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RESEARCH ARTICLE
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# Antioxidative effect of yak milk caseinates hydrolyzed with three different proteases

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## Abstract

**Aim**: Yak milk is a type of milk that people are less familiar with due to its remote geographical location which may have significant effects on composition, microbiota and hydrolytic outcome. Present work was designed with the aim to evaluate the antioxidative effect of peptides derived from yak milk caseinate on hydrolysis with three different proteases.

**Materials and Methods**: In this investigation Yak milk casein was hydrolyzed by three commercially available proteases (Trypsin, Pepsin and chymotrypsin). These hydrolysates collected at different hydrolysis times (30 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min, 240 min, 270 min, 300 min, 330 min and 360 min) were assayed for their antioxidant activity with respect to the effect of incubation period.

**Results**: Among all the enzyme hydrolysates, the tryptic hydrolysates showed highest antioxidant activity followed by chymotryptic hydrolysates. Further, the peptide samples showing highest activity 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.

**Conclusion**: The results indicated that yak milk casein could be a resource to generate antioxidative peptides and be used as multifunctional active ingredients for many value-added functional foods as well as a traditional food protein.

**Key words**: antioxidant, hydrolysates, bioactive peptide, hydrophilic, casein

## Introduction

Food proteins, besides their nutritional roles, contain peptide sequences encrypted in their primary structures that are capable of modulating specific physiological functions. These protein fragments, termed bioactive peptides, are inactive within the sequence of the precursor protein, and could be released through enzymatic hydrolysis *in vivo* or *in vitro*. After release, they might exert opioid, antihyper-tensive, immuno-modulatory, antibacterial, and antioxidant activities, among others, with potential applications in food science, technology, and nutrition [1-4].

Milk proteins are considered important sources of bioactive peptides that could be released through enzymatic hydrolysis by digestive (gastrointestinal), fermentation, and proteolysis employing enzymes derived from microorganisms or plants [4,5]. These proteins, mainly caseins, are commercially available in large amounts at a high degree of purity and at low price which, from a technological aspect, make them attractive in the search for bioactive peptides [2]. Therefore, the properties of milk protein-derived bioactive peptides have been thoroughly investigated, and an increasing number of such molecules have been identified in milk protein hydrolysates and also fermented dairy products [6,7]. Investigations usually report the production of biologically active peptides through the hydrolysis of caseins isolated from bovine milk. However, milk caseins from different species (yak, for instance), and the high genetic variability of milk proteins, might originate different bioactive peptides [8,9]. The most common way to generate bioactive peptides is through enzymatic hydrolysis of whole protein molecules, and the bioactivity of such hydrolysis-generated peptides appears to be inherent to size and specific amino acid sequences [2,5]. Proteolytic enzymes from various sources (animal, plant, microbial) have been successfully employed in the production of these molecules [6,7].

In the current study, three different commercially available proteases were employed in the hydrolysis of sodium caseinate obtained from yak milk, under conditions simulating human digestive tract.The biological activities (antioxidant) of the protein hydro-lysates were then assessed, particularly considering the effect of incubation period, with the aim of evaluateing its potential for food applications.

## Materials and Methods

Isolation of casein: Raw milk samples were collected from yak in the higher regions of Himalaya in

Table-1. Conditions employed for hydrolysis of yak caseinate

| 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 |

Table-2. Antioxidant activity of the hydrolysates

| Incubation Period (minutes) | Percent inhibition (significant differences between all groups) | | |
|---|---|---|---|
| | Trypsin | Pepsin | Chymotrypsin |
| 30 | 47.53±1.23 | 31.53±1.66 | 40.97±2.01 |
| 60 | 50.93±1.15 | 38.87±1.47 | 45.43±0.81 |
| 90 | 54.47±1.48 | 41.37±0.71 | 48.9±1.2 |
| 120 | 60.2±1.87 | 43.37±0.67 | 55.63±1.89 |
| 150 | 63.7±1.32 | 45.9±1.27 | 58.23±1.33 |
| 180 | 67.0±0.97 | 48.1±1.69 | 61.6±1.49 |
| 10 | 69.3±1.27 | 51.2±1.31 | 64.2±0.77 |
| 270 | 73.63±0.18 | 54.53±0.7 | 68.71±0.44 |
| 300 | 74.9±0.72 | 56.2±2.01 | 70.71±1.26 |
| 330 | 75.15±0.44 | 57.62±0.2 | 73.31±0.27 |
| 360 | 77.5±1.51 | 58.6±1.77 | 74.12±1.98 |

Uttarakhand. Casein was prepared from the collected milk sample using the method of isoelectric precipitation. Immediately after collection, milk was defatted by centrifuging twice at 5000 g for 20 min at 4℃ 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 [10].

Hydrolysis of casein: Casein prepared isoelectrically was treated with three different enzymes according to the method of Abubakar et al. [11] and Pihlanto-Leppala et al. [12] with some modifications (enzyme: substrate ratio is taken as 1:100) (Table-1). Initially, casein was incubated for 30 min, 60 min, 90 min, 120 min, 150 min (so as to simulate human digestive tract conditions where protein takes maximum about 120 minutes to get digested) and after that for 180 min, 240 min, 300 min and 360 min to access the effect of higher incubations. The degree of hydrolysis in various samples was estimated by using Hull's method [13].

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. [14] with some modifications. Each sample assay is carried out in triplicate & data are represented as a mean of three values along with the standard deviation.

Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) of hydrolysates: The hydrolysates showing maximum activity were resolved on RP-HPLC for separation of different fractions of peptides following the procedure given by Hernandez-Ledesma et al. [15]. All reagents for HPLC were prepared using HPLC grade water and solvents. These including acetonitrile used for preparation of solvents were membrane (0.22 µ PVDF, Millipore, SA, France) filtered and degassed using Millipore filtration assembly.

## Results

The total protein content of casein as estimated by Lowry's method was found to be 220ug/0.01ml of sodium caseinate [containing 3gm casein/50ml of distilled water]. Degree of hydrolysis (DH) was determined by quantification of protein in these hydrolysates. The higher the DH, the higher the content of released amino groups. DH is reported to affect the biological activity of protein hydrolysates. Therefore, the biological activity of peptides depends on the protein substrate, enzyme specificity, and hydrolysis conditions [16-18]. Degree of hydrolysis found maximum with pepsin treatment. This shows that pepsin utilized more protein as substrate to cause hydrolysis as compared to chymotrypsin and trypsin. Treatment with trypsin yields minimum DH indicating that yak casein is most resistant to this enzyme. Treatment of yak casein with pepsin for 360 min shows highest hydrolysis. The same result of hydrolysis obtained when treated with chymotrypsin and trypsin. It shows that degree of hydrolysis increases with the time of incubation.

The comparative inference of antioxidant status of hydrolysates with reference to different incubation periods were tabulated (Table-2). Tryptic hydrolysates showed highest activity followed by chymotryptic and peptic hydrolysates. The antioxidant activity goes on increasing with the incubation period.

Hydrolysates showing maximum antioxidant activity were further subjected to RP-HPLC. Treatment with trypsin results in 6-8 peaks at 214 nm and 2-3 peaks at 280 nm were observed (Figure 1(a) & 1(b)). However, the peptic hydrolysate shows 3-4 peaks at 214 nm and

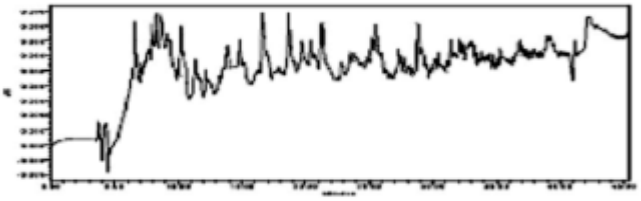

Fig.1(a): Elution profile of tryptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 214nm

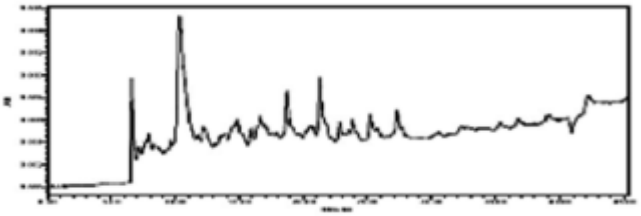

Fig.1(b): Elution profile of tryptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 280nm

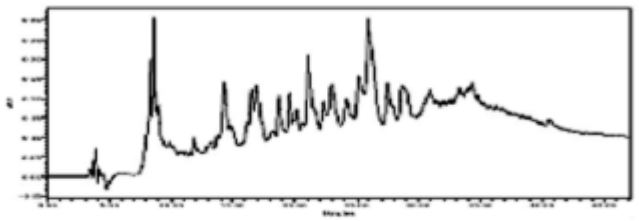

Fig.2(a): Elution profile of peptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 214nm

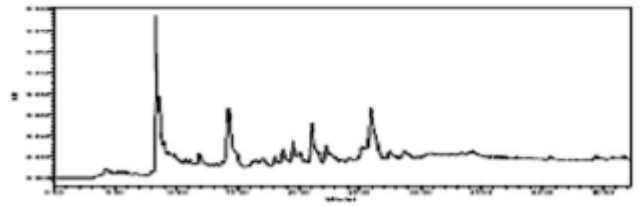

Fig.2(b): Elution profile of peptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 280nm

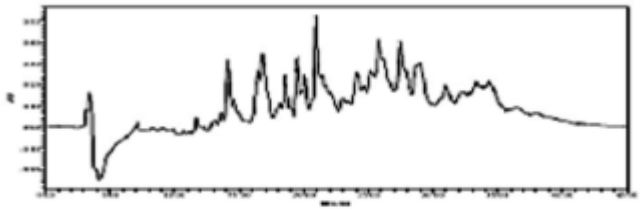

Fig.3(a): Elution profile of chymotryptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 214nm

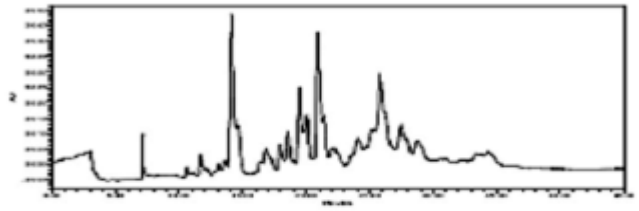

Fig.3(b): Elution profile of chymotryptic hydrolysate (2 hrs) on C18 column (RP-HPLC) at 280nm

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)). In all these chromatograms only the major peaks showing significant rise from the baseline has been considered and taken into count.

## 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 [16-18]. DH found to be maximum with pepsin treatment, while in case of cow and buffalo caseins DH is usually high with trypsin. This difference can be attributed to difference in amino acid sequence of yak casein from that of cow and buffalo casein e.g., in yak casein there is glycine and lysine at 192 and 59 positions in $\alpha s^1$ casein while in cow and buffalo its glutamate and glutamine respectively [19]; similarly glycine, threonine and isoleucine at 33, 47 and 130 position in $\alpha s^2$ casein in yak while its glutamate, alanine and threonine respectively in cow and buffalo [20]; and alanine in yak κ casein at 148 position while its aspartate in cow and buffalo casein [21]. This shows that pepsin utilized more protein as substrate to cause hydrolysis as compared to chymotrypsin and trypsin.

Scavenging activities of yak 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. Proteolysis of food proteins is usually reported to enhance the DPPH-scavenging activity of hydrolysates [7]. 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 [22]. Here also, the higher DPPH-scavenging activity was evidenced with higher degree of hydrolysis. Nevertheless, this is not always observed [23]. Specifically, bovine casein hydrolysates obtained with diverse proteolytic enzymes were shown to possess lower DPPH activity than the whole protein [24].

Maximum percentage inhibition was shown by peptic hydroysates. 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 was found to increase with the time of incubation in case of all the enzymes.

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 (aqueous) peaks represent the presence of hydrophilic peptides and for solvent B (organic) peaks represent hydrophobic peptides.

All the enzyme hydrolysates produce peaks mainly in the region of solvent B indicating the presence of hydrophobic peptides at both the wavelengths. So from the graph it can be concluded that above said hydrolysates consist of mainly hydrophobic peptides which were aromatic and/or non-aromatic in nature. From the observed pattern of DPPH-scavenging activity, caseinates 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. Histidine, Phenylalanine, Tyrosin, Tryptophan among other aromatic and hydrophobic amino acids, seem to be involved in the antioxidant activity of protein hydrolysates [7,16,25].

## Conclusion

Yak 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 yak 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 yak caseinate hydrolysates are under investigation.

## Authors' contributions

SK planned the entire work and carried out the laboratory analysis with help of AS. UVST helped in interpretation of HPLC graphs. VSC provided the yak casein. All authors read and approved the final manuscript.

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## References

1. FitzGerald, R.J., Murray, B.A. and Walsh, D.J. (2004) Hypotensive peptides from milk proteins. *J Nutr.*, 134: 980S–988S.
2. Haque, E. and Chand, R. (2008) Antihypertensive and antimicrobial bioactive peptides from milk proteins. *Eur Food Res Technol.*, 227: 7–15.
3. Sarmadi, B.H. and Ismail, A. (2008) Antioxidative peptides from food proteins: a review. *Peptides.*, 31: 1949–1956.
4. Silva, S.V. and Malcata, F.X. (2005) Casein as source of bioactive peptides. *Int Dairy J.*, 15: 1–15.
5. Korhonen, H. (2009) Milk-derived bioactive peptides: from science to applications. *J Func Foods.*, 1: 177–187.
6. Korhonen, H., and Pihlanto, A. (2006) Bioactive peptides: Production and functionality. *Int Dairy J.*, 16: 945–960.
7. Phelan, M., Aherne, A., FitzGerald, R.J. and O'Brien, N.M. (2009) Casein-derived bioactive peptides: biological effects, industrial uses, safety aspects and regulatory status. *Int Dairy J.*, 19: 643–654.
8. Minervini, F., Algaron, F., Rizzello, C.G., Fox, P.F., Monnet, V. and Gobbetti, M. (2003) Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from Lactobacillus helveticus PR4 proteinase-hydrolyzed caseins of milk from six species. *Appl Environ Microbiol*., 69: 5297–5305.
9. Benkerroum, N. (2010) Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. *Int J Dairy Technol*., 63: 320– 338.
10. Lowry. O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with folin-phenol reagent. *J. Biol. Chemistry*., 193: 265-275.
11. Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y. and Itoh, T. (1998) Structural analysis of new antihypertensive peptide by proteinase K digestion. *J.Dairy Sci*., 81:3131-3138.
12. Pihlanto-Leppala, A., Koskinen, P., Piilola, K., Tupasela, T. and Korhonen, H. (2000) Angiotensin -1 converting enzyme inhibitory properties of whey protein digest: Concentration and characterization of active peptide. *J.Dairy Res.*, 67:53-64.
13. Hull, M.E. (1947) Studies on Milk Proteins. II. Colorimetric Determination of the Partial Hydrolysis of the Proteins in Milk. *J. Diary Sci.*, 30 (11): 881-884.
14. Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995) Use of free radical method to evaluate antioxidant activity. *LWT Food Sci Technol.*, 28: 25–30.
15. Hernandez-Ledesma, B., Recio, I., Ramos, M. and Amigo, L. (2002) Preparation of ovine and caprine ß-lactoglobulin hydrolysates with ACE inhibitory activity: Identification of active peptides from caprine ß-lactoglobulin hydrolyzed with thermolysin. *Int. Dairy J.*, 12: 805-812.
16. Sarmadi, B.H. and Ismail, A. (2010) Antioxidative peptides from food proteins: a review. *Peptides.*, 31: 1949–1956.
17. Hogan, S., Zhang, L., Li, J., Wang, H. and Zhou, K. (2009) Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef. *Food Chem.*, 117: 438–443.
18. Zhang, L., Li, J. and Zhou, K. (2010) Chelating and radical scavenging activities of soy protein hydrolysates prepared from microbial proteases and their effect on meat lipid peroxidation. *Bioresour Technol.*, 101: 2084–2089.
19. Grosclaude, F., Mahé, M.F. and Mercier, J.C. (1974) Comparaison du polymorphisme génétique des lactoprotéines du zébu et des bovins. *Annales de Génétique et de Sélection Animale*, 6: 305-329.
20. Grosclaude, F., Mahé, M.F., Mercier, J.C., Bonnemaire, J. and Teissier, J.H. (1976b) Polymorphisme des lactoprotéines de bovinés népalais. II. Polymorphisme des caséines as-mineures; le locus as2-Cn est-il lié aux loci as1-Cn, ß-Cn et -?Cn? *Annales de Génétique et de Sélection Animale* 8(4): 481–491.
21. Sulimova, G.E., Badagueva, IuN., Udina, I.G. (1996) Polymorphism of the kappa-casein gene in populations of the subfamily Bovinae. *Genetika*. 32 (11): 1576-1582.
22. Mao, X.Y., Cheng, X., Wang, X. and Wu, S.J. (2011) Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis. *Food Chem.*, 126: 484–490.
23. Chang, C.Y., Wu, K.C. and Chiang, S.H. (2007) Antioxidant properties and protein compositions of porcine haemoglobin hydrolysates. *Food Chem.*, 100: 1537–1543.
24. Rival, S.G., Boeriu, C.G. and Wichers, H.J. (2001) Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *J Agric Food Chem*., 49: 295–302.
25. Saiga, A., Tanabe, S. and Nishimiura, T. (2003) Antioxidant activity of peptides obtained from porcinemyofibrillar proteins by protease treatment. *J Agric Food Chem*. 51:3661–3667.

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