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Research Article

ANTIOXIDATIVE PROPERTY OF COW MILK CASEINATES HYDROLYZED WITH DIFFERENT PROTEASES

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ABSTRACT

Fundamental studies have opened a new field of research related to bioactive substances derived from food, and the pioneering work in this field has been done in the last decade on the basis of substance-orientated food and nutritional science. Milk proteins are considered important sources of bioactive peptides that could be released through enzymatic hydrolysis by digestive (gastrointestinal) enzymes, by fermentation, and by proteolysis employing enzymes derived from microorganisms or plants. These peptides are inactive within the protein sequence, requiring enzymatic proteolysis for release of the bioactive fragment from the proteins precursor. These molecules could be potentially employed in health and food products. In this investigation cow milk caseinate hydrolysates obtained with three different proteases studied with respect to the effect of incubation period on their activity.

After treating the caseinate with different enzymes, each enzyme hydrolysates were estimated for their degree of hydrolysis and finally analyzed for their antioxidant activity by DPPH method. Peptic hydrolysates of cow casein showed highest antioxidant activity followed by tryptic hydrolysates.

Peptic hydrolysates of 2hr incubation showed highest activity. Further, the peptide samples showing highest activity upto 2hrs of incubation were subjected to RP-HPLC for their partial characterization. Tryptic and peptic hydrolysates produced peaks mainly in the region of hydrophillic solvent indicating the presence of hydrophillic peptides/peptides. However, in general all the hydrolysates show fairly good antioxidant activity.

Thus, it could be concluded that bioactive peptides from the cow casein possesses good antioxidant property which has useful implications in food science & nutrition in the future prospective.

Keywords: Antioxidant, Immunomodulatory, Bioactive peptide, Casein, DPPH.

INTRODUCTION

Bioactive substances of food origin can be defined as components (genuine or generated) of consumption ready food which may exert regulative activities in the human organism beyond basic nutrition. A great diversity of food derived bioactive substances having a non-nutrient character can be considered as an aid in maintaining good health. Based on this concept, food researchers are presently considering different bioactive substances of food origin as health enhancing ingredients, for example for use in functional foods/FOSHU (Foods for Specified Health Use), which can be consumed to reduce the risk of disease or to enhance a certain physiological function.

Such bioactive peptide fragments originating from milk protein casein by enzymatic proteolysis should be taken into account as potential modulator of various regulatory processes in the body [1]. Research carried out during the last ten years has shown that casein is an important source of various biologically active peptides. These peptides are of particular interest in food science and Nutrition. These biological activities include antioxidant, opioid agonist and antagonist peptides, hypotensive peptides (which inhibit angiotensin-1 converting enzyme), mineral binding, immunomodulatory, antibacterial, anti-inflammatory, antiulcer and antithrombotic peptides [2, 3]. Out of these activities the antioxidant peptides gained importance for further utilization because of their immense implications in various areas of food science and nutrition. Further the antioxidant, antihypertensive and antibacterial activity of chemically synthesized bioactive peptides derived from Bovine & Ovine casein hydrolysates were already studied [4].There is significant data available about the antihypertensive and immunomodulatory activity of bovine [5] & ovine milk derived casein hydrolysates. However, there is very little data available about the antioxidant activity of cow milk casein derived bioactive peptides after treatment with proteases, under conditions simulating human digestive tract, particularly considering the effect of incubation period.

MATERIALS & METHODS

Isolation of casein

Raw milk samples were collected from local breeds of cow in the locality (Nanda Ki Chowki, Dehradun, Uttarakhand). Casein was prepared from the collected milks using the method of isoelectric precipitation. Immediately after collection, milk was defatted by centrifuging twice at 5000 g for 20 min at 4°C in a refrigerated centrifuge. The milk was filtered via four layers of cheese cloth and fat separated was discarded. The filtrate was diluted with equal volume of double distilled water (DDW); pH adjusted to 4.6 with 1N HCl and the mixture was stirred for 30 minutes. 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, re-precipitated and washed 3-4 times with distilled water. The wet casein, after thorough washing with distilled water, was air-dried by spreading on a sheet of filter paper at room temperature. The concentration of protein in various caseins formed was estimated by Lowry's method [6].

Hydrolysis of casein

Casein prepared isoelectrically was treated with two different enzymes according to the method of Abubakar *et al*.[7] and Pihlanto-Leppala *et al*.[8] with some modifications (enzyme: substrate ratio is taken as 1:100) [Table 1].

Table 1: Conditions employed for hydrolysis of different caseins

Enzyme	Buffer	pH	Enzyme/Substrate (w/w)	Temp (°C)
Pepsin	0.05 M HCl	2.0	1:100	37
Trypsin	0.05 M Tris HCl	8.0	1:100	37
Chymotrypsin	0.02M Ammonium Acetate	8.0	1:100	25

Initially incubated for 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min, 120 min, 135 min and 150 min (so as to simulate human digestive tract conditions where protein takes maximum 120 minutes to get digested) and after that for 4hrs and 6hrs incubation period. The percent hydrolysis in various samples was estimated by using Hull's method [9].

Assay of Antioxidant activity

Antioxidant activity was measured using 2, 2-Diphenyl- 1-picrylhydrazyl (DPPH) radical-scavenging assay as described by Brand-Williams et al.[10]. Each sample assay is carried out in triplicate & data are represented as a mean of three values along with the standard deviation.

RESULTS

Research efforts have been focused on the generation of bioactive peptides from a myriad of food sources, including milk and dairy products, egg, soy and meat, envisaging potential utilization by the food industry. In particular, investigations have been carried out to obtain bioactive peptides through the hydrolysis of bovine milk caseins [11, 12, and 13]. In the current study, the antioxidant activities of cow caseinate hydrolysates were investigated, on which there are relatively few studies in the literature.

The dry weight of casein was found to be 2.42gm/100ml. of cow milk. It was reported that cow milk contain 2.49 gm of casein/100ml of milk. The values reported were near about the range of the cited values. The total protein content of casein was found to be 264ug/0.1ml of sodium caseinate [containing 3gm casein/50ml of distilled water].

Degree of hydrolysis was determined by quantification of protein in these hydrolysates. Treatment of cow casein with pepsin for 6hr incubation shows highest hydrolysis. The same result of hydrolysis obtained when treated with trypsin. It shows that degree of hydrolysis goes on increasing with the time of incubation.

The comparative inference of antioxidant status of hydrolysates with reference to different incubation periods were tabulated [Tables 2].

Table 2: Antioxidant Activity of the Hydrolysates:

Incubation Period (In Minutes)	Percent Inhibition		
	Trypsin	Pepsin	Chymotrypsin
15	17.17±1.06	24.53±1.78	21.27±1.38
30	21.57±0.80	30.57±1.72	25.17±1.86
45	28.07±1.15	34.43±0.97	29.43±0.70
60	32±1.9	41.56±1.72	32.6±1.34
75	36.2±1.87	47.63±2.77	36.2±1.65
90	40.27±1.72	53.73±1.19	40.67±1.82
105	45.43±1.60	58.03±1.59	44.13±1.43
120	49.33±1.76	61.0±1.95	48.4±1.41
135	54.6±0.66	66.37±1.90	51.6±0.75
150	50.93±2.05	64.87±1.11	49.1±1.4
240	45.23±1.67	58.2±1.75	46.47±1.32
360	49.57±1.40	61.1±1.9	43.37±1.22

Peptic hydrolysates showed highest activity followed by chymotryptic and tryptic hydrolysates. The antioxidant activity goes on increasing with the incubation period up to 120 – 150 minutes, and after that decreases with further increase in incubation period.

Hydrolysates showing maximum antioxidant activity up to 2hrs of incubation were further subjected to Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC). At 2hr incubation with trypsin of cow casein, 6-8 peaks at 214 nm and 2-3 peaks at 280 nm were observed (Figure 1(a) & 1(b)).

However, the peptic hydrolysate of 2hr incubation, shows 3-4 peaks at 214 nm and 2-3 peaks at 280 nm (Figure 2(a) & 2(b)). In case of chymotryptic hydrolysates, results are almost same as in peptic hydrolysates (Figure 3(a) & 3(b)).

DISCUSSION

The degree of hydrolysis (DH) measures the content of peptide bonds cleaved in the substrate by a proteolytic agent (proteases, in the current case): the higher the DH, the higher the content of released amino groups. DH is reported to affect the antioxidant activity of protein hydrolysates. Therefore, the biological activity of peptides depends on the protein substrate, enzyme specificity, and hydrolysis conditions [14, 15, and 16]. Degree of hydrolysis found maximum with trypsin treatment. This show that trypsin utilized more protein as substrate to cause hydrolysis as compared to pepsin.

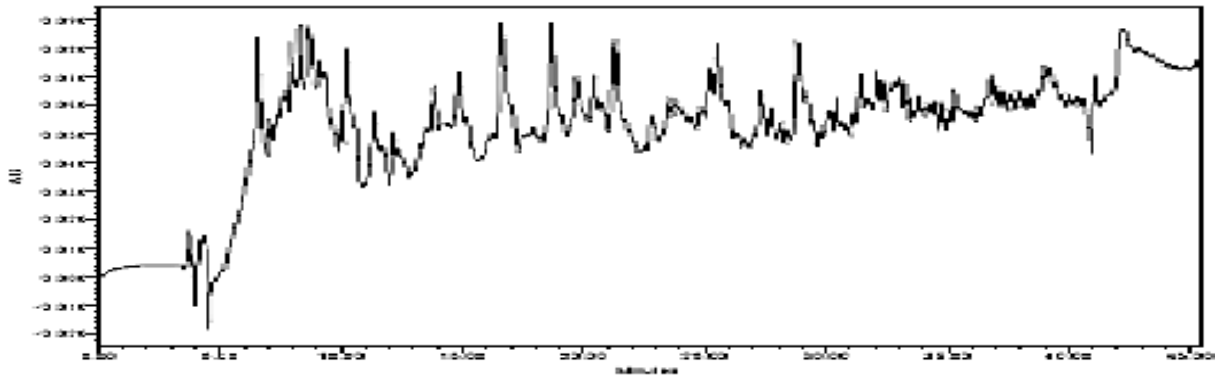

Fig.1 (a): Elution profile of tryptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 214nm

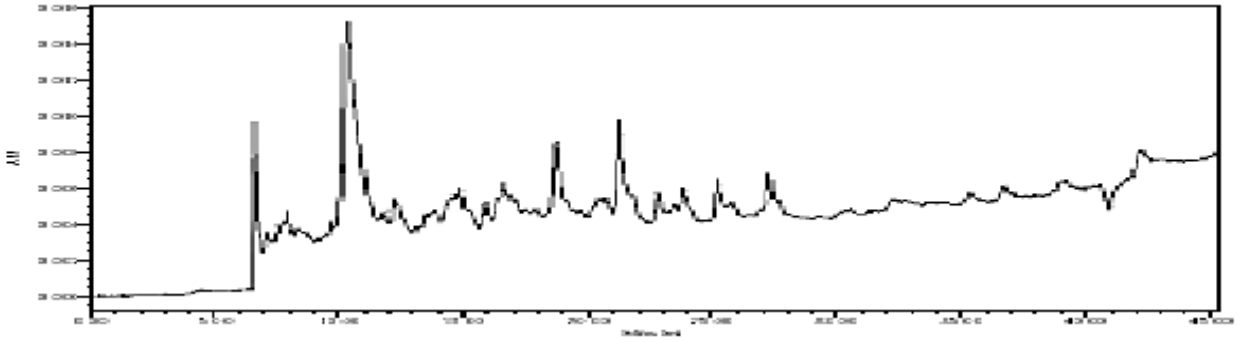

Fig.1 (b): Elution profile of tryptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 280nm

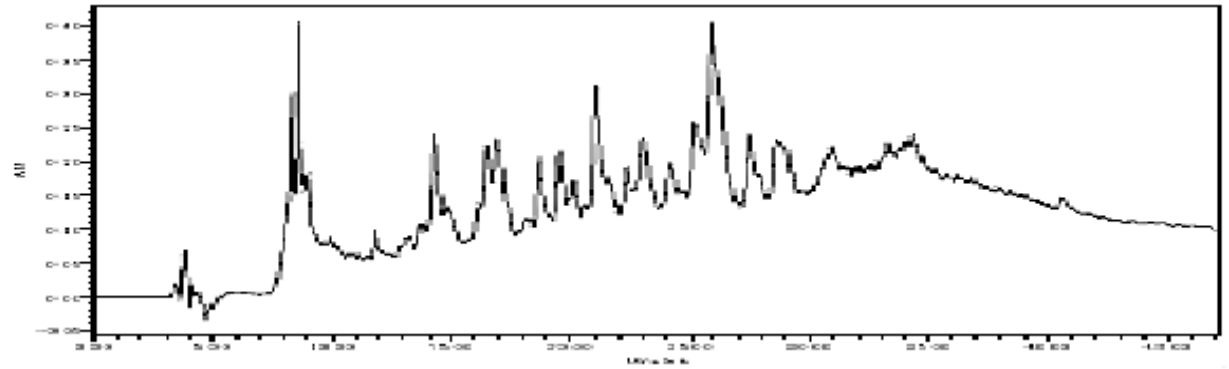

Fig.2 (a): Elution profile of peptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 214nm

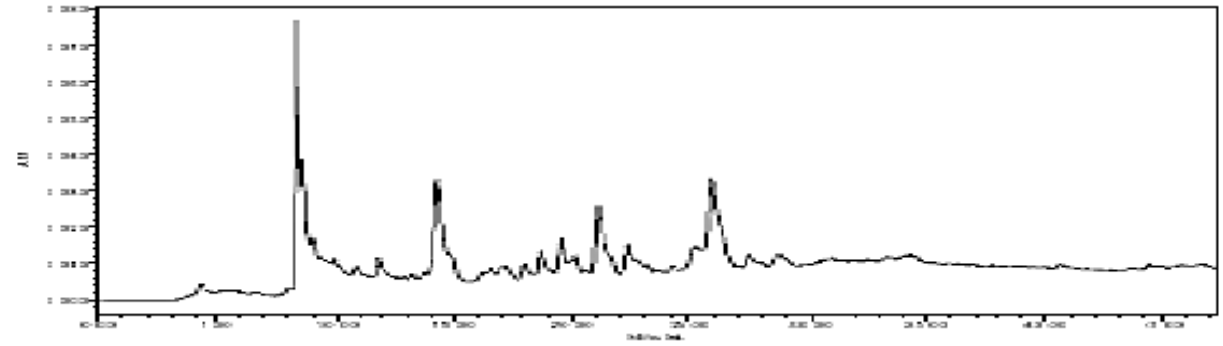

Fig.2 (b): Elution profile of peptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 280nm

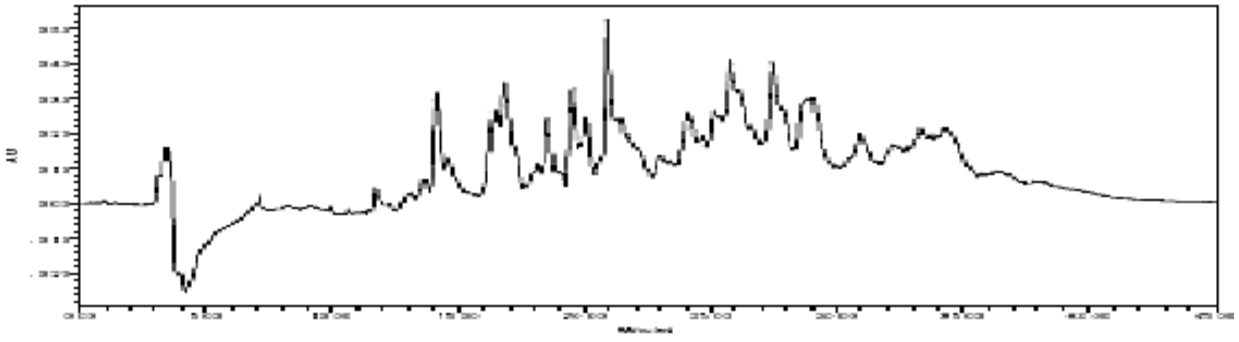

Fig.3 (a): Elution profile of chymotryptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 214nm

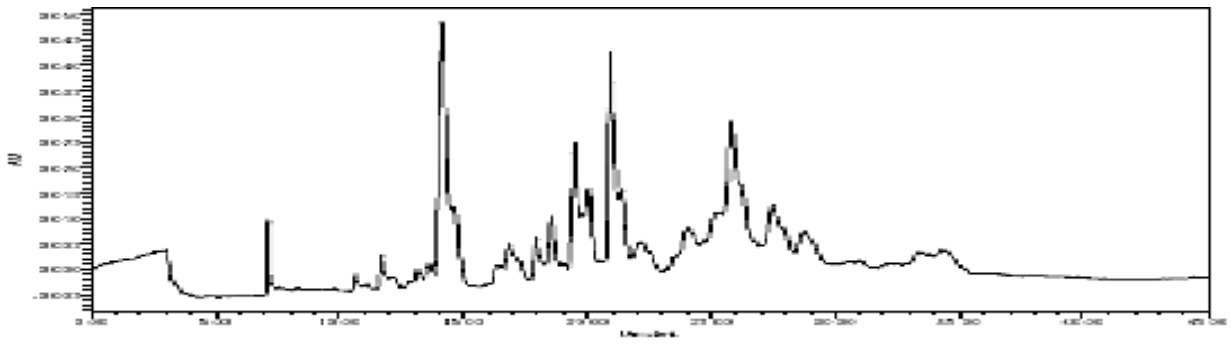

Fig.3 (b): Elution profile of chymotryptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 280nm

Peptides and protein hydrolysates, obtained from the proteolysis of various food proteins, are reported to possess antioxidant activities. Antioxidant mechanisms include radical-scavenging (both hydrogen-donating capability and free radical quenching) activity, inhibition of lipid peroxidation, metal ion chelation, or a combination of these properties [14, 17]. Antioxidant activities might protect biological systems against damage related to oxidative stress in human disease conditions. These antioxidant peptides and hydrolysates might also be employed in preventing oxidation reactions (such as lipid peroxidation) that leads to deterioration of foods and foodstuffs [15, 16]. Antioxidants from protein hydrolysates might confer nutritional value besides functional/physiological properties, which are additional advantages over the synthetic counterparts [18, 19].

Scavenging activities of cow caseinate hydrolysates were determined using DPPH radicals. DPPH is a free radical that accepts an electron or a hydrogen radical, becoming a stable molecule. For this reason, it is employed as a substrate to evaluate the antioxidant activity of peptides and protein hydrolysates. Results varied widely along with hydrolysis time, and a relationship between hydrolysis time and DPPH activity could not be established; however, the higher DPPH-scavenging activity was evidenced after 1 h of hydrolysis. Proteolysis of food proteins is usually reported to enhance the DPPH-scavenging activity of hydrolysates [12]. The DPPH-scavenging activity of yak milk protein hydrolysates obtained with Alcalase was observed to increase during the hydrolysis process for up to 7 h [20]. Nevertheless, this is not always observed [21]. Specifically, bovine casein hydrolysates obtained with diverse proteolytic enzymes were shown to possess lower DPPH activity than the whole protein [17].

Maximum percentage inhibition was shown by peptic hydrolysate. Based on the result, hydrolysates release short peptides having the antioxidant activity to relevant level which is directly related to incubation period or time of hydrolysis. In general scavenging activity goes on increasing till 150 minutes of incubation in case of treatment with all the enzymes, but in case of 4 hr and 6 hr incubation the activity declines.

Partial characterization of peptides showing maximum activity was done by RP-HPLC under two different wavelengths using aqueous as well as organic solvents. Peaks at 214 nm showing the presence of non-aromatic, and at 280 nm, peaks represent aromatic amino acid. In the region of solvent A (hydrophobic) peaks represent the presence of hydrophilic peptides and for solvent B (aqueous) peaks represent hydrophobic peptides.

All the enzyme hydrolysates produce peaks mainly in the region of solvent B indicating the presence of hydrophilic peptides at both the wavelengths. So from the graph it can be concluded that above said hydrolysates consist of mainly hydrophilic peptides which were aromatic and/or non-aromatic in nature. From the observed pattern of DPPH-scavenging activity, caseinate contain some substances acting as electron donors that could react with free radicals, converting them into more stable molecules and terminating the radical chain reaction. His, Phe, Tyr, Trp, among other aromatic and hydrophobic amino acids, seem to be involved in the antioxidant activity of protein hydrolysates [14, 12, and 22].

CONCLUSION

Cow caseinate hydrolysates presenting antioxidant activity were produced through hydrolysis with three different protease preparations (pepsin, trypsin and chymotrypsin), under conditions simulating human digestive tract. The bioactivities presented by the protein hydrolysates could have resulted from the synergistic effect of different peptides within the mixture. Such cow caseinate hydrolysates could be useful for food industry applications, aiming to potentially increase the nutritional value and shelf-life of food products, and also in the development of functional foods. The physicochemical characterization and properties of cow caseinate hydrolysates are under investigation.

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