0.1N NaOH drop wise so as to obtain pH of this homogenous solution to 7.2. The final volume was made up to 100ml with double distilled water. The solution of sodium caseinate was stored at 4ºC, till it was processed for further studies. The concentration of protein in various samples was estimated by using the method of Lowery *et al.* (1951).

Treatment with Proteolytic Enzyme

Casein prepared was treated with trypsin enzyme according to the method suggested by Abubakar *et al.* (1998) & Pihlanto-leppala *et al.* (2000). 0.113g of casein was taken (containing 100mg of protein) and dissolved in 20ml TrisHCl buffer (0.05M pH 8.0). To this 100µl of 1% trypsin solution (100mg in 10ml of same buffer) was added to get substrate to enzyme ratio of 100:1 and stirred at 37ºC for 60,120,180,240,300 and 360 min. After completion of each incubation period, enzyme activity was stopped by heating at 100ºC for 5 min. The supernatant and pellet were separated by centrifugation at 10,000rpm for 20 min at 5ºC and supernatant stored at 4ºC in a deep freezer.

Estimation of Partial Hydrolysis of Protein by Hull's method

A stock solution of tyrosine of 0.2 mg/ml was prepared. Different concentrations ranging from 100 µg to 1000 µg were taken. The volume was made upto 6 ml with DDW. To these 10 ml of 0.72N TCA was added and kept at 37°Cfor 15 min. In 5 ml of this aliquot, 15% sodium carbonate in 0.1 N NaOH-Copper Sulphate solutions was added and mixed thoroughly. Finally, 3ml of Folin's Reagent was added. This mixture was kept in dark for 5-10 min for color development. The blue color thus developed was measured at 650 nm. A standard curve plotting tyrosine concentration against O.D. at 650 nm was prepared. For quantification of enzyme hydrolysates, in place of different concentrations of tyrosine, supernatant samples from different incubation periods were taken.

Assay of Anti-Inflammatory Activity

Anti-inflammatory activities of hydrolysates were estimated by using the method of Abe *et al.* (1991) with some modifications. 0.8ml of NIH solution (5.5mg of tri-sodium citrate + 2gm of citric acid + 6.12gm of dextrose dissolved: in 250ml of distilled water) was taken per 5ml of blood in the collection tube, so as to prevent the clotting. To this, 5ml of isotonic saline solution (900mg of sodium chloride dissolved in 100ml of distilled water) added. Mixture was left for 10 minutes and after that centrifuged at 3000rpm for 10 minutes. The supernatant discarded and pellets were washed three times with isotonic saline solution. To washed pellets, 6ml of phosphate buffer (0.26gm of disodium hydrogen phosphate + 1.15gm of sodium dihydrogen phosphate + 900mg of sodium chloride: in 700ml of distilled water and final volume was made to 1000ml with distilled water; pH 7.4) was added for every 4ml of pellets obtained to make the final stock erythrocyte suspension. The experiments were carried out in duplicate pairs. Stock erythrocyte suspension (30µl) was mixed with 5 ml of the hypotonic solution (250mg of sodium chloride dissolved in 100ml of distilled water) containing 100 µl of casein hydrolysate, while the control sample was mixed with hydrolysate free solution. The mixtures were incubated for 30 min at room temperature and centrifuged for 3 min at 1300*g* and the absorbance of the supernatant was measured at 540 nm. Acetyl salicylic acid (200µl/ml) was used as a reference standard. Each sample assay is carried out in triplet and result was represented as a mean of three values ± Standard Deviation.

$$\% \text{ inhibition of haemolysis} = [1 - (OD_1/OD_2)] \times 100$$

Where,

OD_1 = Absorbance of sample
OD_2 = Absorbance of control

Angiotensin Converting Enzyme (ACE) Inhibition Assay

Angiotensin converting enzyme(ACE) inhibition was measured as suggested by Cushman and Cheung (1971) and modified by Hernandez-Ledesman *et al.* (2003).15µl of hydrolysate was added to 110µl of substrate (10Mm Hippuryl-L-Histidyl-L-Leucine in 0.1 M borate buffer containing 0.3M molar NaCl; pH 8.3), and mixed at 37^0C. Then, 25µl of Angiotensin Converting Enzyme (0.2U/ml) was added and incubated at 37^0C for 80 minutes. Reaction was stopped by adding200µl of 1N HCl. Subsequently the hippuric acid formed in the enzymatic process was extracted with 1ml of ethyl acetate (centrifugation at 3,000g for 10 min at 25^0C). An aliquot of 750µl of the upper

organic layer was collected and dried completely by heating at 95^0C for 20 minute; dried material was resuspended in DDW. The negative control was carried out by adding only substrate, ACE and water. The reaction blank was prepared by mixing substrate, HCl and ACE. The product hippuric acid was quantified at 228nm. Each sample assay is carried out in triplet and result was represented as a mean of three values ± Standard Deviation.

$$\%\text{ACE inhibiton} = \frac{A_c - A_s}{A_c - A_b} \times 100$$

Where, A_c= Absorbance of control, A_s=Absorbance of sample, A_b= Absorbance of blank

Results and Discussion

The dry weight of casein was found to be 2.42 gm/100ml of cow milk. The value obtained is near about the cited value (2.6 gm/100ml of milk as reported by Jensen, 1995). The total protein was found to be 264µg/10µlof sodium caseinate.

Degree of Hydrolysis (DH)

The DH value has been gradually increasing with hydrolysis time, reaching maximum at 6h, which is similar to DH of yak casein by Alcalase and Neutrase for 4h (Jiang *et al.*, 2007). The progressions in DH of cow casein were shown in Table 1.

Table 1: Degree of hydrolysis of cow casein

Incubation Period (In min)	% Hydrolysis
60	2.6
120	2.9
180	3.3
240	3.6
300	3.7
360	4

DH measures the content of peptide bonds cleaved in the substrate by a proteolytic agent; higher the DH, the higher the content of cleaved amino groups, thereby affecting the biological activity of hydrolysates. Therefore, the biological activity of peptides depends on the substrate, enzyme specificity and hydrolysis conditions (Sarmadi & Ismail, 2010; Hogan *et al.*, 2009; and Zhang & Zhou, 2010). In the current study, the DH has not increased significantly after 4h of incubation (Figure 1). This suggests that enzyme could not further hydrolyze the remaining bonds, a fact that is directly influenced by enzyme specificity.

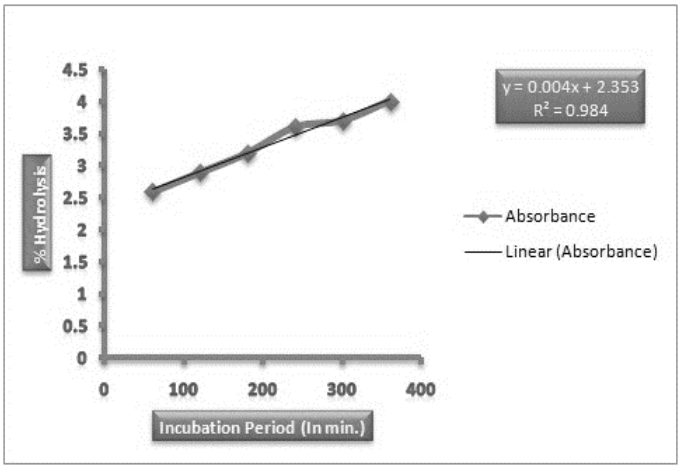

Figure 1: Graph showing degree of hydrolysis of casein

Anti-Inflammatory Assay

Present study demonstrates that all the cow casein hydrolysates can substantially inhibit the haemolysis of erythrocytes induced by hypotonic solution (Table 2). The result obtained could be due to an increase in the surface area/volume ratio of the cells which could be brought about by shrinkage of the cell or expansion of membrane, and an interaction with membrane proteins (Abe *et al.,* 1991).

Table 2: Anti-inflammatory status of casein hydrolysates

Incubation Period (In min)	% Inhibition
60	9.75±1.484
120	25.5±1.414
180	28.4±0.141
240	34.75±0.777
300	29.5±2.41
360	27.55±0.212

Hence, it may be speculated that the cytoprotective effect on erythrocyte membrane may be due to the ability of the casein hydrolysates to alter the calcium influx into the erythrocytes. In general, there is gradual increase in anti-inflammatory activity with the increase in incubation time initially; reaching maximum activity at 240 minutes of incubation, after that the activity decreases slowly (Figure 2).

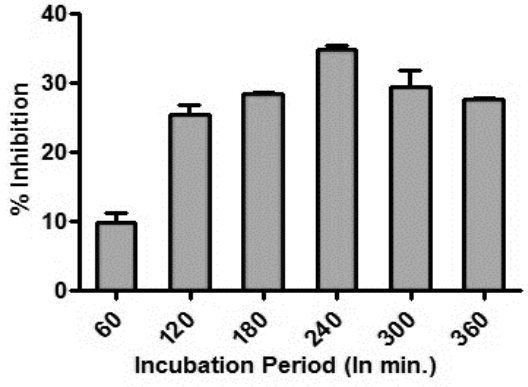

Figure 2: Histogram showing anti-inflammatory activity of casein hydrolysates

Antihypertensive Assay

Casein hydrolysates showed potent ACE inhibitory activity. The inhibition from the peptides was found to be maximum after 3hrsof incubation and after that the activity goes on decreasing slightly (Table 3).

Table 3: Antihypertensive status of casein hydrolysates

Incubation Period (In min)	% Inhibition
60	35.42±2.34
120	56.95±5.028
180	87.08±0.466
240	85.09±2.34
300	66.71±4.91
360	63.24±0.933

C-terminus pro-containing short peptides are supposed to be an appropriate ACE inhibitory peptide released on proteolytic hydrolysis. In the present study, there is sharp increase in ACE inhibitory activity along with the increase in incubation time till 3hrs and then gradual decline in the activity (Figure 3).

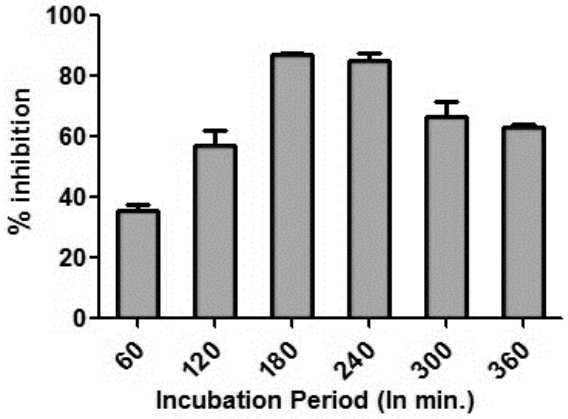

Figure 3: Histogram showing ACE inhibitory activity of casein hydrolysates

This could be due to initially breaking away of inhibitory amino acids and formation of smaller peptides having such types of amino acids which are mainly responsible for the ACE inhibition and finally after 3 hrs on further enzymatic treatment the peptides becomes too short to contain any such amino acids. The results obtained were in accordance with the studies conducted by various researchers previously on buffalo and goat milk caseins (Deepthi & Tandon, 2003).

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Conflict of Interests

There is no conflict of interest.

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