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Casein hydrolysates as natural biological antioxidants produced by different industrial proteases: A review

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Abstract
Enzymatic hydrolysis is a process in which enzymes directly cleave the bond in molecules and leads to formation of degraded products. Careful selection of enzymes and evaluation of parameters are essential to maximize the desired products. Much of the bioactivity in milk is attributed by milk proteins and their constituent peptides. These peptides are inactive within the sequence of the native protein but hydrolysis can release active peptides. According to the World Health Organization (WHO), cardiovascular diseases (CVDs) are the number one cause of death globally, 80% of which occurs in lower and middle income countries with an estimated 23.6 million people likely to die of CVDs, mainly heart disease and stroke, by 2030. So there is need for natural biological antioxidants for reduction of oxidative stress without themselves being destroyed, repairing damage and preventing chronic diseases.

Keywords: Cardiovascular disease (CVD), Casein hydrolysates, world health organization (WHO)

Introduction
Reactive oxygen species and free radicals are produced in our body through life-style activities such as smoking and strenuous exercise and also through insufficient consumption of colorful fruits and vegetables. This can lead to an increase in oxidative stress and in turn can lead to damaged DNA, protein and lipid which finally leads to chronic diseases, such as cardiovascular diseases, asthma, diabetes and osteoporosis. Milk provides the sole source of nutrition in the early stages of life. In addition to its fundamental nutritional role, the bio functional potential of milk is now being recognized. Bioactive peptides can be released naturally from proteins during the digestion process in the gastrointestinal tract which possess limited antioxidant activity due to specificity of gastrointestinal proteases (Martinze *et al*. 2013; Pihlanto, 2006 & 2010) [23, 29, 30]. The composition of hydrolysates mainly dependent on the protein substrate, Proteolytic enzyme, physicochemical conditions: pH, hydrolysis time and temperature of reaction (Parmar and Bajaj 2014) [26]. *In vitro* enzymatic hydrolysis of milk proteins can modify the bio-functional properties of the generated hydrolysates. Enzymatic hydrolysis cause three distinct effects: Decrease in molecular weight, increase in the number of ionizable groups, exposure of hydrophobic groups (Panyam and Kilara., 1996) [9]. Enzyme catalyzed hydrolysis under milder conditions, making substrates less damaging. Most widely used Industrial enzymes Alcalase, Flavourzyme and Neutrase derived from microorganisms (*B Licheniformis*, *A Oryzae* and *B amyloliquefaciens* respectably) as well as enzymes from plant (e.g. papain) and from animal sources (e.g., Pepsin, Trypsin & Chymotrypsin) for antioxidative peptides generation (Kristinsson & Rasco, 2000) [18]. Alcalase, Flavourzyme and Neutrase have active pH range 6-8 with optimum temperature 50^0C but for Pepsin, Trypsin and Chymotrypsin pH range varied as 1-4, 7-9, 8-9 respectably at 37^0C Alcalase Pepsin, Trypsin Chymotrypsin and Neutrase are endopeptidase that breaks peptide bonds of non terminal amino acids (i.e. within the molecule) and flavourzyme is an exopeptidase, which break peptide bonds from their end-pieces. Pepsin, alcalase and flavourzyme have specificity for hydrophobic-COOH groups, Trypsin and Chymotrypsin have specificity for lys-arg-COOH and phe-tyr-trp-COOH respectively (Qian *et al*., 2007; Liu & Zhao., 2010) [32, 22]. Casein hydrolysates generated with different proteases exhibited varying antioxidant activities (Phelan, *et al*., 2009) [28]. Present review mainly focuses on antioxidative hydrolysates produced by Hydrolysis of Casein using different industrial Proteases.

Major essencial characteristics of antioxidative casein fractions

Antioxidants can inhibit oxidation by different mechanisms such as scavenging of free radicals, cheltion of metal ions etc (valko *et al*., 2007; Welch *et al.,* 2002) [39-40]. Antioxidant activity of milk proteins depends on physicochemical properties of the amino acid. Amino acids with an aromatic ring structure such as His, Trp, Tyr, Phe and Pro can donate a proton to electron deficient radicals (Chen *et al.,* 1998) [6]. The presence of hydrophobic and aromatic amino acid residues is a determinant factor in radical scavenging (Ajibola *et al.,* 2011, Parmar and Bajaj 2014) [1, 26]. Hydrophobic residues Val, Leu and Tyr can enhance the solubility of peptides in a lipid matrix improving the accessibility to hydrophobic radical species or polyunsaturated fatty acids (Qian *et al.,* 2008) [31]. Met and Cys are effective in quenching of free radicals due to their ability to donate sulphur hydrogen (Ajibola *et al.,* 2011; Xiong, 2010) [1, 41]. Antioxidative peptides have molecular weights ranging from 500 to 1800 Da usually composed of 3 to 16 amino acid residues (Kim *et al.,* 2007; Jun *et al*., 2004; Ranathunga *et al.,* 2006; Chen, 2003) [16, 15, 34, 7]; Among these peptides, some contained hydrophobic amino acids Val or Leu at the N-terminus and Pro, His, or Tyr in sequences (Chen *et al*., 1995) [5]. Peptide with a pro-his –his sequance showed the greatest antioxidant activity among the tested peptides (Pihlanto, A., 2006) [29] For this reason there are multiple ways to measure the antioxidant activity of a compound and the use of different assays to confirm the antioxidant activity (Huang *et al*., 2005) [14]. Based on electrophoretic separation and amino acid sequence four major families of casein have been identified: αs1, αs2, β, κ. (Farrel *et al*., 2004) [11]. Both buffalo and cow β-CN have the same amino acid sequence in some of the region. The differences in amino acid sequence among buffalo and cow casein also observed in all the casein fractions as listed in table 1. The differences between the amino acid positions in the sequence plays, an important role to change the properties of specific casein fractions. As sequence of cow and buffalo casein varied at position 4, 14, 42, 105, 115, 119, 148 for α_{s1}-CN, 2, 29, 144, 147, 157, 170, 176, 186, 199 for α_{s2}-CN and 25, 41, 68, 92,148 for β-CN. Major feature of buffalo α_{s1}-casein with respect to the homologous cow casein is the lack of phosphorylation at site 115, as a result of the substitution PSer115—>Leu. Buffalo α_{s1}-casein, α_{s2}-casein and β-casein (β-CN) have similar phosphoserine clusters as in their cow counterparts. Therefore, proteolysis of buffalo casein fractions would yield phosphopeptides similar to that obtained from cow milk caseins (Hernandez *et al*., 2011) [13]. For the generation of antioxidative peptides, there has to be more hydrophobic amino acid in the sequence, buffalo casein fractions have more hydrophobic amino acids compared to cow fractions. In α_{s1}-CN at position 74, 174, 178 hydrophobic amino acids (ile, pro, leu) present in buffalo casein sequence, but at same position for cow casein asn, thr, ser amino acids present. In α_{s2}-CN at position 175, 194 hydrophobic amino acids (ala, ile) present in sequance but at same position for cow casein thr amino acid present. In β-CN at position 92,148, hydrophobic amino acids (val, his) present in sequence but at same position for cow casein ile, pro amino acids present, this mainly decrease the chances for generation of hydrophobic amino acids from cow casein.

Table 1: Impact of casein sequence variations on buffalo casein fractions (Farrel *et al*., 2004) [11]

Residue Variations		Impact of sequence on buffalo casein properties
cow	Buffalo	
(α_{s1}-CN)		
glu (14) pser (115) glu (148)	Gly Leu Gln	Negative charge decrease
his (4) lys (42) lys (105) arg (119)	Gln Thr Asn Gln	Positive charge decrease
asn (74) thr (174) ser (178)	Ile Pro Leu	Increase in hydrophobicity
(α_{s2}-CN)		
his (2) his(29)	Asn Asn	Positive charge decrease
tyr (186) asn (199)	His Lys	Positive charge increase
ile (144) ile (147)	Val Phe	Hydrophobicity decrease
thr (175) thr (194)	Ala Ile	Hydrophobicity Increase
asp (157)	Glu	Polarity decrease
his (170)	Arg	Polarity increase
trp (176)	Leu	No change
(β-CN)		
his (25)	Arg	Negative charge increases
met (41)	Thr	Increase in hydrophobicity
lys (68)	Asn	Negative charge increases
ile (92)	Val	Increase Hydrophobicity
pro (148)	His	Increase Hydrophobicity

Effect of different industrial proteases (Animal/ plant/ microbial sources) for generation of Antioxidative casein Hydrolysates

Bioactive peptides released by enzymatic hydrolysis achieve a therapeutic effect as nutraceuticals. Industrial enzymes, from microorganisms (Alcalase, Flavourzyme and Protamex) from plant (e.g. papain) and from animal sources (e.g., Pepsin, Trypsin & Chymotrypsin) widely used for production bioactive peptides (Kristinsson & Rasco, 2000) [18]. Most of work has been done by using digestive enzymes pepsin, Trypsin, pancreatin, Chymotrypsin for *In vitro* digestion of proteins. Cow whole casein hydrolysed by pepsin and corolase pp gives 0.80µmol (TEAC) but for αs, β, κ casein fractions activity was 1.06, 0.62, 0.44 µmol (TEAC). Buffalo whole casein hydrolysed by same enzymes gives 1.01, 1.40, 0.91, 0.72 µmol (TEAC) respectively, which was comparatively high for buffalo casein hydrolysates (Bajaj *et al*., 2010). 3kDa fractions of pepsin digest from buffalo αs, β, κ casein fractions give 2.23, 2.38, 2.23, 2.26 µmol (TEAC) and for corolase pp 3.21, 2.95, 3.19, 2.86 µmol (TEAC). Buffalo whole sodium caseinate hydrolysed by alcalase, flavourzyme alone and combination gives 22mM, 19mM and 31mM (TEAC) respectively (Parmar *et al.,* 2015) [25]. Pepsin digestion of cow αs1-CN fraction hydrolysates provide peptides (1.tyr–phe–tyr–pro-glu–leu, 2.phe–tyr–pro–glu–leu, 3.tyr–pro glu–leu, 4.pro–glu leu, 5.glu–leu) with strong superoxide and DPPH scavenging activity (Suetsuna *et al*., 2000) [37] on the other hand Trypsin digestion of cow β -CN fraction hydrolysates provide peptides, βCN(98–105) [val–

lys–glu–ala–met–pro–lys], βCNf(177–183)[ala–val–pro–tyr-pro–gln–arg], βCNf(169–176) [lys–val–leu–pro–val–pro–glu–lys] and βCNf(170-176) [val–leu–pro–val–pro–glu–lys] have 0.095,1.0,0.99,1.05 DPPH radical scavenging activity (Rival *et al*.2001) [35] and βCaseinf(169-176) [lys-val-leu-pro-val-pro-gln-lys] have ORAC activity. Alcalase derived hydrolysates contain AO peptides with shorter amino acid sequences and more resistant to digestive enzyme (Sarmadi and Ismail 2010) [36]. Twenty five peptides were identified from with crude proteinases from *L. helveticus* NCDC288 described as antioxidative peptides (Ramesh *et al.*, 2011) [33]. Casein phosphopeptides effective against H_2O_2-induced stress (Laparra *et al*., 2008) [20] and metal chelators as they bind pro-oxidant metals, such as iron and inhibit lipid oxidation (Kim *et al*., 2007) [16]. Pepsin and pancreatin digest of human milk β casein fraction (154-160) [trp–ser–val–pro–glu–pro–lys] provides 1.7 µmol TE antioxidative capacity (Hernandez-Ledesma *et al.*, 2007) and Peptides tyr–gly–tyr–thr–gly–ala, ile–ser–glu–leu–gly–trp have 5169 and 4479 µM TE antioxidative capacity respectively (Tsopmo, *et al*., 2009) [38]. Pepsin and Chymotrypsin digest of ovine milk κ casein fraction (98-105) [his-pro-his-pro-his-leu-ser-phe] gives 1.5mg/ml TE antioxidative capacity (Gomez *et al*., 2008) [12]. Neutrase digest of goat whole casein gives 244.275 µg/ml DPPH activity, 81.41 µg/ml hydroxyl radical scavenging and 0.449 µg/ml ABTS activity which was mainly due to five isolated peptides val-tyr-pro-phe, phe-pro-tyr-cys-ala-pro, phe-gly-gly-met-ala-his, phe-gly-gly-met-ala-his, tyr-val-pro-glu-pro-phe (Li *et al.,* 2013) [21]. Yak casein hydrolysates treated with pepsin, chymotrypsin and Trypsin, out of three proteases Tryptic hydrolysates showed highest activity followed by chymotryptic and peptic hydrolysates (Kumar *et al*., 2013) [19].

Conclusion

Casein hydrolysates, generated by alone or cocktail of different food grade enzymes is a emerging approach to link the innovations to commercial levels, but important factors which should be evaluated before selection of enzymes are cost of purified enzymes, their availability, thermal stability. This makes the implementation to a great success.

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