EVALUATION OF ANTIOXIDANT PEPTIDES ENRICHED CASEIN HYDROLYSATE AND THEIR APPLICATION IN WHEY BASED MODEL SYSTEM

THESIS SUBMITTED TO
ICAR- NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE

MASTER OF TECHNOLOGY
IN
DAIRY CHEMISTRY
BY

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Approved by:

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ABSTRACT

Milk proteins are the important source of bioactive peptides. The ability of peptides to interact with radical species or to inhibit oxidative reactions could lead to the development of novel functional ingredients relevant in health promotion and disease prevention. Buffalo milk casein shows high homology to that from cow milk casein, therefore, can serve as a potential precursor for bioactive peptides with diversified functionality. In the present study hydrolysis of buffalo casinate (5% w/v) was carried out using commercial food grade proteases alcalase, flavourzyme alone and in combination. Based on optimized condition of hydrolysis under pH-Stat, three types of hydrolysates were prepared CH1(Alcalase-flavourzyme), CH2 (alcalase) and CH3 (flavourzyme) . Hydrolysate (CH1) showed significantly ($p<0.05$) higher antioxidant activity (AO) as compared to CH2 and CH3.Maximum AO was retained in UF (1kDa MWCO) fractions.

Based on separation with size exclusion chromatography, it was observed that CH1 with 1-0.5 kDa peptides covering 14.49% area of chromatogram showed maximum AO i.e. 5.45 mM TEAC/mg peptideunlike CH2 and CH3 with 1.22% and 36.5% respectively. UF fractions (3 kDa and 1kDa) of CH1, CH2 and CH3 hydrolysates were subjected to RP-HPLC, the peak fractions were pooled and identified for antioxidant activity. 1 kDa fraction of CH 1 hydrolysate showed higher antioxidant activity in peak 4 which on further subjecting to HPLC-TOF-ESI separation four potential antioxidant peptides were identified. On amino acid analysis of 1kDa fraction of hydrolysates showed high content of proline, leucine, isoleucine, valine, alanine phenylalanine and histidine, which might be responsible for high antioxidant activity.

The incorporation of casein hydrolysate in whey drink (0.5%) raised their antioxidant activity nearly fourfold as compared to control. The product retained over 85% level of initial antioxidant activity after storage for 3 weeks at 4°C.Casein hydrolysate with good nutritional, biological and techno functional properties can be effectively used in formulated foods as biofunctional ingredients.

ऑक्सीकरणरोधी पेप्टाइड्स संवर्धित कैसिइन जलाअपघटनजो का मूल्यांकन तथा व्हेय पेय पदार्थ आधारित मॉडल में उपयोग

दुग्ध प्रोटीन जैवसक्रिय पेप्टाइड्स के महत्वपूर्ण स्रोत हैं । स्वास्थ्य संवर्धन और रोग की रोकथाम में प्रासंगिक पेप्टाइड्स की क्षमता रेडिकल प्रजातियों के साथ परस्पर प्रभाव के लिए या ऑक्सीडेटिव प्रतिक्रियाओं को बाधित करके नव कार्यात्मक सामग्री के विकास के लिए नेतृत्व कर सकते हैं ।भैंस दुग्ध कैसिइन से गाय दुग्ध कैसिइन की उच्चअनुरूपता का पता चलता है इसलिए विविधकार्यक्षमता के साथ जैवसक्रिय पेप्टाइड्स के लिए एक संभावित अग्रदूत के रूप में कार्य कर सकते हैं । वर्तमान अध्ययन में भैंस caseinate की जल-अपघटन में(w/v 5%) वाणिज्यिक खाद्य ग्रेड प्रोटिएजों alcalase अकेले flavourzyme अकेले और संयोजन का उपयोग किया गया था। पीएच-स्टेट के तहत जल-अपघटन की अनुकूलित हालत के आधार पर जलाअपघटनजो के तीन प्रकार CH1(Alcalase-flavourzyme) CH2(alcalase) और CH3 (flavourzyme) तैयार किये गए थे। CH2 और CH3 की तुलना में जलाअपघटनजो (CH1) ने महत्व के साथ उच्च (पी <0.05) ऑक्सीकरणरोधी सक्रियता (एओ) प्रदर्शित की अधिकतम ऑक्सीकरणरोधी सक्रियता (एओ) अतिनिस्पंदित (1के.डी.ए कट-ऑफ) भागों में बरक़रार रही ।
आकार अपवर्जन वर्णलेखन द्वारा पृथक्करण के आधार पर CH1 के 1-0.5 केडीए पेप्टाइड्स जो वर्णलेख के 14.49% क्षेत्र को आच्छादित करते हैं में अधिकतम ऑक्सीकरणरोधी सक्रियता 5.45 mM TEAC/mg पेप्टाइड्स के साथ CH2 और CH3 के विपरीत क्रमश: 1.22% और 36.5% प्रदर्शित करते है । CH1, CH2 और CH3 जलाअपघटनजो के यूएफ घटकों (1 के.डी.ए और 3 के.डी.ए) को आरपी-एचपीएलसी द्वारा पृथक्करण के आधार पर, शिखर घटकों का एकत्रीकरण किया गया और ऑक्सीकरणरोधी सक्रियता के लिए पहचान की गई। CH1 जलाअपघटनजो के 1के.डी.ए घटक के शिखर घटक 4 मे अधिकतम ऑक्सीकरणरोधी सक्रियता प्रदर्शित हुई जिसे एचपीएलसी-TOF-ईएसआई द्वारा पृथक्करण के आधार पर चार संभावित एंटीऑक्सीडेंट पेप्टाइड्स की पहचान की गई है। जलाअपघटनजो की 1kDa घटक का अमीनो एसिड विश्लेषण पर प्रोलाइन, लुसिने , इसोलुसिने, वेलिन, एलनाइन, फेनिलएलनिन और हिस्टडीन की उच्च मात्रा प्रदर्शित हुई जो उच्च ऑक्सीकरणरोधी सक्रियता के लिए जिम्मेदार हो सकता है।

व्हेय पेय पदार्थ (0.5%) में कैसिइन जलाअपघटनजो के समावेश से कंट्रोल की तुलना में ऑक्सीकरणरोधी सक्रियता मे लगभग ४ गुना की उन्नति हुई ।उत्पाद 4 डिग्री सेल्सियस पर 3 सप्ताह के लिए भंडारण के बाद प्रारंभिक ऑक्सीकरणरोधी सक्रियता के 85% के स्तर पर बरकरार रखा ।पोषणिक,जैविक और तकनीकी कार्यात्मक गुणों के साथ कैसिइन जलाअपघटनजो को प्रभावी ढंग से जैवकार्यात्मक सामग्री के रूप में तैयार खाद्य पदार्थों में इस्तेमाल किया जा सकता है।

INTRODUCTION

Milk has long been consumed and promoted for its nutritional properties, specifically as rich source of protein, energy, minerals, especially calcium and vitamins. Among all the constituents of milk, protein is of paramount significance for its unique physiochemical and functional attributes. Caseins are one of the most important and complex proteins in milk representing approximately 80% of the total protein fraction. Milk proteins, like a number of other food proteins, contain sequences that, when liberated as peptides by fermentation or enzymatic hydrolysis, exert beneficial physiological effect. Such bioactive peptides can affect the cardiovascular system by exerting antithrombotic, antihypertensive, opioid, antioxidant, or immunomodulatory effects among others (Korhonen and Pihlanto, 2006; Phelan *et al*. 2009).

Formation of free radicals, such as superoxide anion radical and hydroxyl radical, is an unavoidable consequence in aerobic organisms during respiration. These radicals are very unstable and react rapidly with other groups of substances in body, leading to cellular and tissue injury. Under normal conditions, antioxidant defence systems can remove reactive species through enzymatic (like superoxide dismutase and glutathione peroxidase) and non-enzymatic antioxidants (such as antioxidant vitamins, trace elements, coenzymes and cofactors) (Johansen *et al*., 2005). Food supplements containing antioxidants components may be used to help the human body to reduce free radicals (Zommara *et al*., 1996; Oxman *et al*., 2000; Terahara *et al*., 2001; Kullissar *et al*., 2003).

Antioxidant peptides are particularly interesting because they can potentially prevent or delay oxidative deterioration of foods and thus prolong shelf-life (Ledesma *et al*. 2005; Pihlanto, 2006; Suetsuna *et al*. 2000). Antioxidant compounds can inhibit oxidation by different mechanisms, such as scavenging of free radicals, a major source of oxidation (Valko *et al*., 2007), or chelation of metal ions(e.g., Fe^{2++}), an initiator of oxidation (Welch *et al*. 2002). For this reason there are multiple ways to measure the antioxidant activity of a compound and the use of different assays is preferable to confirm the activity

(Huang *et al.,* 2005). It is also important, when testing potential antioxidants, to include evaluation methods with specific food related oxidation substrates (Becker *et al*., 2004).

Casein hydrolysate used as a functional ingredient in health-promoting foods. From functional perspective, casein hydrolysates are highly soluble over a wide pH range and contribute emulsifying, foaming, gelling, properties to food and beverages system. The presence of bitter peptides in hydrolysate poses a challenge for their utilization in functional beverage.

A limited number of studies conducted to date for assessing the antioxidative potential of buffalo casein hydrolysates (Kumar *et al.,* 2010, Shanmugam *et al*., 2014). The major buffalo milk proteins show high homology to that from cow milk proteins, therefore, these are the potential precursors for bioactive peptides with diversified functionality. However, the presence of encrypted bioactive peptide in a protein does not mean that it can be obtained from the digestion of this precursor by proteolytic enzymes, e.g., digestion of buffalo β casomorphine containing fragment (f 49-68) with pancreatic juice did not yield β casomorphine (Petrilli *et al*. 1987). Further, minute substitutions/additions or deletions of amino acids in primary structure of buffalo casein might produce changes in the production of bioactive peptides with potential bioactivity.

Therefore the present study has been formulated to optimize the process for biofunctional buffalo casein hydrolysates with high antioxidant activity and their characterization with respect to their biological property and utilization for the development of functional whey beverage with the following objectives:

1. Optimization of condition for production of AO peptides enriched buffalo casein hydrolysate using pH-stat auto-titrator
2. Evaluation of Techno-functional properties of AO enriched buffalo casein hydrolysate
3. Evaluation functionality of whey beverage enriched with buffalo casein hydrolysate

REVIEW OF LITERATURE

Milk forms a rich source of biologically active components in particular its protein fraction is known to encompass many health promoting effects. Milk protein derived biologically active peptides are currently the subject of intensive research. These bioactive peptides are sequence of amino acids that are encrypted within the primary structure of milk proteins, requiring proteolysis for their release from precursor. Biological activities associated with such peptides include immune-modulatory, antibacterial, anti-hypertensive, antioxidative and opioid-like properties (Miesel, 2004). Proteolytic activation of bioactive sequence by enzymatic hydrolysis has great advantage of using different proteolytic enzyme to enrich foods with bioactive substances. Thus, proteolytic enzyme could potentially generate a large variety of peptides, including bioactive sequences based on the specificity of proteolysis.

2.1 Bioactive Peptides

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts and Weiler, 2003). Bioactive peptides are encrypted within the sequence of proteins. They are released by the action of proteolytic enzymes, and have an impact on cells or tissues of the body. Bioactive peptides derived from milk protein have been associated with a range of different health-related effects. There is a great potential in bioactive peptides for optimizing nutrition-based interventions in the prevention of lifestyle diseases (Figure 2.1) such as cardiovascular diseases, type-2 diabetes, hypertension, or obesity. Two well-described effects of milk peptides are antioxidant capacity and angiotensin-1 converting enzyme (ACE) inhibition (FitzGerald and Meisel, 2000; Pihlanto, 2006). Studies have shown that both these activities are highly dependent on the amino acid composition, as well as the specific amino acid sequence of the peptides (Chen *et al.*, 1998). Since proteolytic enzymes hydrolyze peptide bonds according to their pre-determined specificity, a given combination of protein and protease will result in a unique combination of peptides.

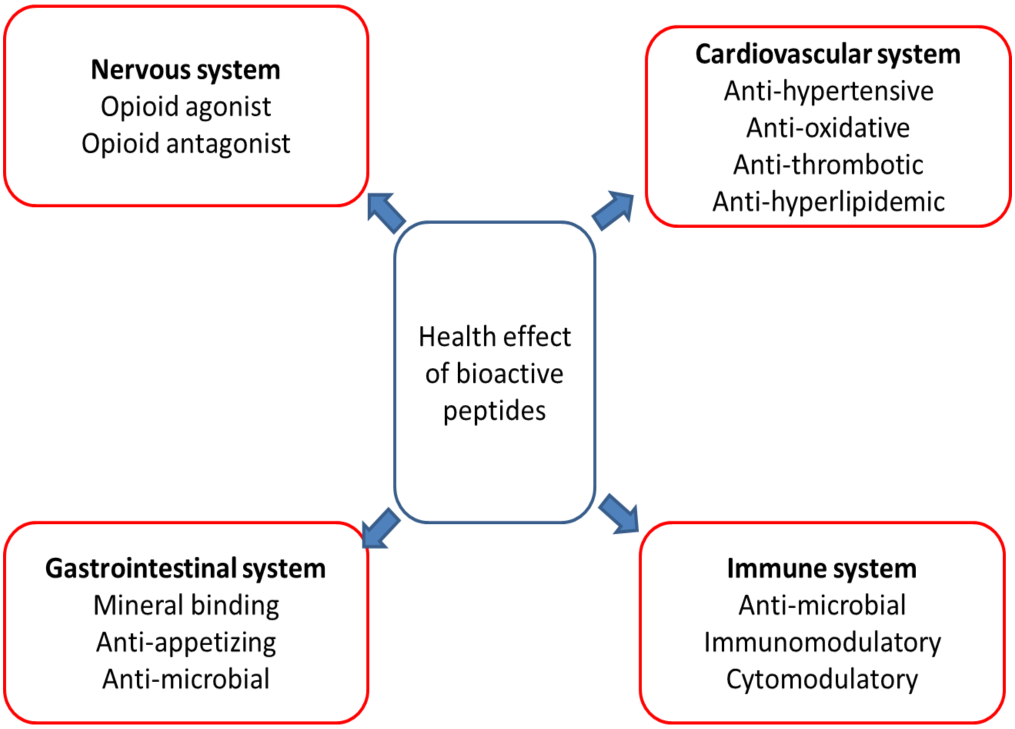

Figure 2.1: Physiological effects of food derived bioactive proteins on major body systems.

2.2 Production of bioactive peptides

Bioactive peptides are formed from precursor inactive proteins and can be released by following four ways (Meisel, 2001).

1 Enzymatic hydrolysis with digestive enzymes
2 Fermentation with proteolytic starter cultures
3 Hydrolysis by enzymes derived from proteolytic microorganisms
4 Combination of fermentation and hydrolysis

2.2.1 Enzymatic hydrolysis

Hydrolysis of proteins involves the cleavage of peptide bonds to give peptides of varying sizes and amino acid composition. There are a number of types of hydrolysis; enzymatic, acid or alkali, hydrolysis. Chemical hydrolysis is difficult to control and reduces the nutritional quality of products (Celus *et al.*, 2007) destroying L-form amino acids and producing toxic substances such as lysino-alanine (Lahl and Braun, 1994). Enzymatic hydrolysis works without destructing amino acids and by avoiding the extreme temperatures and pH levels required for chemical hydrolysis, the nutritional properties of the protein hydrolysates remain largely unaffected (Celus *et al.*, 2007). Enzymatic hydrolysis of protein occurs under mild processing conditions of pH 6-8 and temperature 40-60ºC, which minimizes side reactions (Clemente, 2000).

Many well-known bioactive peptides have been generated *in vitro* by the action of digestive enzymes, mostly pepsin and chymotrypsin. Other digestive enzymes derived from plant, animal and microbial as well as enzymes derived from bacteria and fungi are also used for the production of bioactive peptides from various sources. These proteases which can be classified based on their origin, i.e. animal, plant or microbial origin, their mode of catalytic action, i.e. endo- or exo-activity, or on basis of their catalytic site. Endoproteases cleave amide bonds within the protein chain, contrary to exoproteases that remove terminal amino acids from proteins or peptides, either at the C-terminus (carboxypeptidases) or at the N-terminus (aminopeptidases). The nature of the catalytic site of proteases differs according to the active group that will form the enzyme/substrate intermediate. The active group can be either an amino acid, i.e. serine, cysteine or aspartic acid, or a metallo group, most often zinc (Adler-Nissen, 1993). Alcalase represents a good candidate for the industrial production of casein hydrolysate because of its endoprotease with broad specificity and its low cost. It is a serine alkaline protease produced by a selected strain of *Bacillus licheniformis*. The enzyme has a pH optimum for activity between pH 7 and 9 and broad pH stability. Alcalase was observed to have a high specificity for aromatic (Phe, Trp, and Tyr), acidic (Glu), sulphur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) residues (Dany Doucet *et al*., 2003).A number of studies have reported potent AO activity of alcalase derived hydrolysates (Peng *et al*., 2010). Compared to other enzyme hydrolysates, Alcalase derived hydrolysates contain a higher yield of AO peptides with shorter amino acid sequences and appear to be more resistant to digestive enzymes (Sarmadi and Ismail, 2010). Protein hydrolysates have a bitter taste and the elimination or reduction of this bitterness is essential to make the hydrolysates acceptable to consumers. The bitterness of protein hydrolysates is attributable to their hydrophobic amino acid content (Schaafsma, 2009) and the release of these amino acids by exoproteases can reduce bitterness (Pedersen, 1994).The hydrolysis degree and amino acid compositions have been reported to determine the antioxidant activity of hydrolysates (Kong and Xiong, 2006).

Type of protease	Name	Source	pH-range
Serine protease	Trypsin Chymotrypsin	Porcine, bovine	7-9 8-9
	Alcalase	*Bacillus Licheniformis*	6-10
Cysteine proteases	Papain	Papaya latex	5-8
	Ficin	Ficus latex	5-8
Aspartic protease	Pepsin	Porcine, bovine	1-4
	Aspergillopeptidase A	*Aspergillus satoi*	2-5
	flavourzyme	*Aspergillus oryzae*	5-8
Metallo protease	Neutrase	*Bacillus amyloliquefaciens*	5-7
	Thermolysin	*Bacillus thermoproteolyticus*	7-9

Table 2.1 Different commercial Proteases used for hydrolysis of food proteins (Adler-Nissen, 1993; Whitaker, 1994; Uhlig, 1998)

2.3 Degree of hydrolysis-

The actual number of peptide bonds cleaved during the reaction, or degree of hydrolysis (DH) is a useful way of monitoring the extent of protein degradation. DH is defined as the percentage of the total number of peptide bonds in a protein which have been cleaved during hydrolysis (Adler-Nissen, 1986).Several methods of monitoring the DH during protein hydrolysis have been described i.e. pH-stat, OPA, osmometry, soluble nitrogen content, and the trinitro- benzene-sulfonic acid (TNBS) method. The principle of the pH-stat technique is that when hydrolysis is carried out at neutral or alkaline conditions, dissociation of protons from the free amino groups released is favoured (Adler-Nissen, 1986). The liberation of protons into the surrounding medium leads to a reduction in the pH of the reaction mixture. The number of peptide bonds cleaved can be estimated from the amount of base required to maintain a constant pH during the reaction (Adler-Nissen, 1986).The OPA method is based on the specific reaction between OPA and primary amino groups, in the presence of a thiol to form 1-alkylthio-2-alkyl-substituted isoindoles (Medina *et al*., 1990b). The isoindoles formed can be quantified spectrophotometrically at 340 nm.

2.4 Antioxidant activity

Antioxidants are molecules that protect biological systems either by inhibiting or preventing the oxidation of substrate by free radicals (Serafini, 2006). The free radicals are chemical species that contain an unpaired electron in the outer orbital (Cheesman and Slatter, 1993). They are highly reactive as the unpaired electron in the outer orbit allows attraction of electrons from other molecules resulting in another free radical that is capable of reacting and damaging another molecule (Clarkson and Thompson, 2000). Free radicals such as superoxide (O_2^-), $OH^\bullet$ and other oxygen-derived species (H_2O_2, and hypochlorous acid) are formed constantly in the human body during oxygen metabolism in addition to exposure to oxidants such as air pollutants, ozone, oxides of nitrogen, tobacco smoke, and motor vehicle exhaust (Clarkson and Thompson, 2000). The free radicals can attack all macromolecules, causing lipid peroxidation (oxidative destruction of PUFAs in cell membranes), and DNA damage and protein degradation during oxidatively stressful events. Of these, unsaturated lipids are the most susceptible (Clarkson and Thompson, 2000).

Enzymatic antioxidants include the most important intracellular superoxide dismutase (SOD), glutathione peroxidase (GPx), which protects against low levels of oxidative stress and catalase (CAT), and the non-enzymatic antioxidants like ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavanoids (Perez-Campo *et al*., 1994; Thannickal and Fanburg, 2000; Nordberg and Arnér, 2001; Valko *et al*., 2006). Recent studies have shown that peptides with antioxidant activity can be released by food proteins such as milk casein (Suetsuna *et al*., 2000), whey protein (Hernandez-Ledesma *et al*., 2005) and egg proteins (Davalos *et al*., 2004). Proteins have excellent potential as antioxidant additives in foods because they can inhibit lipid oxidation through multiple pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems. Milk proteins are a good source of antioxidative peptides which plays a major role in enhancing aging induced loss of defense system against oxidative stress, inhibiting peroxidation of lipids in foods and living system (Korhonen and Pihlanto, 2006). The identification and development of food-derived peptides with antioxidant properties has attracted increased attention due to heightened safety concerns over the use of synthetic antioxidants and the consumer’s preference for naturally derived products.

2.4.1 Antioxidant activity of caseins

Milk proteins and peptides can act as antioxidants via primary and secondary mechanisms (Kitts, 2005). The former involves the process of antioxidants accepting free radicals to delay the initiation step, or interrupting the propagation step in auto-oxidation. The latter, however, includes the chelation of transition metals, replenishment of hydrogen to the primary antioxidants, oxygen scavenging and deactivation of reactive species (McClements and Decker, 2000). Caseins and other milk proteins are excellent precursors of AO peptides (Haque *et al.,* 2008; Su *et al.,* 2012; Power *et al.,* 2013). Clausen *et al.* (2009) demonstrated that caseins were quantitatively the major radical scavenging species in milk. Intact casein has been shown to possess AO activity against thiobarbituric acid reactive substances (TBARS) and in a model linoleic acid oxidation system (Laakso, 1984). Caseins have been shown to be antioxidants in phosphatidylcholine liposomes, where their ability to chelate copper and scavenge free radicals by tryptophan residues inhibited the formation of conjugated dienehydroperoxides and hexanal. Caseins were also antioxidants when ferric (50 μM) and ascorbic acid (500 μM) induced the oxidation of arachidonic acid in phosphatidylcholine liposomes. Higher amount of phosphoseryl residues in the casein subunits is generally correlated with higher antioxidant activity; however, dephosphorylation of caseins caused only a partial loss of antioxidant activity, indicating that caseins may also act as free radical scavengers (Bennett *et al.,* 2002). Kumar *et al.* (2010) fractionated whole casein and its fractions (α_{s1}-, β-, κ-) both from buffalo and cow milk and evaluated their radical scavenging activity against DPPH and found that among casein fractions, highest value was observed for α_{s1}-casein fraction while lowest for κ-casein fraction. Further, buffalo whole casein had greater DPPH IC_{50} compared to cow whole casein. Based on DPPH radical scavenging activity, significant ($p<0.05$) difference between casein and its fractions both from cow and buffalo was observed. The radical scavenging activity of different casein fractions both from buffalo and cow milk followed the order of α_{s1}- >β- >κ-casein.

2.4.2 Antioxidant activity of casein hydrolysate

Irshad *et al.* (2012) studied the antioxidant activity of bovine casein hydrolysate prepared by trypsin alone and by the combination of pepsin with trypsin at different time intervals. Hydrolysate were fractionated through 10 kDa and 1 kDa which showed radical scavenging effect 17.3% and 24.4% respectively, while 15.5 % and 19.6% was observed in case of combination. DPPH radical scavenging activity of 1 kDa fraction was highest and peptides of less than 1 kDa possess the potential to be used in physiologically functional foods or pharmaceutical industries.

Diaz *et al.* (2003) reported that enriched phosphorylated peptides from casein effectively inhibit lipid oxidation in corn oil in water emulsions. These peptides were able to inhibit lipid oxidant at pH 3.0 and 7.0 and antioxidant activity was not affected by calcium upto 100 mM. They also observed that casein and less purified sources of phosphors peptides also inhibited lipid oxidation and suggested that casein and casein peptides inhibit lipid oxidative reactions under the conditions expected in foods.

Xue-Ying *et al*. (2011) showed that Yak casein hydrolysate can reduce free radical production as compared to intact yak casein and hydrolysate prepared after 7 hr hydrolysis with alcalase shows the highest anti-radical activity. Hogan *et al*. (2009) also reported that hydrolysate/peptides from milk protein possessed oxygen radical absorbance capacity (ORAC) and DPPH scavenging activities.

Alcalase might be more effective in producing low molecular weight CPP with the high level of phosphorus content from casein than trypsin due to the broad specificity of alcalase. The antioxidant capacity of CPP in terms of ORAC, ROO- activity decreased with increasing pH from 3 to 8 in concentrations tested and was positively correlated with its reduction capacity. The antioxidant capacity of CPP in terms of ORAC OH- activity at 50 ug/ml increased with increasing pH to 7 and then decreased, and its metal chelating activity on ferrous ion increased linearly with increasing pH from 3 to 5, then slowly increased until pH 8, indicating that the ORAC OH- activity of CPP might be attributed to the scavenging activity on hydroxyl radical itself as well as the chelating activity on transition metal ions (Kim *et al*., 2007).

Caseins (α_{S1-}, α_{S2-}andβ-) have polar domains that contain phosphorylated serine residues and characteristic functional domain,SerP-SerP-SerP-Glu-Glu- of caseinphosphopeptides (CPP's).These anionic domains are effective cationchelators that form complexes with calcium, iron and zinc (Bennett *et al.,*2000). CPP's in the aqueous phase could be a source of natural AO and chelators to control lipid oxidation in food emulsions by binding and taking transition metal away from the emulsion droplet (Diaz *et al.,* 2003). The proposed mechanism of AO activity of CPP's is linked to the presence of phosphate groups originating from the phosphoserine residues in close proximity to the peptide chain.

Dąbrowska *et al*.(2013) determine potential application of a serine proteinase derived from Asian pumpkin for obtaining biologically active peptides from caseinate. Casein hydrolysis by three doses of the enzyme (50, 150, 300 U/mg of protein) was monitored for 24 hours. The peptides obtained after enzymatic hydrolysis of casein showed the ability to reduce iron ions in the FRAP test in all analyzed variants. The highest activity, 96.15 μg Fe^{3+}/mg were detected in the hydrolysate obtained with the enzyme dose of 300 U/mg after 24 h. The analyzed hydrolysate showed the chelating activity also at the beginning of the reaction at the level of 348.80 to 510.36 μg Fe2+/mg.

Gobba *et al*. (2014) microfiltered goat milk and fractions were hydrolysed with subtilisin, trypsin or both. The retentate treated with subtilisin was most active regarding radical scavenging capacity (SC_{50}), while permeate treated with subtilisin exhibited the best iron chelation capacity (IC_{50}) and prevent the secondary lipid oxidation products formation. Li *et al*. (2013) also found that high ABTS radical scavenging activity (SC50) in goat casein hydrolysate produced with a combination of neutral protease and alcalase. Castro *et al*. (2014) studied the antioxidant activity of whey protein hydrolysate prepared by *Aspergillus oryzae* LBA 01(AO) with two commercial proteases from *A.oryzae* (CAO)and *Bacillus licheniformis* (CBL).The antioxidant activity of hydrolysate was 424.32 and 16.39 μmol TEAC per gm for the ORAC and DPPH assays with substrate concentration of 8.0% and addition of 70.0U of protease per mL of reaction. The fractions obtained by ultrafiltration of AO hydrolysate with molecular weights below 3kDa showed the strongest antioxidant activities and the fractions with molecular weights between 3 and 10 kDa showed the strongest DPPH scavenging activity. They suggested that the ultrafiltration can be used as a subsequent step to concentrate the active compounds.

Correa *et al*.(2011) showed that Ovine caseinate hydrolysates presenting antioxidant, antihypertensive and antimicrobial activities were produced through hydrolysis with a novel microbial protease preparation. The bioactivities presented by the protein hydrolysates could have resulted from the synergistic effect of different peptides within the mixture. Such ovine 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 food. Intarasirisawat *et al*. (2012) studied the antioxidative and functional properties of protein hydrolysate; hydrolyzed by alcalase 2.4L with different degree of hydrolysis (DH 5-50%) using various non-cellular antioxidant assays. Metal-chelating activity and superoxide-scavenging activity increases with increase in DH. Enzymatic hydrolysis increases the protein solubility (>80%). However, the highest emulsion activity indexes and foam stabilities of the hydrolysate are observed at low DH.

Contreras *et al*. (2011) investigated the antioxidant activity of hydrolysates from whey proteins concentrate (WPC) with thermolysin. 3 kDa permeate derived from WPC hydrolysate showed highest ORAC-FL values ranged from 0.832 to 2.321 μmol trolox per mg protein and 2.321 μmol trolox per mg protein for radical scavenging activity which is higher than that of corresponding to the non-ultrafiltered hydrolysate. Hydrolysate with antioxidant properties preventing lipid oxidation via radical scavenging activity could be used in food systems as a natural additive.

2.4.3 Antioxidant peptides

Proteins from certain foods have been found to have the scavenging activity of active oxygen species. Although the mechanism remains to be elucidated, the primary sequence may contribute to generate the activity. As an example, milk casein was reported to inhibit lipoxygenase-catalyzed lipid autoxidation. Quenching of free radicals by oxidation of amino acid residues in casein was proposed. Because the casein peptide has scavenging activity of hydroxyl and DPPH radical, it may be expected that it may also function as an antioxidant in vivo. The dipeptide derived from casein showed very high superoxide scavenging activity and this finding is especially promising because the dipeptides can be directly absorbed *via* the intestinal dipeptide transporter. However, free amino acids could not substitute for peptide as the antioxidant, suggesting that the primary structure of peptide molecules played a role (Suetsuna *et al*., 2000). Kansci *et al*. (2004) reported that β-casein tryptic hydrolysis exhibited antioxidant activity against iron/ascorbate induced oxidation of rich in PUFA phospholipid liposomes as shown by decreased amounts of conjugated dienes, TBARS and total volatile compounds. These effects were mainly due to the β-caseinophosphopeptide (f 1-25). The presence of a "negative cluster" of phosphoseryl residues at the positions 15, 17, 18 and 19 enables iron chelation.

Shanmugam *et al*. 2014 scrutinised the antioxidative property of peptides from buffalo casein digested with pepsin, trypsin, chymotrypsin alone and in combinations. Pepsin-trypsin digested hydrolysates have highest antioxidant activity as compared to other hydrolysate, The 1 kDa permeate of pepsin-trypsin hydrolysates was resolved into 16 fractions by RP-HPLC. Fraction containing highest antioxidant activity on custom sequencing yielded 4 peptides, of which the synthetic peptide VLPVPQK possessed the highest TEAC (5.71±0.59 μmol mg-1).

Gobba *et al*. (2014) identified three peptides AWPQ matching αs_2-CN f(176-179), QPPQP matching β-CN f(146-150) and VYPF corresponding to β-CN f(59-62) can be considered as new antioxidant peptides due to the presence of tryptophan, tyrosine and proline. Gupta *et al.* (2009) identified two peptides

VKEAMAPK matching f (98-105) of β-casein and HIQKEDVPSER corresponding to αs_1-casein f (80-90), which shows antioxidant activity comparable to commercial chemical antioxidants (BHA, t-BHQ, Ferulic acid).

Sequence	Casein fragment	Mode of hydrolysis	Activity	Reference
YAKPA	κ-cn f(61-65)	Papain	Antioxidative	Bezerra *et al*., 2013
YAKPVA	κ-cn f(61-66)			
INNQFLPYPY	Cn- fraction			
NPWDQVK	αs2-cn f(123-129)			
YFYPEL	α_{S1}-cn f (144-149)	Pepsin	Radical scavenging activity. Superoxide anion radical scavenging activity, Hydroxyl radical	Suetsuna *et al*., 2000.
FYPEL	α_{S1}-cn f (145-149)			
YPEL	α_{S1}-cn f (146-149)			
PEL	α_{S1}-cn f (147-149)			
EL	α_{S1}-cn f (148-149)			
AYFYPEL	α_{S1}-cn f (143-149)		ORAC	Contreras *et al*., 2009
YQKFPQY	α_{S2}-cn f (89-95)			
FALPQYLK	α_{S2}-cn f(174-181)	Pepsin	Radical scavenging activity(ORAC)	Lopez-Exposito *et al*., 2007
PYVRYL	α_{S2}-cn f(203-208)			
IQY	κ-cn f(28-30)			
YVL	κ-cn f(30-32)			
RPKHPIKHQGL PQEVL	α_{S1}-cn f (1-23)	Yoghurt	n.i.	Farvin *et al*., 2010
VPYPQ	β-cnf(169–173)	Trypsin,	Antioxidant	Tsopmo *et al*., 2011
YPNSYP	κ-cnf(31–36)			
NPYVPR	κ-cn f(53–58)			
VLPVPQKKVLP VPQK	β-cn f(162-177)	Trypsin, clostripain, and subtilisin	Linoleate oxidation	Rival *et al.,* (2001)
VPYPQ	β-cn f(178-182)	Yoghurt	DPPH radical-scavenging activity, Fe^{2+} ion-chelating capacity	Farvin *et al*., 2010
KAVPYPQ	β-cn f(176-182)			
KVLPVPE	β-cn f(169-175)			
GVRGPFPII	β-cn f(200-208)			
IPIQY	β-cn f(26-30)			

lys-val-leu-pro-val-pro-gln-lys	β-CN f(169–176)	Trypsin	ORAC	Dı´az and Decker (2004)
WSVPQPK	β-cnf(154-160)	Pepsin and pancreatin	ABTS	Herna´ndez-Ledesma *et al.* (2007)
TVQVT	k-cnf(161-165)	Yoghurt	DPPH radical-scavenging activity, Fe^{2+} ion-chelating capacity	Farvin *et al*., 2010
TVQVTST	k -cnf(161-167)			
TVQVTSTAV	k -cnf(161-169)			
VIESPPEIN	k -cnf(152-160)			
ARHPHPHLSFM	κ-cn f(96-106)	Fermentation	DPPH radical scavenging activity	Kudoh *et al*., 2001

Table 2.2 Antioxidant peptides identified from milk proteins

Microorganisms /Enzymes	**Protein fragment**	**Peptide sequence**	**Bioactivity**	**References**
B. subtilis ATCC 6051	n.a	Peptides with molecular weights between 6.5 kDa	Antioxidant Antihypertensive	Limón *et al*. (2015)
Trypsin, clostripain, and subtilisin	β-cn f(162-177)	VLPVPQKKVLPV PQK	Linoleate oxidation	Rival *et al*., (2001a)
Corolase PP	β-lg f(145-149) β-lg f(19-29) β-lg f(42-46)	MHIRL WYSLAMAASDI YVEEL	Radical scavenging activity(ORAC)	Hernandez-Ledesma *et al*. (2005)
Fermented milk	β-cn f(199-208)	GPVRGPFPII	ABTS	Hernandez-Ledesma *et al*., (2005)
L. delbrueckii,	β-cn f(178-182)	VPYPQ	DPPH	Farvin *et al*.,

S. thermophiles	β-cn f(176-182) β-cn f(169-175) β-cn f(200-208) β-cn f(26-30)	KAVPYPQ KVLPVPE GVRGPFPII IPIQY	radicalscavenging activity, Fe^{2+} ion-chelating capacity	(2010)
Cheddar cheese	β-cn f(98-105)	VKEAMAPK	DPPH radical scavenging and superoxide scavenging	Gupta *et al*., (2010)
Human milk simulated digestion	–	YGATGA ISELGW	Radical scavenging activity(ORAC)	Tsopmo *et al.,* (2011)
Bifidobacteriumlonm		RELEELNVPGEIVE	ABTS radical scavenging activity	Chang *et al*., (2013)
Thermolysin	β-lg f(87-91) β-lg f(110-116) β-lg f(71-75) β-lg f(95-101) β-lg f(151-155) β-lg f(72-79)	LNENK SAEPEQS IIAEK LDTDYKK FNPTQ IAEKTKIP	Radical scavenging activity(ORAC)	Contreras de Mar *et al*., (2011)

Table 2.3 Antioxidant peptides from milk protein by fermentation and digestive proteases

2.5 Structure activity relationship

Preliminary mechanistic studies suggest that the AO activity of proteins and peptides is related to their amino acid composition, sequence/ structure and hydrophobicity (Chen *et al.,* 1998).

2.5.1 Size of peptides

AO activity is mainly contributed by 3 kDa fraction (Kumar *et al.,* 2010; Castro and Sato, 2014). Chen and Li, (2012) studied the effect of molecular weights on the survivability of casein–derived antioxidant peptides after the simulated gastrointestinal digestion and reported that peptides above 3 kDa were easily digested by gastric digestion than those below 3 kDa molecular weights, between 3 and 10kD showed the strongest DPPH scavenging activity (Zhao *et al*, 2010). Orally administered casein derived antioxidant peptides of <1 kDa confers promising antioxidant activity (Chen and Li, 2012).

2.5.2 Amino acid composition

The mechanism of AO activity can be categorised by the physicochemical properties of the amino acid. For example, amino acids with an aromatic ring structure such as His (imidazole group), Trp (indolic group), Tyr (phenolic group), Phe (aromatic amino acid) and Pro (hydroxyl radical scavenger) can donate a proton to electron deficient radicals (Chen *et al.,* 1998). The presence of hydrophobic and aromatic amino acid residues in peptides is a determinant factor in radical scavenging (Ajibola *et al.,* 2011). Met and Cys are effective in quenching of free radicals due to their ability to donate sulphur hydrogen (Xiong, 2010; Ajibola *et al.,* 2011).

Amino Acid	**Mechanisms**	**Formed Product**
Cysteine (Cys)	(1)Abstracting hydrogen from SH group and dimerizing radicals (2)Abstracting hydrogen from SH group, then reacting with O2 and isomerizing	(1) Cystine (2) Oxy acids
Histidine (His)	Attacking $HO^{\cdot}$ or oxidizing (decomposing) its imidazole ring	2-oxo-histidine
Leucine (Leu)	Attacking $RCO^{\cdot}$ of susceptible fatty acids	Leu-NH2
Methionine (Met)	Various reacting including radical and non-radical	Methionine sulfoxide
Tryptophan (Trp)	Attacking $HO^{\cdot}$ or one-electron oxidizing ring	*N*-formylkynurenine, kynurenine,5-

		hydroxytrytophan,7-hydroxytryptophan
Tyrosine(Tyr)	(1)Attacking $HO^{\cdot}$ or one-electron oxidizing of aromatic ring (2)Attacking $HO^{\cdot}$ or one-electron oxidizing of tyrosine then dimerizing radicals HOCl (3) Chlorinating of tyrosine (4)Reacting with nitrogen species (5)When $O2^{\cdot -}$ presents, forming tyrosine phenoxyl radical	(1)3,4-dihydroxy-phenylalanine (2)Di-tyrosine (3)3-chlorotyrosine (4)3-nitrotyrosine (5)Tyrosine hydroperoxides and subsequent materials
Phenylalanine (Phe)	(1)Attacking $HO^{\cdot}$ or one-electron oxidizing of aromatic ring (reacting with nitrogen species) (2) Dimerizing	(1)*o-*, *m-* tyrosine (2) Dimers of hydroxylated aromatic amino acids

Table 2.4: Antioxidant Mechanisms of Selected Amino Acids and their Formed Products. *(Davies and Dean, 1997; Mendis et al., 2005; Wade and Tucker, 1998)*

2.5.3 Position of amino acids

AO activity is not solely dependent on the presence of specific amino acid residues. The location of the amino acids within the sequence of the protein/peptide will determine their accessibility and susceptibility to proteolytic release. As peptide bonds are cleaved, there can be an increased availability of free amino acids, thus providing a source of protons or electrons to maintain a high redox potential. Proteinase specificity will influence the characteristics of the resultant hydrolysate. For example, chymotrypsin has been used to generate hydrolysates of whey protein with AO activity. This enzyme is specific toward bonds containing aromatic or hydrophobic residues at the C-terminus and generates hydrolysates with AO amino acids at the C-terminus (Pihlanto, 2006). Pepsin hydrolysis will expose hydrophobic residues as pepsin is specific towards hydrophobic and aromatic amino acids, which facilitates electron transfer from these peptides (Zhu *et al.,* 2008). For a given amino acid or peptide to act as an AO, it must be preferentially oxidised compared to other compounds in the food matrix. The oxidative reactivity of a variety of amino acids was evaluated by photochemical oxidation with $OH^{\cdot}$and it was found that amino acid reactivity was in the following order Cys>Trp, Tyr > Met >Phe> His >Ile >Leu> Pro (Sharp *et al.,* 2004).

Quantitative structure activity relationship (QSAR) is a statistical modelling approach that has been used to identify the structural characteristics of AO peptides. This approach found that N and C terminus amino acids of the peptide are important predictors of AO activity (Li *et al.,* 2011). The hydrophobic properties of N terminal amino acids are important and will increase the AO activity of the peptide. To date more than half of the identified AO peptides derived from milk protein have a hydrophobic residue at the N terminus. The electronic charge properties (i.e. net charge, molecular polarity) of the C terminal amino acid are also an important predictor of AO activity. This position is a polar domain and is affected by its electrostatic potential with the amino acids Trp, Glu, Leu, Ile, Met, Val, and Tyr frequently present. The second amino acid adjacent to the C terminus is suggested to be the major contributor to AO activity and if this amino acid has large hydrogen bonding and steric properties and low hydrophobicity, this will increase the potential AO activity. This makes acidic or basic amino acid residues (Asp, Glu, His, Arg, Lys) or hydrophilic amino acids (Ser, Thr) preferable in this position. Of the potent casein derived AO peptides, the second amino acid adjacent to the C terminus is typically Glu, Pro, Leu.

Overall, it is likely that the AO activity of food protein derived peptides is determined by the combined and synergistic action of some or all of the mechanisms detailed above rather than a single predominant factor. Synergistic action may arise due to interaction of AO compounds with different mechanisms of action or interaction of AO peptides with different polarities, which may be distributed in different phases of a food matrix (Laguerre *et al.,* 2007). Therefore, when characterising the AO activity of protein hydrolysates, evaluating each type of activity may be important to gain a better understanding of the mechanisms involved.

2.6 Methods to evaluate antioxidant activity

There are a variety of methods to assess total antioxidant activity (Huang *et al*., 2005; Prior *et al*., 2005). Methods used to determine antioxidant capacities can roughly be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET), depending upon the reactions involved. The majority of HAT based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azocompounds. These assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and crocin bleaching assays. ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. The degree of colour change is correlated with the sample‘s antioxidant concentrations. ET-based assays include the total phenols assay by Folin-Ciocalteu reagent (FCR), Trolox equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), total antioxidant potential‖ assay using a Cu (II) complex as an oxidant, ABTS and DPPH (Hu *et al*., 2010).

2.6.1 DPPH (Free Radical Scavenging Activity Assay)

The scavenging effect of hydrolysate on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured according to the method of Zhidong *et al*., (1992) with little modification. The DPPH radical (Figure 2.2) is one of stable organic nitrogen radicals and they bear a deep purple colour (Lin *et al*., 2010). This assay is based on the measurement of the reducing ability of antioxidants toward DPPH•. The ability can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance. Antioxidant assays are based on measurement of the loss of DPPH colour at 515 nm after reaction with test compounds (Mendis *et al*., 2005), and the reaction is monitored by a spectrometer. The percentage of the DPPH remaining is calculated as when a DPPH stock solution is mixed with a compound that can donate an electron, the DPPH radical will be reduced simultaneously with the loss of its violet colour. However, it is expected that a residual pale yellow color from the picry1 group will remain present (Molyneux, 2004).

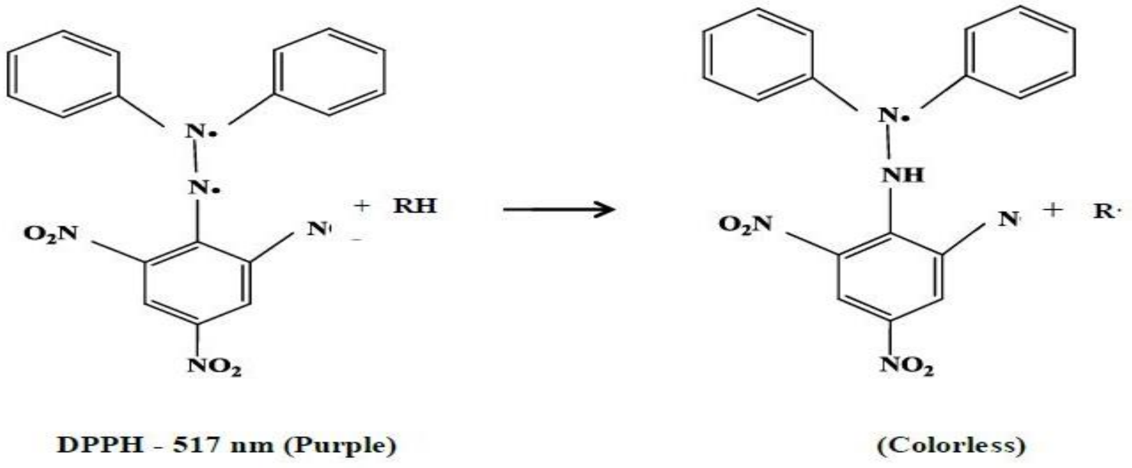

Figure 2.2: The scheme for scavenging the DPPH Radical by an antioxidant.

2.6.2 Oxygen radical absorbance capacity (ORAC) assay

The assay measures the oxidative degradation of the fluorescent molecule after being mixed with free radical generators, such as azoinitiator as compounds. Azo-initiators are considered to produce the peroxyl radical by heating, which damages the fluorescent molecule, resulting in loss of fluorescence. Antioxidant is able to protect the fluorescent molecule from oxidative degeneration. The degree of protection will be quantified using a fluorometer. Oxygen radical absorbance capacity (ORAC) (Figure 2.3) assay has found even broader application for measuring the antioxidant capacity of botanical samples (Cao *et al*., 1993) and biological samples, the ORAC (oxygen radical absorbent capacity) method (Cao *et al*., 1993; Prior and Cao,1999) with subsequent development (Huang *et al*., 2002; Ou et al., 2001) was more sensitive than the iron-based assay (FRAP) (Ou *et al*., 2002) and may meet those needs. Originally the ORAC assay developed by Cao *et al* 1993. used 2, 20-azo-bis (2-amidinopropane) dihydrochloride (AAPH) as the radical source and phycoerythrin as the target molecule as shown in the (Figure 2.3).

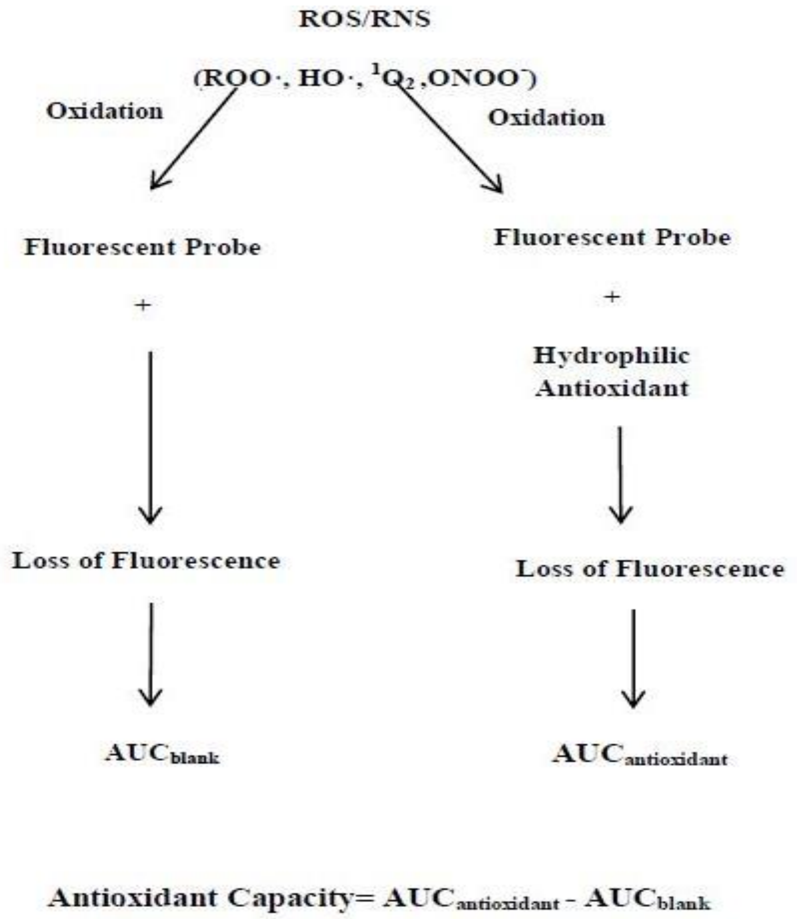

Figure 2.3: Mechanism of ORAC assay

2.6.3 ABTS Radicals Scavenging Activity Assay

The ABTS [2, 2'-azinobis-(3- ethylbenzothiazoline-6-sulfonic acid)] assay (Re *et al*., 1999). It is a spectrophotometric method based on the quenching of the ABTS radical cation, a blue/green chromophore, resulting in decreased absorbance after the addition of antioxidants. The method was first suggested by Miller *et al.,* (1993) to test biological samples and then was widely applied for testing food and natural water-soluble phenolics. $ABTS^{\bullet+}$ has a strong absorption in the range of 600-750 nm can be easily determined spectrophometrically. $ABTS^{\bullet+}$ is stable, but it reacts energetically with a H-atom donor such as phenolics, being converted into a non-coloured form of $ABTS^{\bullet+}$. The quantity of $ABTS^{\bullet+}$consumed due to reaction with phenolic containing sample, expressed as trolox equivalent antioxidant capacity (TEAC).TEAC for individual antioxidant is the number of $ABTS^{\bullet+}$radical-cation consumed per molecule of antioxidant (Rice-

Evans *et al.,* 1996).ABTS$^{\bullet+}$is generated by reaction with potassium persulfate as shown in (figure 2.4).

One assay used was a modification of the FRAP procedure, which measures the ferric ion reducing capacity. It is a spectrophotometric method based on the measurement of the formed coloured complex of ferrous ion and 2, 4, 6- tri (2'-pyridyl)-1, 3, 5-triazine.

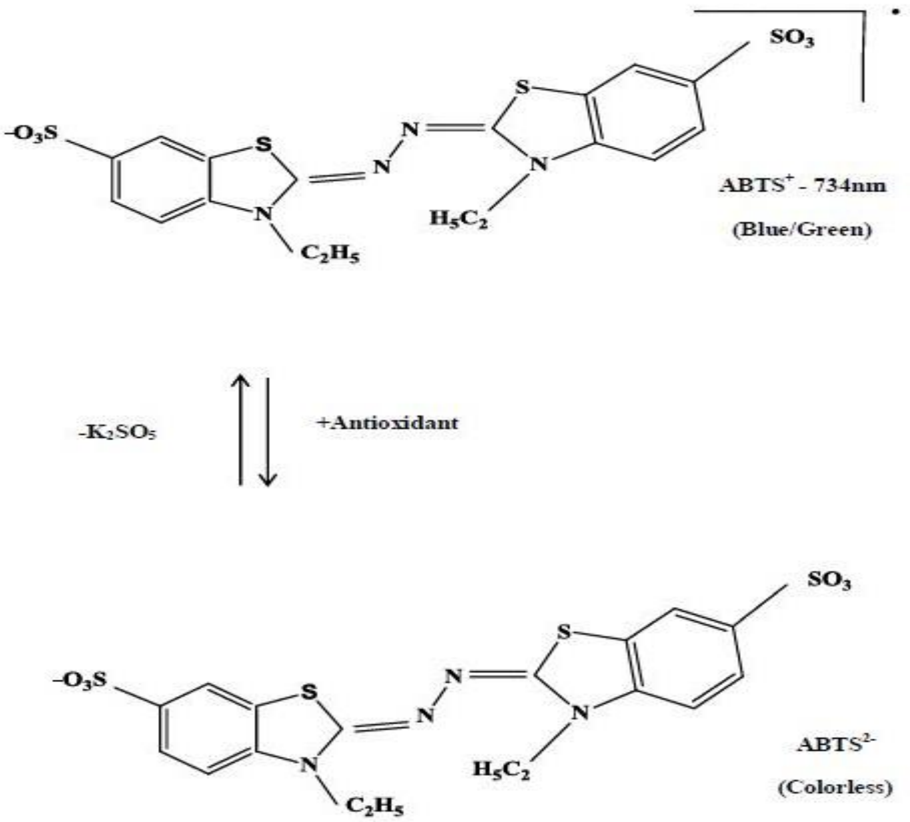

Figure 2.4: The Scheme for Scavenging the ABTS Radical by an Antioxidant.

2.7 Separation and Identification of peptide sequences

The isolation and purification techniques are very important in bioactive peptides research. Most of the protein isolation and purification techniques can be applied for bioactive peptide separation. However, because of relatively small size and molecular weight, special consideration should be given to bioactive peptides purification. Chromatography is the most powerful technique to isolate and purify bioactive peptides. Based on different properties of peptides, different chromatographic techniques have been developed. Among them, HPLC is the most commonly used separation method. Commercially available reversedphase columns allow for rapid separation and detection of the peptides from a mixture, whereas normal phase liquid chromatography is used preferentially for the separation of hydrophilic peptides. Reverse- phase HPLC indicates the hydrophilicity of peptides. Because several peptides may have similar retention times, it is not fully reliable to use the retention time to identify peptides even if the target peptide sequence is known and standards has been run. In this case, UV spectra (usually from 200 to 300 nm) may provide extra information to facilitate peptide identification. HPLC and size-based analysis cannot give direct amino acid sequence information. For unknown peptides, amino acid analyzer and protein sequencer are commonly used to determine amino acid composition and sequence. Automatic protein sequencer based on Edman degradation method is still widely used in peptide sequencing (Li *et al.* 2002; Chen *et al.* 2003; Kuba *et al.* 2003; Motoi and Kodama 2003; Megias *et al.* 2004). Mass spectrometry methods, such as triple stage Model API-III (Gibbs *et al.* 2004), ESI-MS/MS (Gupta *et al*., 2010) and MALDITOF- MS (Rejtar *et al.,* 2004) together with database search are becoming more and more popular.

2.8 Techno- functional properties

Buffalo casein hydrolysate has a variety of functional applications. The term functional properties are often used in relation to physico-chemical properties of proteins. It is important to study the functional properties of hydrolysates because molecular changes occur during hydrolysis may alter techno functional behaviour of the hydrolysates than to that of intact protein in terms of solubility, viscosity, gelling, sensory properties, emulsion and foam properties (Panyam and Kilara, 1996; Nielsen, 1997; Caessens *et al*., 1999b). Sodium caseinate (NaCas) is a dairy ingredient developed to enhance functionalities such as water solubility, emulsifying and foaming properties, as well as encapsulation capabilities (Pan *et al*., 2013; Sánchez and Patino, 2005). Sodium casinate has also been recently explored as a novel stabilizer and absorption enhancer of hydrophobic protein nanoparticles (Luo *et al*., 2013; Zhang *et al*., 2014).

2.8.1 Solubility

Generally, the solubility at the isoelectric point (pI) of proteins increases with hydrolysis time, which is mainly due to reduction in molecular weight and the increase in the number of polar groups (Chobert *et al*.,1988b; Nielsen, 1997; Slattery and Fitzgerald, 1998).However, the decrease in solubility after hydrolysis due to protein molecules exposes more hydrophobic group (Liu *et al*., 2010).The solubility of hydrolysates differs with the enzyme type and hydrolysis time (Luo *et al*., 2014). Caseinates are very soluble at pH values above and below the pI (pH 4-5). Consequently at these pH values the solubility of hydrolysates is similar to or slightly lower than that of intact caseinates (Chobert *et al*., 1988a; Chobert *et al.*, 1988b; Svenning *et al*., 1993; Slattery and Fitzgerald, 1998). The solubility of casein hydrolysates prepared with papain having different DH in the pH range of 2 to 9 was highly soluble over a wide pH range with more than 90% solubility (Pralea Daniela *et al*.,2011).Liu *et al*.,(2010) reported that casein hydrolysate prepared from porcine blood plasma protein (PPH) with alcalase at different DH has higher solubility (>90%) over a wide pH range (pH 2 to 9) as compared to non-hydrolysed plasma protein since smaller peptides resulting from hydrolysis are more polar than polypeptides in non-hydrolysed proteins.

2.8.2. Emulsion and foaming properties

The functional properties of milk proteins may be improved by limited proteolysis. Hence the limited proteolysis of caseinate reduces its emulsifying capacity, increases specific foam volume but reduces foam stability and increases heat stability. Emulsion and foam properties of hydrolysate were similar or inferior to those of the parental proteins. Foam-stabilizing ability of casein hydrolysates also depended on the MWD of hydrolysates, but higher molecular weight peptides, *i.e.* larger than 7 kDa, were required to obtain good foam stability (Ven *et al.,* 2002).

The emulsifying properties of hydrolysate at pH 7.0 were evaluated as EAI and ESI. When compared to NaCas, both EAI and ESI were generally improved for most hydrolysate samples, with exceptions of ESI for hydrolysates produced by papain for more than 1 h and EAI and ESI of the hydrolysate produced by pancreatin for 24 h (Luo *et al*., 2013).

The peptides with low molecular weights probably had a reduced amphiphilicity required to produce a good emulsion. Turgeon *et al*. (1991) also showed that hydrolysis resulted in a drastic loss of emulsifying properties. Although small peptides diffuse rapidly towards the interface, they are less efficient in stabilizing emulsions because they may not readily agglomerate to produce a fat globule membrane due to charge repulsions.

Hydrolysis of casein and whey protein generally resulted in increased foam-forming ability of the hydrolysate compared to the parental proteins (Britten *et al*., 1994; Ludwig *et al*., 1995; Lieske and Konrad, 1996; Caessens *et al*., 1999b). In two studies whey protein hydrolysate were fractionated with ultrafiltration over a 10 kDa membrane (Althouse *et al.*, 1995; Mutilangi *et al*., 1996). For almost all hydrolysates it appeared that the permeate fraction, with peptides having a MW <10 kDa, formed a higher foam overrun than the original protein. The foam-forming behaviour of the retentate fraction decreased for some hydrolysates, although for trypsin, chymotrypsin and alcalase the results differed between the two studies. The effect of hydrolysis on foam stability seems to depend on enzyme specificity and degree of hydrolysis (Althouse *et al.*, 1995; Ludwig *et al.*, 1995; Mutilangi *et al.*, 1996; Slattery and Fitzgerald, 1998). In case of tryptic whey protein hydrolysates, it was shown that the fraction with peptides having MW <10 kDa had better foam-stabilizing properties than the peptides with MW >10 kDa (Althouse *et al.*, 1995; Mutilangi *et al.*, 1996). Other hydrolysate fractionated over a 10 kDa membrane, showed for both the high and low molecular weight fractions either increased or decreased foam stability relative to WPI.

From the above discussed literature it can be concluded that for emulsion stability and emulsion-forming ability peptides with relative high molecular weights are important, while for foams low molecular weight peptides may also contribute to the stability. For foam-forming ability the lower molecular weight fraction also seems to contribute to the formation process. Although a general idea about the peptides important for the techno-functional behaviour is obtained from these studies, only a few molecular weight fractions have been studied which solely give an estimation of the molecular weights that are important. Most of the studies reporting on techno-functional properties of hydrolysates only give the DH as an indicator for the hydrolysate, but as already discussed, this parameter is just a very rough indicator for the hydrolysate. An overall correlation between DH and techno-functional properties of hydrolysate cannot be deduced from the results reported in literature.

Overall, proteolytic treatment may provide a practical approach for increasing the use of casein hydrolysate in beverages because of improvements in solubility and stability at different pH and temp (Dickinson *et al*., 1987).

2.8.3 Surface hydrophobicity (S_0)

Hidalgo *et al.* (2011) prepared sodium caseinate (NaCas) hydrolysate by extracellular protease from Bacillus sp. P45 after 2 h of hydrolysis surface hydrophobicity (S_0) decreased as time increased due to higher exposure of hydrophilic groups in the protein surface. Papain and trypsin treated hydrolysate srevealed the decrease in S_0 with an increase in hydrolysis time (Luo *et al*., 2014). While the S_0 of the pancreatin treated hydrolysateswas increased significantly with the increase of hydrolysis time in the first 4 h due to the initial production of peptides more hydrophobic than caseins. Surface hydrophobicity of protein hydrolysates can significantly affect their water solubility (Rao *et al*., 2013).

Liu *et al*.,(2010) hydrolysed porcine blood plasma protein (PPH) prepared with alcalase, the surface hydrophobicity of PPH with different DHs was significantly affected by pH, the highest value was reached at pH 3.0, and higher DH had lower surface hydrophobicity ($P < 0.05$). At pH 7.0, the hydrophobicity of 0%, 6.2%, 12.7% and 17.6% of DH were 64.83%, 26.34%, 6.38% and 4.53% (photons), respectively. Due to shortening of peptide chains, proteolysis is accompanied by gain or loss in hydrophobicity, depending mainly on the nature of the hydrolysed protein and molecular weight size of the formed peptides (Calderon *et al*., 2000). Enzymatic hydrolysis by alcalase was accompanied by decrease of surface hydrophobicity since peptides released from the native structure of porcine plasma protein reveal great flexibility, which helped them to adopt a conformation with hydrophilic groups exposed more towards outside.

2.9 Utilization of hydrolysate in the products

Casein hydrolysate contributes low level of fat and lactose to their products which makes them ideal ingredients for formulating sugar free, low fat or non-fat dairy products. From functional perspective, buffalo casein hydrolysate are highly soluble over a wide pH range and contribute emulsifying, water binding, foaming, gelling, and film forming properties to food and beverages system. Commercial dairy products containing bioactive peptides as shown in Table 2.5

Brand name	**Type of product**	**Bioactive peptides sequence**	**Health function claims**	**Manufacturer**
Calpis	Sour milk	Vall-Pro-Pro, Ill-Pro Pro	Reduction of blood pressure	Calpis Co, Japan Calpis Co, Japan
Evolus	Calcium enriched fermented milk drink	VPP, IPP from _-β- CN and k-CN	Blood pressure reduction	ValioOy, Finland
Bio PURE GMP	Whey protein isolate	k-CN f(106–169)	Prevention of dental caries, influence the clotting of blood, protection against	Davisco, USA
PRODIET F200/Lactium	Flavored milk drink, confectionery,	α-s1-CN (91–100)	Reduction of stress effects	Ingredia, France
Festivo	Fermented low-fat hard	α -s1-CN (1–9),	No health claim as	MTT Agrifood Research,

	cheese	α -s1-CN (1–7), α-s1-CN (1–6)	yet	Finland
Cysteine Peptide	Ingredient hydrolysate	Milk protein derived peptide	Aids to raise energy level	DMV International,
C12 peptide	Ingredient hydrolysate	Casein derived Peptide	Reduction of blood pressure	DMV International, Netherlands
Capolac	Ingredient	Casein derived Peptide	Helps mineral absorption	Arla Foods Ingredients, Sweden
PeptoPro	Ingredienthydrolysate	Casein derived Peptide	Improves athletic performance and muscle	DMV International, Netherlands

Table 2.5: Commercial dairy products and ingredients with health or function claims based on bioactive peptides. (Korhonen, 2006)

2.9.1 White bread and carrot cream

Hydrolysates produced by enzymatic hydrolysis of chia protein-rich fraction were sequentially hydrolysed with alcalase for 60 min followed by flavourzyme. The chia hydrolysates with the highest degree of hydrolysis (DH) were added to white bread and carrot cream resulted in products with improved ACE-inhibitory activity (141.29 -297.68; 0.24-1.71 mg protein/mL) compared to the controls. Antioxidant activity increased from 10.21 mmol/L-mg in the Carrot cream control treatment to 17.52 - 18.88 mmol/L-mg in the treatments containing the chia protein hydrolysates (Segura-Campos *et al*., 2013)

2.9.2 Milk beverage:

The incorporation of whey protein hydrolysates in the skim milk (1-2%) raised its antioxidant activity from 21% to 88% and ACE inhibitory activity was 35 times higher than the skim milk (Kishore, 2009).

2.9.3 Chocolate-flavoured whey protein concentrate beverage

WPC hydrolysate was produced using fungal protease and papain, a beverage was formulated with hydrolysed WPC, skim milk powder, cocoa, liquid glucose, sugar and vegetable fat. Results revealed that the protein content of WPC was 75.6% and decrease slightly with enzyme treatment (69.6%),enzymatic hydrolysis of whey proteins caused an increase in the foam volume initially and then a decrease with time. The water absorption capacity of WPC increased on enzymatic hydrolysis with fungal protease and papain and was highest for a hydrolysis time of 60 min (Sinha *et al.,* 2007). This can be attributed to dissociation of proteins into smaller subunits, which have more water binding sites (Castimopoolas *et al*., 1970). The emulsification capacity, in contrast, reduces on enzyme hydrolysis in both samples. The emulsifying capacity of proteins is related to their capacity to lower interfacial tension between the hydrophobic and hydrophilic components in foods. Similar results on emulsifying capacity were also obtained by other researchers and have been attributed to dry heat treatment (Rahma *et al*., 1998), partial hydrolysis (Sekul *et al*., 1978), and enzyme modification (Bhagya *et al*.,1999).

2.9.4 Ice cream:

Utilizing whey protein in ice cream reveals that the level of total protein, ash, specific gravity, freezing points, viscosity and weight per gallon increased (Shenana *et al*., 2007). In ice cream, MPC formulation gives higher mix viscosity, larger amount of fat destabilization, homogeneous ice crystal and greater shape retention compared to control (Alvarez, 2005). In ice cream, 1% whey protein hydrolysate gives higher overrun, better melting resistance, sensory and antioxidant properties. The antioxidant activity and overrun is raised by 55 and 26.6% resp. than that of control (Nidhi, 2010).

2.9.5 Yoghurt

Sodini *et al.* 2004 studied the physical properties and the microstructure of yoghurts containing probiotic bacteria, and supplemented with casein and whey protein hydrolysates at a concentration ranging from 0.25 to 4 g/L. In comparison to control yogurt, hydrolysate supplemented yogurts have decreased complex viscosity and graininess which may be due to the more open and less branched structure of supplemented yogurt. The addition of hydrolysates also reduced fermentation time. The difference in fermentation time between milks with different levels of added hydrolysates could partially explain the differences in microstructure and physical properties of the final yoghurts.

2.9.6 Flavoured milk

Mann *et al*. 2015 reported that flavoured milk enriched with antioxidative whey protein hydrolysates (WPHs) prepared by using three commercial proteases flavouzyme, alcalase and corolase PP. Among three hydrolysate whey protein hydrolysates prepared using corolase showed maximum antioxidant activity (1.42±0.12) was higher than the WPC (0.19±0.01).WPC and WPHs and 2 % addition in chocolate flavour milk showed increase in antioxidant activity up to 42 % as compared to control. These result suggest that WPH could be used as natural bio functional ingredients in enhancing antioxidant properties of food products.

MATERIALS AND METHODS

3.1 Preparation of whole casein

Fresh whole pooled buffalo milk(collected from the Cattle Yard, National Dairy Research Institute, Karnal, Haryana) was defatted using refrigerated centrifuge (Sorvall, Model RC 6 Plus, Thermo scientific) at 4,000 rpm for 30 min at 4°C to separate cream from milk. The defatted milk was diluted in 1:1 ratio with distilled water. The pH of defatted milk was adjusted to 4.6 with 1 N HCl at 20°C with slow stirring to precipitate casein. The mixture was held at room temperature for 30 min for clear separation of casein and whey. The precipitated casein curd was separated from the whey by filtering through four layered muslin cloth. The casein precipitates were washed several times with distilled water and re-precipitated to remove whey trapped in the curd mass. The excess water was removed by squeezing through muslin cloth and wet casein dissolved in 1 N NaOH adjusts pH to 7.5. Caseinate was subjected to freeze drying.

3.2 Enzymes

The commercial enzyme, Alcalase 2.4 L FG (P4860, endoproteinase from *Bacillus licheniformis*) and flavourzyme 500 L (P6110, endoprotease and exopeptidase from *Aspergillus oryzae*) were purched from SIGMA (St. Louis, MO, USA). All the enzymes were stored at 4ºC.

3.3 Enzymatic hydrolysis of buffalo sodium caseinate

3.3.1 Using pH-stat autotitrator

Fresh solution of buffalo sodium caseinate (2.5 % and 5.0 % w/v) was prepared in distilled water. This solution was heated at 85°C for 15 min to inactivate the proteases already present in the solution before the addition of enzymes and kept it for overnight for hydration. Enzymatic hydrolysis was performed with alcalase, flavourzyme and alcalase followed by flavourzyme for sequential hydrolysis. Temperature and pH was corresponding to values previously optimized in the lab. Enzymatic hydrolysis was performed in a reaction vessel (50 ml) equipped with a stirrer, thermometer and a pH electrode. The enzyme was added in the solution after optimum pH was adjusted, and then pH of solution was kept constant at a specific time of hydrolysis using1 N NaOH. Samples were drawn at a regular interval of time and amount of alkali consumed during hydrolysis was determined for calculation of degree of hydrolysis (% DH). The enzymatic hydrolysis was stopped by heating the solution at 95°C for 10 min (Tauzin *et al.,* 2003). After cooling, hydrolysates were stored at -20°C for further use.

3.3.2 Using Shaking water bath

Based on optimization using pH-stat autotitrator, production of hydrolysate of buffalo sodium caseinate (5% w/v) (4x250 ml) was done using shaking water bath. Fresh solution of buffalo casein was prepared as described above (3.3.1). The enzyme was added in the solution and hydrolysed over shaking water bath (Jeotech) at 110 rpm at constant temperature by maintaining constant pH manually using 1 N NaOH. During the hydrolysis, samples were drawn at a regular interval of time. The enzymatic hydrolysis was stopped by heating the solution at 95°C for 10 min (Tauzin *et al.,* 2003). Centrifuge the solution at 12,000 rpm for 15 min to remove unhydrolyzed part and supernatant were stored at -20°C for further use. The hydrolysate prepared using combination of alcalase-flavourzyme was labelled as CH1, using alcalase as CH2 and using flavourzyme as CH3. The hydrolysates were evaluated for peptide content and antioxidant activity (as mentioned in part 3.6 and 3.7.1).

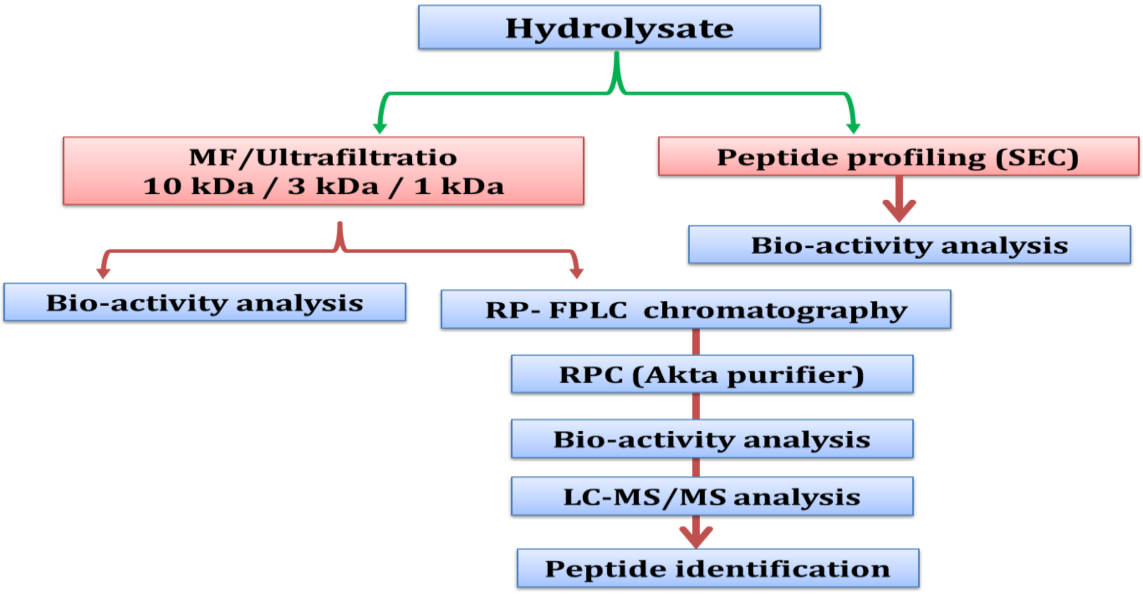

Figure 3.1: Schematic diagram of process for analysis of hydrolysate

3.4 Microfiltration and ultrafiltration

After thawing, hydrolysate were centrifuged at 12000 rpm for 10 min at 4ºC in a refrigerated centrifuge (Kubota centrifuge, Tokyo, Japan) and the supernatant was further micro filtered (0.45 µ). Micro filtered hydrolysates were further ultrafiltered by Stirred ultrafiltration cell (Millipore Cop. Bedford, USA)with 10 kDa (PLGC06210),followed by 3 kDa (PLBC06210) and 1 kDa (PLCC06210) molecular weight cut-off membrane (MWCO) under nitrogen pressure (< 2 kg/cm^2). After ultrafiltration, the membrane was washed with 0.1 N micro filtered (0.45 µ) NaOH (approx. 3 times) and finally rinsed with distilled water (approx. 3 times). It was then removed from holder and stored in 10%ethanol at 4°C for further use. The permeate fractions of ultrafiltration were collected, lyophilized (Model: Ecospin 3180 C, Hanil science industrial, Koria) and stored at -20ºC until use. Membrane was cleaned using 1N NaOH.

3.4 Degree of hydrolysis

3.5.1 OPA method:

Degree of hydrolysis for hydrolysate was analyzed by method given by Nielsen *et al*. (2001) with a slight modification.

3.5.1.1 Reagents:

a) **Sodium tetra borate: (100mM):** Dissolve 3.9 gm of sodium tetra borate in distilled water and make up volume to 100 ml**.**

b) **SDS (20%):** Take 20 gm sodium dodecyl sulfate was dissolved in distilled water and make up volume to 100 ml.

c) **O-Phthalaldehyde (OPA):** 80 mg OPA was dissolved in 2ml ethanol.

d) **OPA Reagent**: Immediate before use, add 50 ml of sodium tetraborate, 5ml of SDS , 2ml OPA Reagent and 200µl β-mercaptoethanol and make up the volume to 100 ml .

3.5.1.2 Procedure:

The assay was carried out by adding 400µl of sample and 3ml of OPA reagent. After vortex, incubated for exactly 2 min at room temperature and the absorbance was measured at 340 nm using double beam UV-Vis spectrophotometer (Model UV-2700, Shimadzu, Tokyo, Japan). L-serine (0.9516 meq/L) was used as positive control and distilled water as negative control.

3.5.1.2 Calculation:

Serine $-NH_2 = (A_{340Sample} - A_{340\ Blank}) / (A_{340Standard} - A_{340Blank}) * 0.9516$ meqv /L * S * D * (P/V)

$DH\% = h/h_{tot} * 100\%$

Where,

D = Sample dilution

P = Protein content

H = $(serine-NH_2 - \beta)/\alpha$ meqv /g protein

α, β and h_{tot} constants are1.039,0.383,and 8.2 respectively

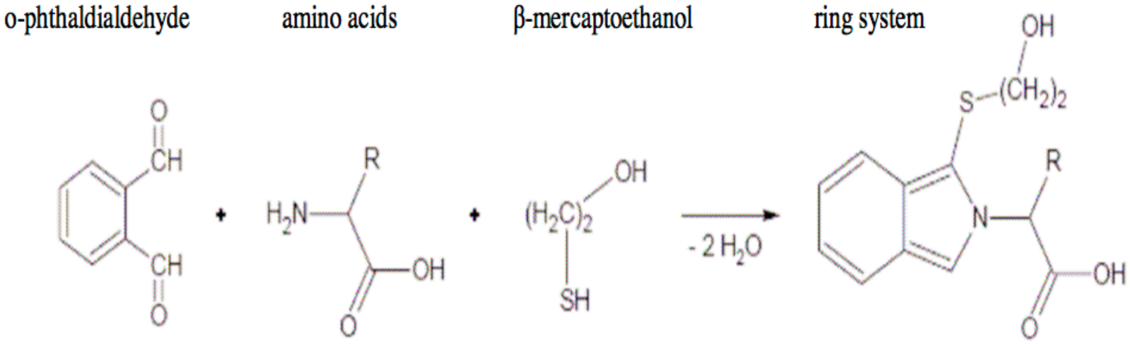

Figure 3.2: Mechanism Of OPA Assay to detect free amino acids and peptides present in a solution

NOTE- *Samples prepared with 2.5% and 5.0% protein were diluted to 100 and 200 times respectively for analysis.*

3.5.2 pH-stat Method

Quantification of DH by the pH stat method was carried out using data obtained with DH-analyzer (Model: TitroLine®7000, SI Analytics GmbH, Germany). Percentage DH was calculated using the following formula (Adler-Nissen, 1986):

3.5.2.1 Reagent:

a) NaOH (1N): Take 40 gm sodium hydroxide was dissolved in distilled water and make up volume to 1000 ml.

3.5.2.2 Calculation:

$$\mathbf{DH\% = 100\ B * Nb * (1/\alpha) * (1/MP) * (1/h_{tot})}$$

Where,

B = Base consumption in mL

Nb = Normality of the base

α = Average degree of dissociation of the α -NH2 groups

MP = Mass of protein being hydrolysed (g)

h_{tot} = The total number of peptide bonds in the protein substrate (meqv/ g protein)

The degree of dissociation (α) for the α -NH2 groups was calculated as follows

$$\alpha = \frac{10^{(\mathrm{pH}-\mathrm{pK})}}{1 + 10^{(\mathrm{pH}-\mathrm{pK})}},$$

where,

pK is the average dissociation value for the α-amino groups liberated during hydrolysis, the average pK value for the α-amino groups of peptides and proteins is 7.1(Adler-Nissen, 1986).

3.6 Protein Estimation

Protein determination in samples of casein, casein Hydrolysate was carried out according to the method of Lowry *et al* (1951) as given below.

3.6.1 Reagents:

a) Copper sulphate solution (1% w/v)

Dissolved 1.0 g of copper sulphate in distilled water and made up the volume to 100 ml.

b) Sodium potassium tartrate solution (2% w/v)

Dissolved 2.0 g of sodium potassium tartrate in distilled water and made up the volume to 100 ml.

c) **Sodium carbonate solution (2% w/v) in 0.1 N NaOH**

Dissolved 2.0 g of sodium carbonate and 0.4 gm NaOH in distilled water and made up the volume to 100 ml.

d) **Alkaline reagent:**

To 100 ml of alkaline reagent 0.5 ml reagent A and 0.5 ml reagent B in 100 ml reagent C. This reagent was prepared fresh as and when required.

e) **Folin's reagent:**

To 10 ml of Folin and Ciocalteau's phenol reagent (Loba), added 10 ml of distilled water. This dilution was done immediately before use.

3.6.2 Procedure

To 0.5 ml of suitably diluted sample, added 5 ml of alkaline reagent (e). Mixed the contents rapidly and allowed to stand for 10 min Thereafter, 0.5 ml of Folin reagent (f) was added, and mixed immediately and allowed to stand at room temperature for 30 min. The blue colour developed was measured by taking absorbance at 660 nm on Specord-2700 against blank of 0.5 ml distilled water processed under identical conditions of sample. Protein content of the sample was determined from a standard curve of bovine serum albumin (50µg to 500µg).

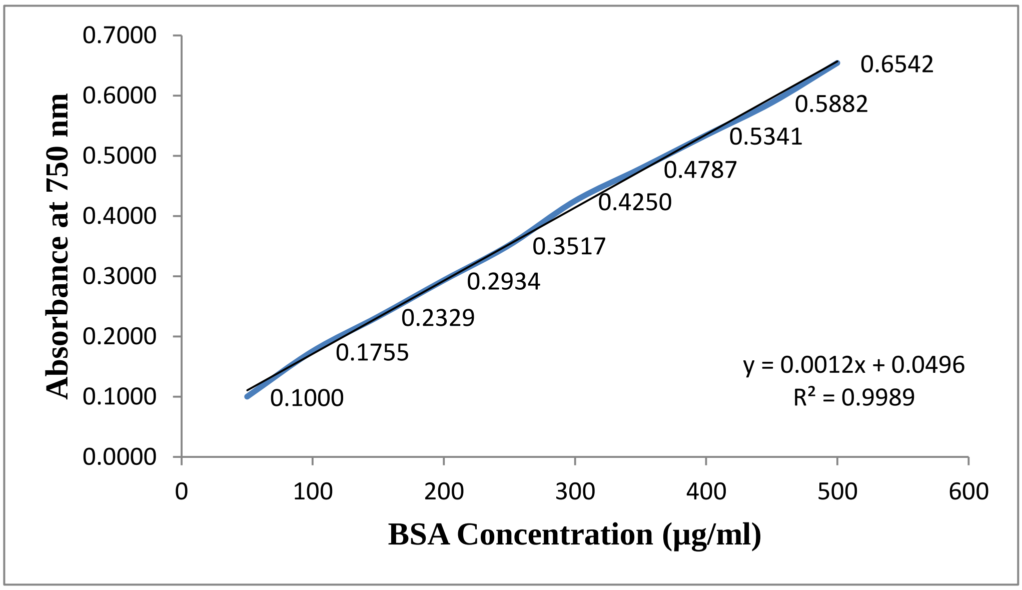

Figure 3.3: Standard curve of bovine serum albumin (BSA)

NOTE- *Samples prepared with 2.5% and 5.0% protein were diluted to 100 and 200 times respectively for analysis.*

3.7 Bioactivity evaluation

3.7.1 Antioxidant activity by ABTS radical scavenging activity

The antioxidant activity was determined using $ABTS^+$ (2, 2- azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay developed by Re *et al.* (1998) with some modifications described by Tachakittirungrod *et al*. (2007).

3.7.1.1 Materials

2, 2- azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (A1888) and 6-hydroxy. 2, 5, 7, 8 – tetra methyl chroman-2-carbocyclic acid (Trolox) (23881-3) were purchased from Sigma (St. Louis, MO, USA). Potassium persulphate, sodium chloride, potassium chloride, disodium hydrogen orthophosphate and potassium dihydrogen orthophosphate were purchased from HiMedia (Mumbai, India).

3.7.1.2 Reagents

a) Potassium persulphate solution (140 mM)

1.892 gm of potassium persulphate was dissolved in double distilled water and the volume was made up to 50 mL freshly prepared before use.

b) ABTS stock solution [2, 2'- Azinobis (3-ethylbenzo-thiazoline)-6-sulfonic acid] diammonium salt (7 mM)

19.2 mg of ABTS was dissolved in 5 mL of double distilled water and was added with 88 μL of 140 mM potassium persulphate solution (2.45 mM final concentration). The mixture was stirred in an Amber color bottle in dark for 12-16 h for production of sufficient free radicals.

c) Phosphate buffered saline (PBS, pH 7.4)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml distilled water, adjusted pH to 7.4 with 1 N HCl and made the volume up to 1 liter with distilled water and filtered through 0.45µ filter.

d) ABTS working solution

1 mL of ABTS stock solution was diluted with phosphate buffer saline till it gave an absorbance of 0.70 ± 0.02.

e) Trolox solution

12.5 mg of Trolox [6-hydroxy. 2, 5, 7, 8 – tetra methyl chroman-2-carbocyclic acid] was dissolved in 10 ml of phosphate buffer saline to obtain 5 mM Trolox solution. It was diluted with distilled water to 500 µM concentration.

3.7.1.3 Preparation of standard curve

180 µL of ABTS working solution and 20 µL PBS was added to a well of 96 well micro plate and initial absorbance was recorded at 750 nm using microplate reader (Model: Infinite F200 Pro, Tecan, Austria). Calibration curve of Trolox, concentration ranging from 60-400 µM was prepared by appropriate dilution of 500 µM Trolox solution. 20 µL of standards were added to 180 µL of ABTS working solution. The contents were mixed for 5 sec and change in absorbance at 750 nm was recorded after 5 min. The standard curve (Figure 3.5)was prepared by plotting concentration (µM) of Trolox (X-axis) v/s % inhibition (Y-axis).

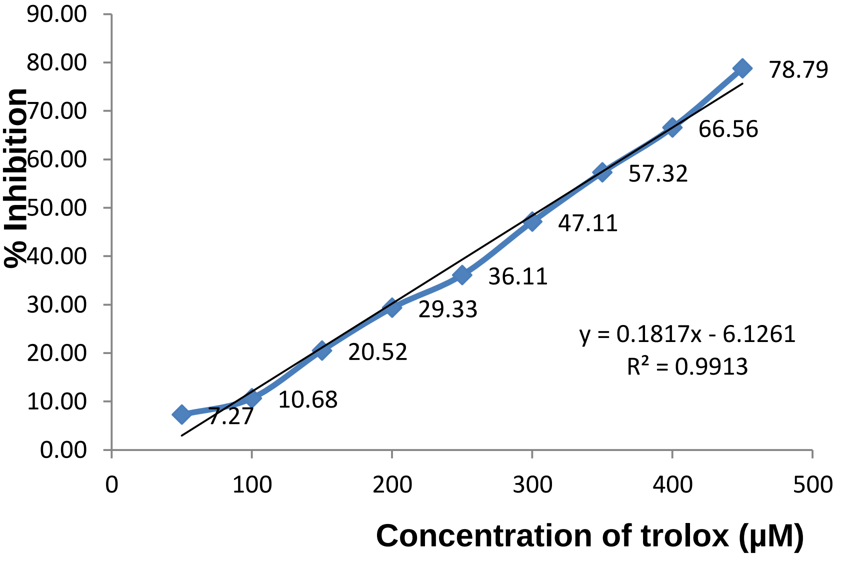

Figure 3.4: Standard curve of Trolox for ABTS radicals

3.7.1.4 Trolox equivalent antioxidant capacity (TEAC)

Samples were diluted to obtain between 20% and 80% inhibition of the blank absorbance. After addition of 20 µL of samples dissolved in PBS (pH 7.4) to each well, 180 µL of diluted ABTS$^{\circ+}$ solution (absorbance adjusted to 0.70 ± 0.02) was added and the absorbance was measured after 5 min spectrophotometrically at 750 nm using a plate reader (Model infinite 200,Tecan, Austria). Appropriate solvent blank was run in each assay. All measurements were performed in triplicate.

3.7.1.5 Calculation

Based on the % Inhibition of absorbance of sample, trolox equivalent was determined from standard curve (Figure 3.5) using the following equation:

$$\mathbf{y = 0.2141x - 1.5635}$$

Where,

y: $\%\text{Inhibition} = \left({(\text{A734control} - \text{A734 sample})}\big/{\text{A734control}} \right) * 100$

x: µM concentration of trolox

The results were expressed as trolox equivalent antioxidant capacity (TEAC) values i.e. µM Trolox equivalent or µM Trolox equivalence per mg of the peptide.

NOTE- *Samples prepared with 2.5% and 5.0% protein were diluted to 100 and 200 times respectively for analysis*.

3.7.2 Antioxidant activity by Oxygen Radical Absorbance Capacity-Fluorescein (ORAC-FL) Assay

ORAC-FL assay was carried out according to Ou *et al.* (2001) following a slight modification as described by Davalos *et al.* (2004).

3.7.2.1 Materials

Disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate were purchased from Hi Media (Mumbai, India). Fluorescein disodium salt (28803) was obtained from Fluka analytical and 2, 2’-azobis (2-

methylpropionamidine) dihydrochloride (AAPH)(440914) was procured from Sigma (St. Louis, MO, USA).

3.7.2.2 Reagents

a) Phosphate buffer (75mM, 7.4pH)

11.5046 g di-sodium hydrogen phosphate, dihydrate, A.R. and 1.616 g of sodium dihydrogen orthophosphate, dihydrate, A.R. were dissolved in 800mL distilled water and the pH was adjusted to 7.4. Final volume was made up to 1 Litre with distilled water and filtered through 0.45 µ filter.

b) Fluorescein disodium salt (117nM)

- **Stock solution (1.17mM)**

22.012 mg of Fluorescein disodium salt was dissolvedin phosphate buffer (75 mM, 7.4 pH) and the volume was made up to 50 mL in a volumetric flask.

- **Working solution (117 nM)**

100 µL stock fluorescein solution (1.17 mM) was diluted to 100 mL with phosphate buffer (75 mM, 7.4 pH) to get a final concentration of 117 nM. This solution was used as a fluorescent probe.

c) 6-Hydroxy-2,5,7,8-tetra-methylchroman-2- carboxylic acid (Trolox®)

12.5 mg of Troloxwas dissolved in 10 mL of phosphate buffer to get 5 mM Trolox solution. It was diluted with distilled water to 500 µM concentration.

d) AAPH {2, 2’-azobis (2-methylpropionamidine) dihydrochloride} (40 mM)

109 mg AAPH was dissolved in phosphate buffer and the volume was made up to 10 mL in a volumetric flask. This solution was used as a peroxyl radical inductor of reaction and was prepared fresh before use.

3.7.2.3 Preparation of standard curve

Standard curve of Trolox solutions ranging from 10-100 µM Trolox was prepared by appropriate dilution of 500 µM Trolox solution. 20 µL of standards (1-10 µM Trolox in final assay volume) were added to120 µL of fluorescein (70 nM of fluorescein in final assay volume) in a fluorescent micro-plate without mixing. After pre-incubation at 37°C for 15 min, 60 µL of AAPH solution (12 mM in final assay volume) was added rapidly to each well using a multichannel pipet to start the oxidation reaction. The microplate was immediately placed in the microplate reader (Model: Infinite F200 Pro, Tecan Austria GmbH, Austria) and the decay in fluorescence was recorded every minute for 80 min with excitation wavelength 495 nm and 520 nm emission. The microplate was automatically shaken prior each reading. Phosphate buffer was used for blank determination. At least three independent assays were performed for each sample. The standard curve (Figure 3.6, 3.7) was prepared by plotting concentration (µM) of Trolox (X-axis) v/s area under curve (AUC) (Y-axis).

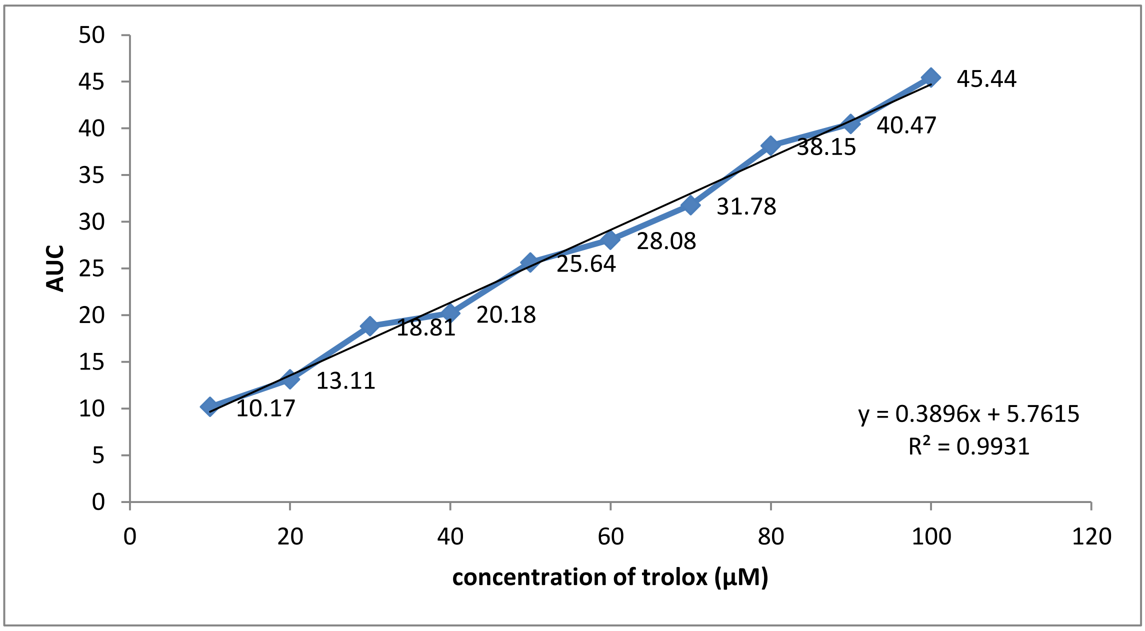

Figure 3.5: Standard curve of Trolox by ORAC method

3.7.2.4 Trolox equivalent antioxidant capacity (TEAC)

Sample (20 µL) and fluorescein (120 µL; 70 nM, final concentration) solutions were placed in the well of the microplate. The mixture was preincubated for 15 min at 37°C. AAPH solution (60 µL; 12 mM, final concentration) was added rapidly using a multichannel pipet. The micro plate was immediately placed in the reader and the fluorescence recorded every minute for 90 min. A blank (FL + AAPH) using phosphate buffer was also carried out in each assay. Three independent assays were performed for each sample.

3.7.2.5 Calculation

Antioxidant curves (fluorescence versus time) were first normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor:

$$fluorescence_{blank,\ t=0} \Big/ fluorescence_{sample,\ t=0}$$

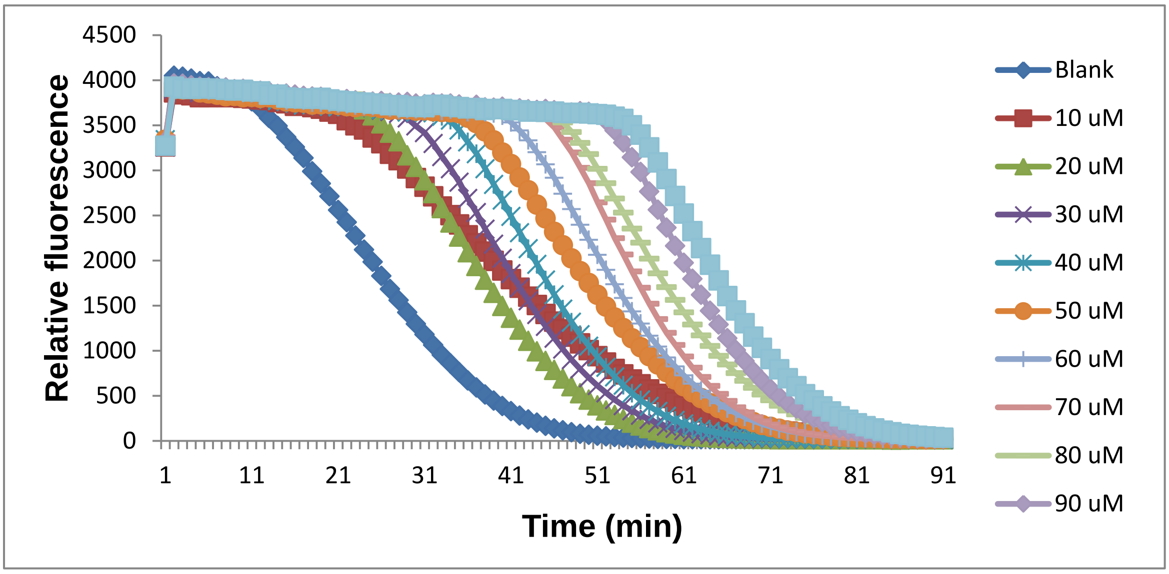

Figure 3.6: The decay of fluorescence intensity of Trolox with time

From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} {}^{fi}\!/_{fo}$$

Where, $fo$is the initial fluorescence reading at 0 min and $fi$is the fluorescence reading at time *i*. The net AUC corresponding to a sample was calculated by

$$\text{Net AUC} = \mathrm{AUC}_{\text{antioxidant}} - \mathrm{AUC}_{\text{blank}}$$

The regression equation between net AUC and antioxidant concentration was calculated. The slope of the equation was used to calculate the ORAC-FL value by using the Trolox curve obtained for each assay. Final ORAC-FL values were expressed as µM Trolox equivalent or µM Trolox equivalent/ mg peptides.

NOTE- *The freeze dried samples were dispersed in distilled water at 1 mg/ml concentration and further diluted up to 100 times for analysis.*

3.7.3 Assay for DPPH radical scavenging activity

Antioxidant capacity based on DPPH (2, 2 diphenyl-1-picryl hydrazyl) radical for peptides analyzed by method given by (Zhidong et al., 2013)

3.7.3.1 Materials

DPPH (2, 2 diphenyl-1-picryl hydrazyl) purchased from Sigma (St. Louis, MO, USA).

3.7.3.2 REAGENTS:

a) **DPPH solution (1mM):** 4 mg of 2, 2-Diphenyl-1-picrylhydrazyl (Sigma) was dissolved in 10 ml of Ethanol. Keep it over night with continuous stirring at 4° C.

3.7.3.3 Procedure

1mililiter of the sample solution/trolox solution was mixed with 1 ml of freshly prepared DPPH solution (0.2mM). The resulting solution was then mixed vigorously and incubated for 30 min at 37^0c temperature by covering the test tube with aluminum foil. The absorbance of the resulting solution was measured at 517 nm using SPECORD-2700 double beam spectrophotometer.

3.7.3.4Calculation:

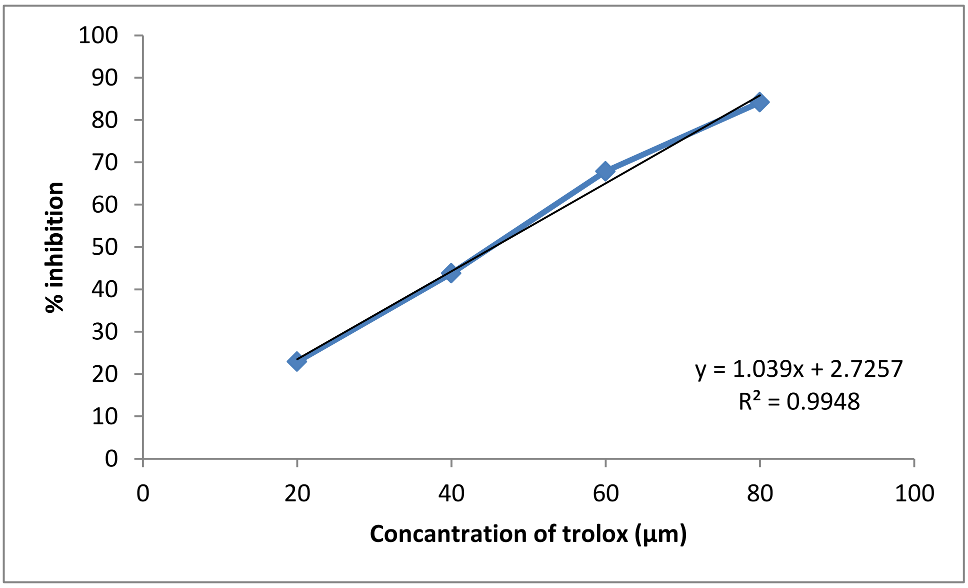

Figure 3.7: Standard curve of Trolox by DPPH method

The results were expressed as

$$\% \text{ DPPH scavenging activity} = 1 - \frac{\text{test sample absorbance}}{\text{blank sample absorbance}} \times 100$$

NOTE- *The freeze dried samples was dispersed in distilled water at 10 mg/ml concentration for analysis*

3.7.4 Assay for Ferric reducing Antioxidant activity:

FRAP activity in samples of casein hydrolysates was carried out according to the method of Benzie and strain (1996) as given below.

3.7.4.1 Materials

Ferric chloride,Glacial acetic acid, TPTZ (2, 4, 6, tri – (2-pyridyl)-5-triazine

3.7.4.2 Reagents:

a) **Ferric chloride (20 mM):** Dissolve 320.4 mg of ferric chloride in 100ml of water.

b) **Glacial acetic acid**: Take 6 ml of glacial acetic acid and make up the volume to 500 ml with distilled water.

c) **TPTZ (2, 4, 6, tri – (2-pyridyl)-5-triazine solution):** Dissolve 31.23mg of TPTZ in 10ml, 40mM HCL.

d) **FRAP Reagent**: Add acetate buffer (0.1M), TPTZ (20mM)and ferric chloride in ratio 100:10:10.

3.7.4.3 Procedure:

Take 90µl of sample. Add 2700µl of FRAP Reagent and 90µl of water. Incubate sample for 30 min at room temperature. Take absorbance at 595nm.

3.7.4.4 Calculation:

Based on the % Increase in absorbance of sample, trolox equivalent was determined from standard curve (Fig. 3.5).

where,

Y is the % increase in absorbance

$$= [(A\ 595\ nm_{control} - A\ 595\ nm_{sample})/A\ 595\ nm_{control}] X\ 100$$

x is the µM concentration of ferrous sulphate

The results were expressed as. µM of $FeSO_4$ / mg of the protein

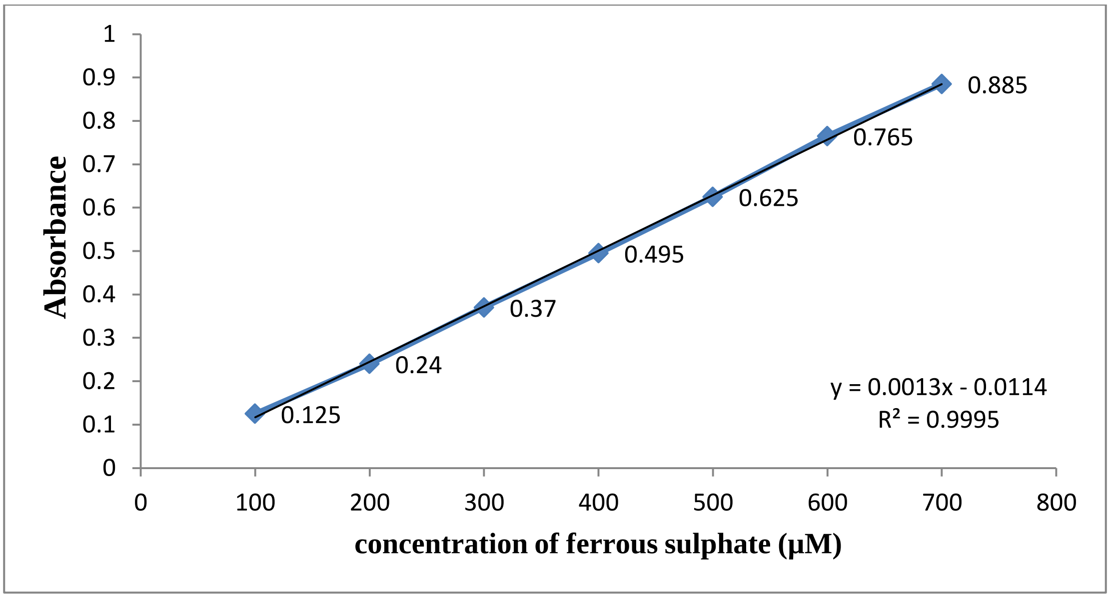

Figure 3.8: Standard curve of FRAP method

NOTE- *The freeze dried samples was dispersed in distilled water at 10 mg/ml concentration for analysis.*

3.7.5 Reducing power

The reducing power of samples was tested according to a method (Wu *et al*., 2003), with slight modifications.

3.7.5.1 Materials

Potassiumferricyanide $K_3Fe(CN)_6$, trichloroacetic acid, ferric chloride, sodium chloride, potassium chloride, disodium hydrogen orthophosphate and potassium dihydrogen orthophosphate were purchased from Hi Media (Mumbai, India).

3.7.5.2 Reagents

a) Potassium ferricyanide(1% w/v)

Dissolved 1.0gmPotassium ferricyanide in distilled water and made up the volume to 100 ml

b) Phosphate buffered saline (PBS, pH 6.6)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 in 800 ml distilled water, adjusted pH to

6.6 with 1 N HCl and made the volume up to 1 liter with distilled water and filtered through 0.45µ filter

c) Trichloroacetic acid (10% w/v)

Dissolved 10gmPotassium ferricyanide in distilled water and made up the volume to 100 ml

d) Ferric chloride (0.1%)

Dissolved 0.1gmFerric chloridein distilled water and made up the volume to 100 ml

3.7.5.3 Procedure

1 ml aliquot of a hydrolysate sample was mixed with 1 ml of 0.2 M PBS and 1 ml of potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50 ^{0}C for 20 min, followed by cooling to room temperature. After adding 2.0 ml of 10% trichloroacetic acid, the mixture was centrifuged at 5,000g for 20 min. The supernatant was transferred and mixed with 0.3 ml of a 0.1% ferric chloride solution and incubate for 10 min at room temperature (21 ^{0}C), the absorbance of the resulting solution was measured at 700 nm using the above UV–vis spectrophotometer (Model: UV-2700, Shimadzu, Tokyo, Japan). Where the control was double distilled water and used in calibration. A calibration curve (figure 3.8) of Ascorbate (20-100 µM) was prepared. Reducing power was expressed as µM Ascorbate equivalent/ mg protein.

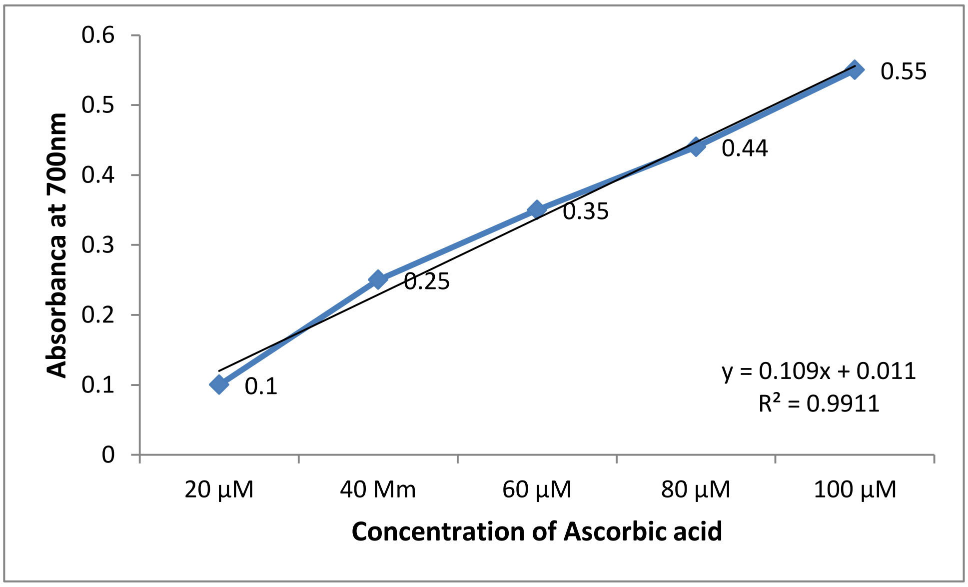

Figure 3.9: Standard curve of reducing power of Ascorbic acid

NOTE-The freezedried samples was dispersed in distilled water at 2 mg/ml concentration for analysis.

3.8 Separation and molecular weight distribution of peptides by gel filtration chromatography

The peptides were isolated from hydrolysates by a HiLoad™ 16/60 Superdex™ 30 prep grade column, 120 mL (16 × 600 mm, GE Healthcare bio-science, Uppsala, Sweden). The column was mounted on ÄKTApurifier™ chromatography system equipped with binary high performance gradient pumps, Pump P-900, Monitor UPC-900 a high precision on-line combined monitor for measuring UV absorption, conductivity and pH, and fraction collector (Frac-920). The system was controlled from UNICORN™ 5.2 software (GE Healthcare, Uppsala, Sweden).

Initially the column was washed with water and then equilibrated with 100 mM ammonium bicarbonate buffer, pH 7.8. The 1 mL of hydrolysates (0.22 µ passed) was injected and isocratic elution was performed at a flow rate of 1.0 mL/min for 1.5 column volume using 100 mM Ammonium bicarbonate buffer, pH 7.8 (Tauzin *et al.,* 2003). All the reagents and solvents were of HPLC grade and were passed through 0.22µ filter,and degassed before use. An internal standards mixture of six components having cytochrome C (13,000 Da) (Sigma cat no- C3131), aprotinin from bovine lung (6511.44 Da) (Sigma cat no- A1153), insulin chain-β oxidized (3495.89 Da) (Sigma cat no- I6383), angiotensin I human acetate (1296.48 Da) (Sigma cat no- A9650), L-glutathione oxidized (612.63 Da) (Sigma cat no-G4376), L-tryptophan methyl ester (254.71 Da) (Sigma cat no-364517) was used to obtain a calibration curve (Figure 3.9). The calibration curve was plotted between K_{av} and Molecular weight. K_{av} for each component was calculated by equation (3.1). This calibration curve was used for calculating molecular weight distribution of peptides in hydrolysates by interpolation of K_{av}. The curve was fitted by linear regression using Graphing software (GraphPad Prism 5.03; GraphPad Software, San Diego, CA, USA).

$$K_{av} = {(V_e - V_o)}\big/{(V_t - V_{o)}} \quad \ldots\ldots\ldots\ldots \qquad (3.1)$$

Where, $K_{av}$is the partition coefficient, V_e is the elution volume for sample, V_o is void volume and V_t is the total volume

$$Y=-0.4544x+1.875$$

$$R^2 = 0.9016$$

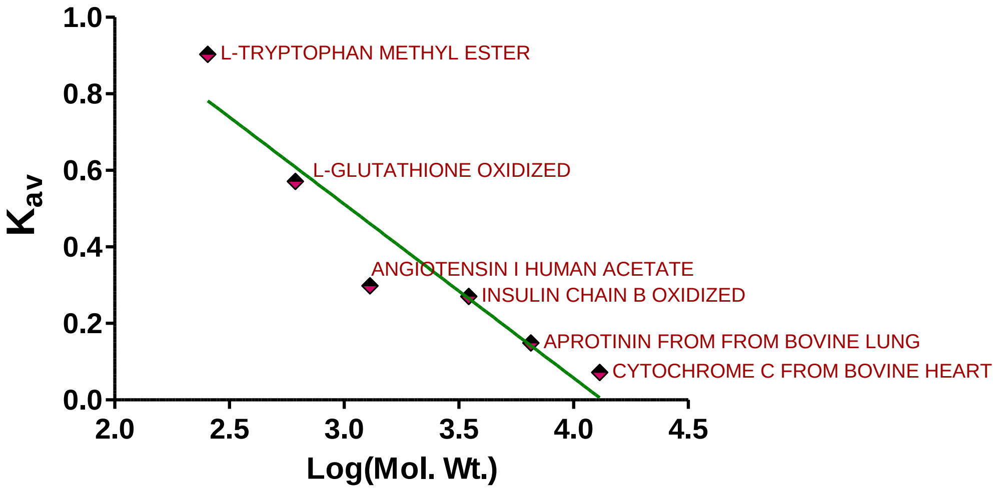

Figure 3.10: Standard curve for determination of molecular weight by SE-HPLC

Note: The peptide peaks collected from gel filtration were lyophilized and dispersed in 200 µl distilled water for further analysis.

3.9 Purification of peptides by semi-preparative RP-HPLC

Ultra-filtered fraction of hydrolysate (CH 1, CH 2, CH 3) were dissolved in HPLC grade water and purified on RESOURCE™ RPC reverse phase C18 column (6.4 X 100 mm, particle size 15 µm, GE Healthcare) with mono disperse underivatized polystyrene/divinyl benzene beads. The column was mounted on ÄKTApurifier™ chromatography system equipped with fixed wavelength 214 nm UV detector, fraction collector and controlled from UNICORN™ 5.2 software (GE Healthcare, Uppsala, Sweden). The elution was run at 0.70 mL/min at room temperature by a binary gradient with acetonitrile as an organic modifier and TFA as ion paring agent. Solvent Acontained 0.1%TFA in HPLC grade water and solvent B contained 0.01% TFA in acetonitrile-HPLC grade water (70:30, v/v). The column was initially equilibrated with 10 column volume (CV) 100 % of solvent A. 500 µL of sample was applied to the column. The unbound fraction was washed with 2 CV of 100% solvent A. The bound fraction was eluted by a gradient of 0–40% B in 25 min, 40- 60% of B in next 10 min and further the gradient was expanded from 60-100% in next 10 min. Finally the elution was completed with 100% B up to 5 min. The column was held at 100% of solvent B for 25 min and re-equilibrated with 10 CV of 100% solvent A. The peaks corresponding to hydrolysate were collected and concentrated for bioactivity evaluation and RP-HPLC-MS/MS (Tauzin *et al*., 2003).

3.9.1 Concentration of the peptides

The solvents in the sample were evaporated by using vacuum concentrator (Model- Ecospin 3180C, Hanil Science International, Korea,) at 1800 rpm. The dried fractions were dissolved in 200 µL distilled water and assessed for its antioxidant and peptide content. The fractions were diluted to 10 times for protein estimation and ABTS assay, 50 times in case of ORAC assay.

3.9.2 Peptide identification

The most active fractions obtained from RP-HPLC purification were analyzed by LC-MS/MS to characterize the peptides responsible for antioxidant activity. Peptides in three RP-HPLC fractions of hydrolysate CH1, CH2 and CH3 were sequenced from Institute of Microbial Technology (IMTECH), Chandigarh, India.. Spectra were analyzed to identify proteins of interest using Mascot sequence matching software [Matrix Science] with taxonomy set to other mammalia.

3.10 Techno functional properties

3.10.1 Solubility measurement

The solubility hydrolysates were measured as a function of pH. The freeze dried sample was dispersed in distilled water at 10 mg/ml, and the pH was adjusted to 3-9 with1N HCl or NaOH. After incubation at different pH for 30 min at room temperature, samples were centrifuged at 14,100 g for 10 min (model 6500, KUBOTA, Tokoyo). The protein content in the supernatant was measured by the Lowry method, as detailed above. The solubility was calculated as the percentage of the protein concentration to that of the corresponding solution at pH 9 (Kasran *et al.,*2012).

$$\% \text{ Solubility} = \frac{\% \text{ Protein at corresponding pH}}{\% \text{ Protein at pH 9.0}}$$

3.10.2 Effect of temperature on solubility

The freeze dried samples of hydrolysate was dispersed in distilled water at 10 mg/ml, and the pH was adjusted to 3-9 with1N HCl or NaOH, After heat treatment at 90^0 C/ 10 min and sterilization temperature (121^0C/ 15 min), samples were centrifuged at 14,100 g for 10 min (model 6500, KUBOTA, Tokoyo). The protein content in the supernatant was measured by the Lowry method, as detailed above. The effect of temperature on solubility was calculated as the percentage of the protein concentration to that of the corresponding solution at pH 9 (as mentioned in part 3.10.1)

3.10.3 Emulsifying properties

Preparation of emulsion with an ultrasonic instrument (model)) consisting of power supply, a sonic convertor and a disruptor horn stepped down to half inch in diameter at the tip. Based on preliminary experiments, the following standard conditions were followed in preparation of test emulsions. For this, different conditions (1, 2, 3%) of samples were taken in 100 ml beakers containing 2.5% soya oil. The power supply of the sonifier and the automatic timer were adjusted to give the desired ultrasonic output for definite time. Each sample was positioned on a wooden block, so that the probe tip of the Sonifier was 1.2 cm, above the inside bottom of the beaker was done for 10 minutes. Immediately after sonication, the probe was rinsed with distilled water and wiped dry between the samples.

3.10.3.1 Emulsifying activity and emulsion stability indexes

Emulsifying activity (EAI) and emulsion stability indexes (ESI) of hydrolysates were determined by the turbidimetric method (Pearce and Kinsella, 1978), sample of the prepared emulsion was withdrawn at 0 and 10 min after emulsion, followed by diluting 500 times with 0.1% SDS solution. Then, the absorbance was measured at 500 nm using the above UV-VIS spectrophotometer. The EAI and ESI were calculated according to

Calculation:

$$\mathbf{EAI\ (m^2/g) = 2T \times A_0 \times dilution\ factor/\ C \times \Phi \times 10{,}000}$$

where T is a constant (2.303 m^2/ml), the dilution factor was 100, C is the protein concentration in the aqueous phase before emulsion preparation (g/ml), Ø is the volumetric fraction of oil (0.25), and A_0 is the absorbance right after emulsion (0 min).

Calculation:

$$\mathbf{ESI\ (min) = A_0 \times \Delta t / \Delta A}$$

Where ΔA is the absorbance decrease from A_0 after Δt (10 min).

3.10.4 Whipping ability

Foaming properties were determined using a household mixer by whipping 75ml of 3 percent (w/v) (De Wit *et al*., 1988). For this, the protein mixtures were maintained at 31°C.

Overrun and foam stability were calculated as follows:

$$\text{Overrun} = (B-A)/ A \times 100$$

$$\text{Stability} = C/A \times 100$$

Where,

A = volume of liquid prior to whipping.

B = total foam volume obtained immediately after whipping

C = volume of liquid retained in the foam after 1 hr at room temperature (i.e., the weight of the foam divided by the density of the liquid)

3.11 Utilization of casein hydrolysate in model food system

whey drink was selected as model food system for the utilization of buffalo casein hydrolysate with high antioxidant property.

3.11.1. Preparation of whey drink

For the preparation of whey drink whey permeate was collected from Modern Dairy, NDRI, Karnal. Zira flavour(Flying bird, India) was purchased from local market.

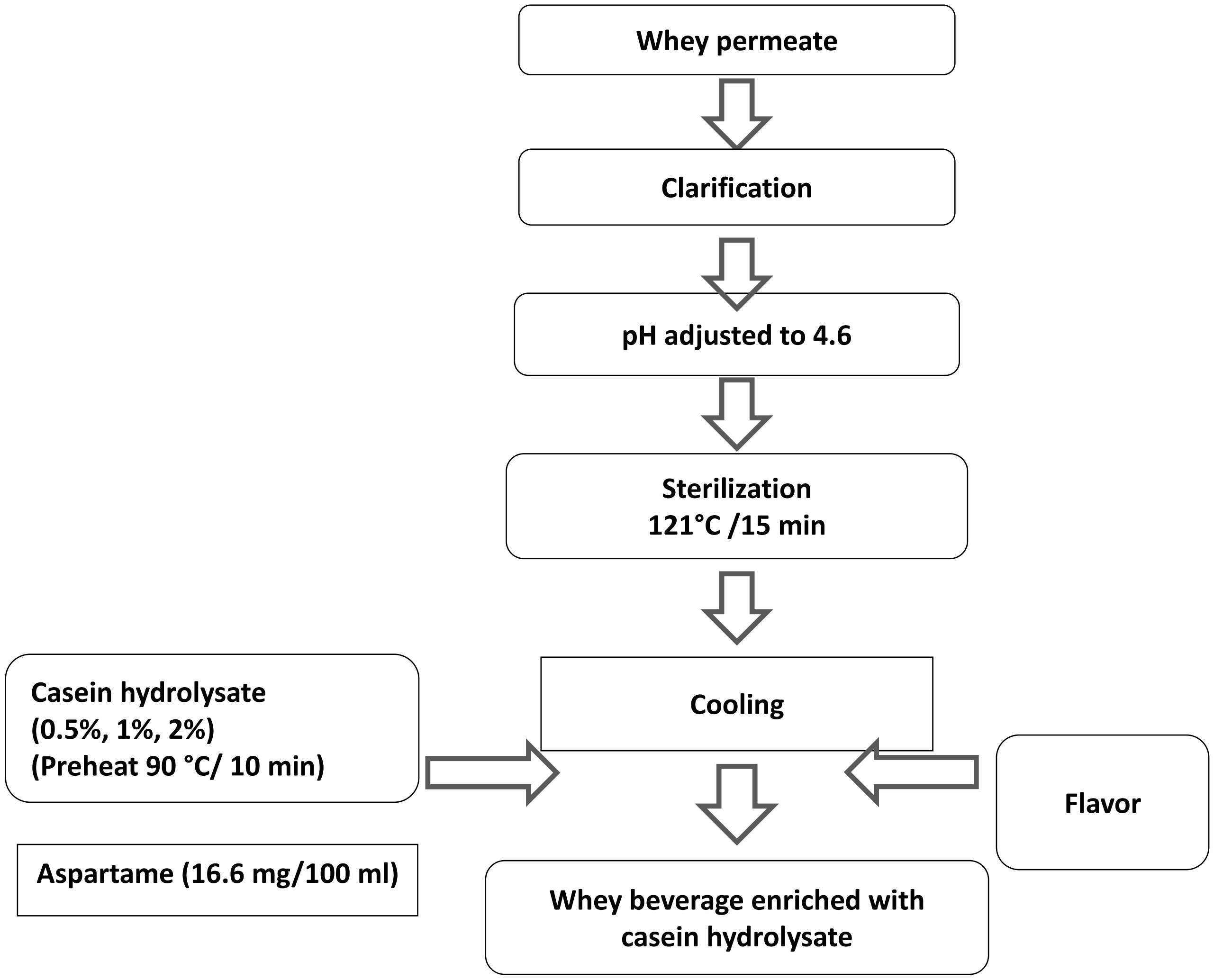

Figure 3.11: Preparation of Whey drink

3.12 Statistical analysis

All the tests were conducted in triplicate. The results obtained were subjected to one-way analysis of variance (ANOVA). Duncan's new multiple range test was performed to determine the significant difference between samples within the 95% confidence interval using Graph pad prism6 software.

RESULTS AND DISCUSSIONS

Enzymatic hydrolysis of buffalo sodium caseinate was performed using commercial proteases alcalase and flavourzyme, alone or in combination to generate antioxidant peptides. The hydrolysis was done under controlled conditions (temperature, pH and stirring) in a 50 ml reaction vessel equipped with a stirrer, thermometer and a pH electrode using DH analyzer (pH–stat auto-titrator). The conditions used for hydrolysis included 3000 units/g protein for alcalase and 2500 units/g protein for flavourzyme at pH 7.5, 50^{o}C as optimized previously in lab. Biological and functional properties of enzymatic hydrolysates of milk proteins can be improved by controlling kinetic conditions during proteolysis.

4.1 Time-dependent degree of buffalo casein hydrolysis:

The enzymatic break down of protein is generally quantified as the degree of hydrolysis (DH) referred to the percentage of peptide bonds cleaved. Different methods have been used to evaluate the DH of the peptide bonds which depended on three essential principles: the amount of nitrogen released by the protein hydrolysis in the presence of a precipitation agent (e.g. trichloroacetic acid), the determination of free α-amino groups and the titration of the released protons. The measurement based on pH-Stat titration show the advantage during hydrolysis as give a direct measure of the amount of alkali used during the course of reaction

The principle of the pH stat technique is that when hydrolysis is carried out at neutral or alkaline conditions, dissociation of protons from the free amino groups released is favoured (Adler-Nissen, 1986). The liberation of protons into the surrounding medium leads to a reduction in the pH of the reaction mixture. The number of peptide bonds cleaved can be estimated from the amount of base required to maintain a constant pH during the reaction (Adler-Nissen, 1986).

The OPA method is based on the specific reaction between OPA and primary amino groups, in the presence of a thiol to form 1-alkylthio-2-alkyl-substituted isoindoles (Medina Hernandez *et al.,* 1990 a,b). The isoindoles formed can be quantified spectrophotometrically at 340 nm.

RESULTS AND DISCUSSIONS

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Based on pH-stat titration, the time dependent hydrolysis of buffalo casein hydrolysates using alcalase, flavourzyme and their combination is presented in Figure 4.1. In case of flavourzyme hydrolysis, initially the reaction is relatively slow and attains DH=5% in 25 minutes , thereafter, the rate of hydrolysis is increased to 10% in 50 minutes and attains the plateau in 60 minutes and kept relative constant for the rest of the hydrolysis reaction. In case of alcalase hydrolysis, it was observed that the DH increased rapidly in the first 40 minutes, due to high protein content and enzyme concentration. The DH reached a plateau after 100 minutes (DH = 19%) and kept relative constant for the rest of the hydrolysis reaction. Similar trend was observed in case of combination of alcalase-flavourzyme combination.

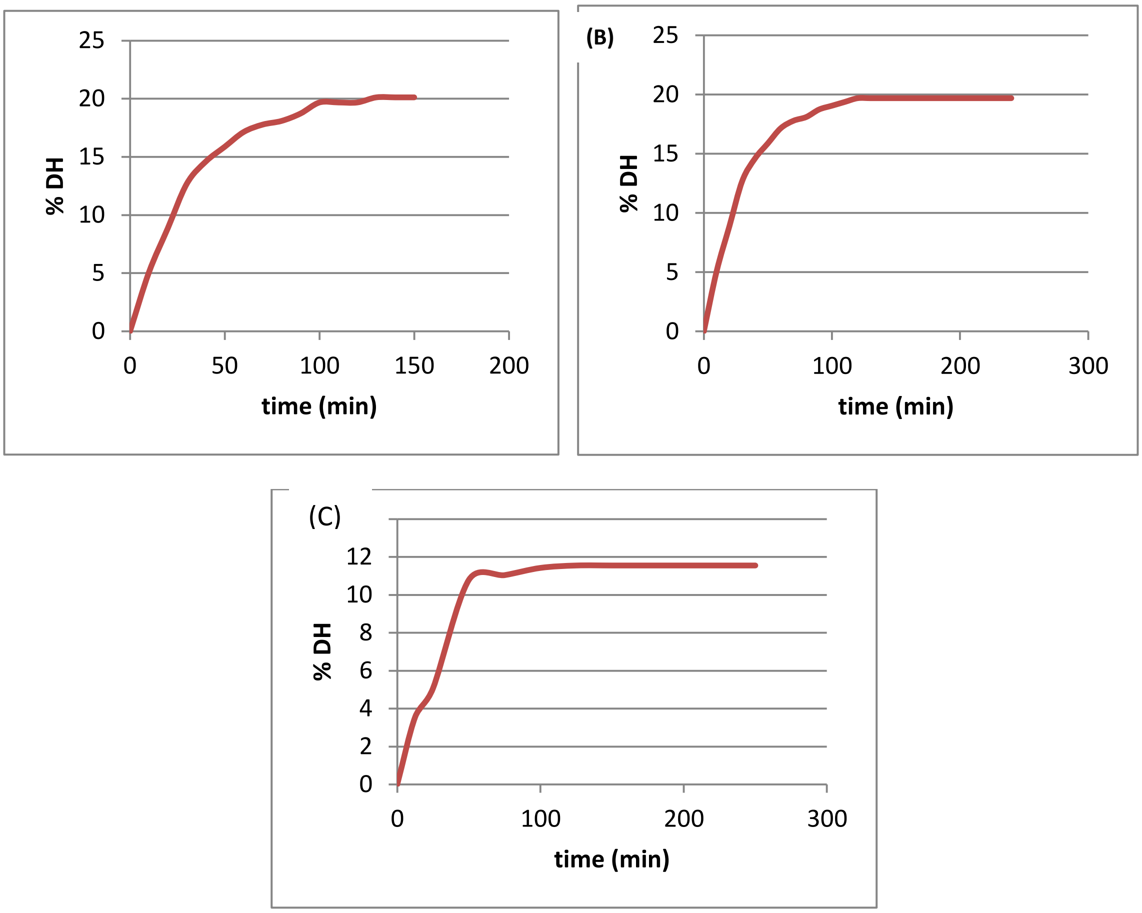


Figure 4.1: Time dependent degree of hydrolysis of buffalo casein hydrolysates A) CH1, B) CH2 and CH3 (pH stat method)

Using OPA method the DH determined for alcalase hydrolysate showed an increase in value from 23.5 to 29.2% as the time period of hydrolysis increased from 120 to 240 minutes unlike pH stat titration method wherein it became constant after 120 minutes. In case of flavourzyme hydrolysis, the OPA method based DH value showed a significant increase in extent of hydrolysis of buffalo sodium caseinate from 120 min to 240 miutes unlike pH stat titration which attains plateau after 60 minutes. Further in case of combination of alcalase and flavourzyme combination with different level of predigestion with alcalase, it was observed that no significant difference was observed in DH value using pH-stat titration, however, a sharp increase in the release of free amino acid as measured by OPA method was observed with passage of hydrolysis time period. The underestimation of DH using flavourzyme by the pH stat method was as expected, since the enzyme preparation is enriched with both endo and exopeptidase activities. Similar observations were made by Spellman *et al* (2003) using exopeptidase rich preparation debitrase HYW20 for hydrolysis of whey protein concentrate. This underestimation is due to the fact that the pK value of the α-amino group is considerably higher for tripeptides, dipeptides and free amino acids, than for polypeptides (Adler-Nissen, 1986). This lead to an under-estimation of the value for 1/α used in the calculation of DH by the pH stat method (see Eq. 1), and a consequent under-estimation of DH. DH values calculated by the pH stat method for hydrolysates generated with exopeptidase-rich preparations would therefore be lower than values obtained by OPA method, as the hydrolysate would contain relatively high amounts of free amino acids. When using a proteinase such as Alcalase 2.4L to generate hydrolysates with moderate DH values (i.e. below 20%), the relative content of free amino acids and dipeptides is likely to be low (Adler- Nissen, 1986). This means that the change in the 1/α value during hydrolysis should be negligible, and DH values obtained by the pH stat would be accurate. In the present study the relative greater DH (28%) might be responsible for the differences in the pH-stat and OPA value observed for alcalase hydrolysate obtained after 240 minutes of hydrolysis.

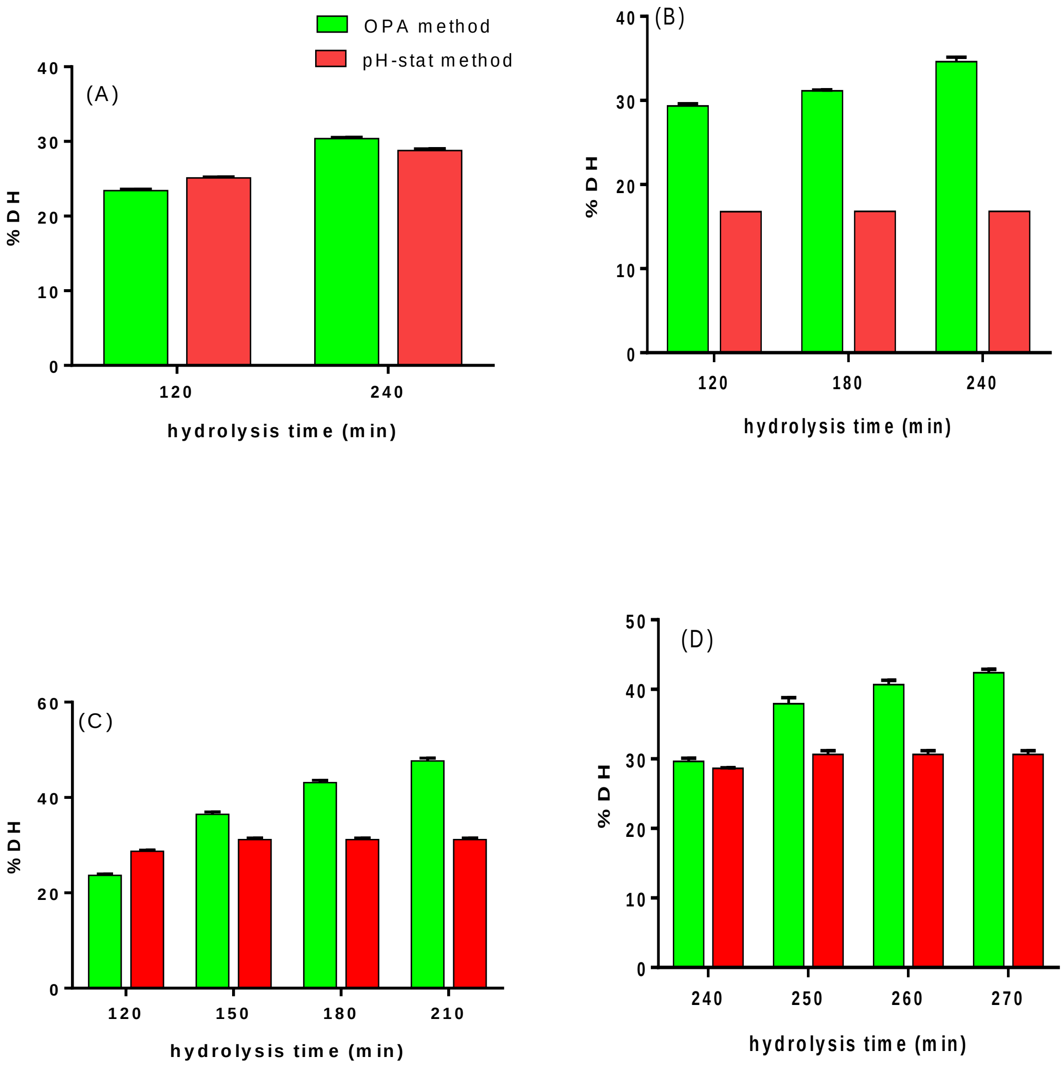


Figure 4.2: Comparative evaluation of methods for measurement of DH A) alcalase hydrolysed buffalo casein B) flavourzyme hydrolysed buffalo casein C) & D) alcalase flvaourzyme combination

4.2 Antioxidant activity of buffalo casein hydrolysate

Effect of hydrolysis of buffalo sodium caseinate using alcalase and flavourzyme alone or in combination (with alcalase pre-digestion at 120 and 240 min, respectively) along with the effect of flavourzyme at varying interval of time was assessed for their effect on antioxidant activity of hydrolysates. The antioxidant activity of hydrolysate was measured using ABTS radical scavenging method and expressed as trolox equivalent antioxidant capacity (TEAC) and presented in figure 4.3 & 4.4

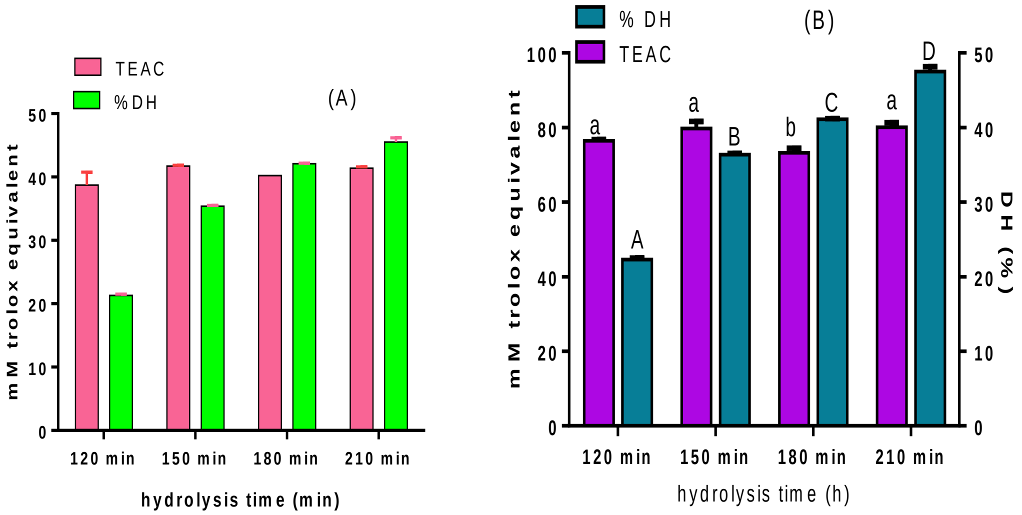


Figure 4.3: Evaluation of antioxidant activity of buffalo casein hydrolysed using alcalase-120 flavourzyme combination (protein concentration A. 2.5% B. 5%)

It was observed that following 2h pre-digestion of 2.5% buffalo sodium caseinate with alcalase, the antioxidant activity corresponded to 38 mM TEAC, which showed a progressive increase with combination of flavourzyme, with consequent increase in degree of hydrolysis (DH) from 24.51 to 36.84%, further no significant ($p<0.05$) difference in antioxidant activity was observed with increase in hydrolysis time up to 210 min, although there was significant difference ($p<0.05$) in DH. Similar trend on hydrolysis of buffalo sodium caseinate at 5% level was observed, with identical level of cleavage of peptide bond following pre-digestion with alcalase for 120 min. A linear increase in the antioxidant activity was observed from 38 mM TEAC to 78 mM TEAC, with increase in the substrate concentration from 2.5% to 5.0%. Further a similar effect was observed on antioxidant activity of hydrolysate following digestion with a combination of alcalase and flavourzyme.

Result as presented in figure 4.4 following pre-digestion for 240 min with alcalase, the antioxidant activity of 2.5% buffalo sodium caseinate remained to the level as observed at 120 min pre-digestion, although the corresponding DH increased. Following combination of flavourzyme there was a progressive increase in antioxidant activity however, no significant difference ($P> 0.05$) on antioxidant activity was observed with passage of time interval although significant difference ($P < 0.05$) on DH was observed. Similar effect on antioxidant activity was observed on increase the substrate concentration to 5% (Figure 4.4), with corresponding increase in antioxidant activity. The value of antioxidant activity nearly doubled as the substrate concentration enhanced from 2.5% to 5.0%.

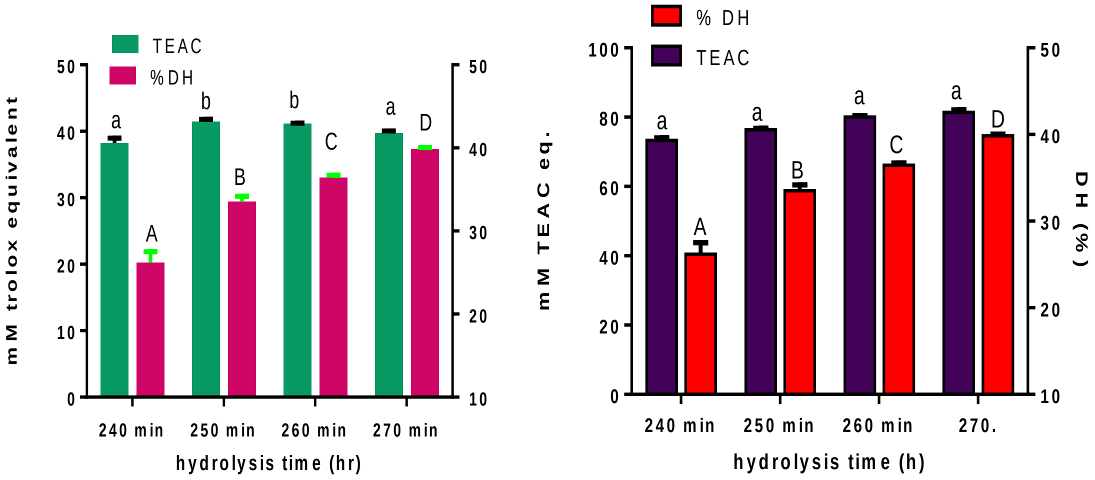


Figure 4.4: Evaluation of antioxidant activity of buffalo casein hydrolysed using alcalase-240 flavourzyme combination (protein concentration A. 2.5% B. 5%)

The degree of hydrolysis measures the content of peptide bond cleaved in the substrate by proteolytic agent; the higher the DH, the higher the content of released amino groups. The size of the peptides is known to be a significant factor in the overall antioxidant activity and functional properties of protein hydrolysates. DH is reported to affect the antioxidant activity of protein hydrolysates. During hydrolysis, peptides with antioxidant properties may be continuously formed and degraded depending on their molecular structure, which is primarily affected by the hydrolysis conditions (Cornelly *et al*., 2002).

Alcalase had a broad specificity for aromatic (Phe, Trp, and Tyr), acidic (Glu), sulphur- containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser) and basic (Lys) residues (Doucet*et al.,* 2003). However, one type of enzyme in a one-type reaction could not achieve a high DH in a reasonable period of time. To obtain a higher DH, the combinations of endoprotease (alcalase) and exopeptidase (flavourzyme) were examined. As flavourzyme is produced by fermentation of selected strains of *Aspergillus oryzae*, which contains both endo- and exo-protease activities, the DH% by flavourzyme in the combination of enzyme systems were higher than those of one-type enzyme (Pedroche *et al*., 2004).

The bioactivity of protein hydrolysates was dependent on protein source, enzyme type, hydrolysis extent and so on (Mota *et al*., 2006). Li *et al.* (2013) also found high ABTS radical scavenging activity (SC50 ≈ 0.5 µg mL-1) in goat casein hydrolysates produced with a combination of neutral protease and alcalase. The higher activity in their study could in part be due to the broader specificity of the two proteases used.

4.3 Antioxidant capacity of buffalo casein hydrolysates and their Ultrafiltred (UF) fractions

On subjecting the supernatant (obtained after centrifugation of hydrolysate) to microfiltration through 0.45 µm filter, significant amount of non-dissolved solids were removed as a means to minimize fouling during UF. The buffalo casein hydrolysate (CH1, CH2 and CH3) were subjected to UF using stirred cell under nitrogen pressure using different molecular weight cut off membrane (MWCO) as 10kDa, 3kDa and 1kDa in sequential manner. The permeate collected on fractionation with different MWCO membranes was freeze dried and dispersed at a concentration of 10mg/ml in distilled water to assess their antioxidant capacity. The measurement of antioxidant capacity was done following different chemical assays based on ABTS, ORAC, DPPH, FRAP as presented in (Figure 4.5) and reducing power in (Figure 4.6)

4.3.1 Antioxidant properties:

4.3.1.1 ABTS radical scavenging activity

Antioxidant activity of the hydrolysates was evaluated by measuring their activity against 2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical In the Trolox equivalent antioxidant capacity (TEAC) assay, preformed ABTS•+ generated by oxidation of ABTS with potassium persulfate, was reduced in the presence of hydrogen donating groups of antioxidants which could be measured by reduction in colour at 750 nm (Re *et al.,* 1999). A significant enhanced ABTS radical scavenging activity was observed for UF 10 kDa permeate compared to total hydrolysate each for CH1, CH2 and CH3. Further, a significant difference ($p<0.05$) in antioxidant activity was observed in 10 kda fractions of CH1, CH2 and CH3 in the order of activity as CH1>CH2>CH3. Further on UF separation of 10 kDa fraction of hydrolysate to 3 kDa and 1 kDa cutoff membranes, a successive increase in antioxidant activity was observed (Table 4.1), which was maximum in 1 kDa fraction in each of the hydrolysate (CH1, CH2 and CH3). Antioxidant activity of 1 kDa fraction corresponded to 2356 µM TEAC/mg for CH1, 1786 µM TEAC/mg for CH2 and 1674 µM TEAC/mg for CH3, respectively.

4.3.1.2 Oxygen radical antioxidant capacity (ORAC):

The ORAC assay measures the damage of fluorescent probe (Fluorescein) by peroxy radicals. The presence of antioxidants inhibits the damage of fluorescent probe by reacting with free radicals which is measured by loss in fluorescence with excitation at 485 nm and emission at 520 nm (Davalos *et al.,* 2004). Based on ORAC assay it was observed that activity of different hydrolysate increased significantly ($P<0.05$) on separation of hydrolysate from 10 kDa, 3 kDa and 1 kDa cutoff UF membrane. There is significant difference ($p<0.05$) in antioxidant capacity observed between CH1, CH2 and CH3, with maximum activity observed in CH 1 and relatively lower activity observed in CH 2. Maximum antioxidant activity was observed in 1 kDa fraction from each kind of hydrolysate. Antioxidant activity increased to a level of 1.19 fold for CH2 and 1.26 fold as compared to CH 3, however, there was no significant difference ($p>0.05$) in activity of 1 kDa fraction prepared from either of hydrolysate preparation.

4.3.1.3 DPPH radical scavenging activity

DPPH is a stable free radical of 2, 2-diphenyl-1- picrylhydrazyl with a deep purple colour whose reaction with other radicals, reducing agents, or compounds capable of hydrogen atom transfer (HAT) leads to loss of colour at 515 nm. The reaction continues more slowly (Apak *et al.,* 2013). The percentage inhibition of DPPH radical scavenging activity increased with separation of hydrolysate using 10 kDa, 3 kDA and 1 kDa cut off UF membrane (Table 4.3 & Fig 4.5). A significant difference ($p<0.05$) between Radical scavenging activity

(RSA) of casein hydrolysates (CH1, CH2 and CH3) was observed with maximum DPPH radical scavenging activity in short peptides i.e. 1 kDa fraction from each of hydrolysate preparation. 1.93 fold in RSA was observed in 1kDa fraction compared to whole hydrolysate in CH 1, while the same for CH 2 was 2.29 fold and 1.86 fold for CH 3. No significant difference ($P>0.05$) in RSA was observed between 1kDa fraction from CH 1 and CH 2 and CH 3 and CH 2.

4.3.1.4 Ferric reducing antioxidant power (FRAP)

The FRAP assay measures the reduction of ferric to ferrous ion at low pH in the presence of antioxidants, which causes a colored ferrous-tripyridyl triazine complex to form (Gliszczynska-Swiglo, 2006). Based on ferric reducing antioxidant power (FRAP) maximum reducing activity was observed in casein hydrolysate (CH1) and no significant difference ($P>0.05$) in activity was observed between CH 2 and CH 3. Further on fractionation with 10 kDa, 3 kDa and 1 kDa cutoff UF membrane, no significant difference ($p<0.05$) in FRAP value was observed between 10 kDa and 3 kDa fraction of CH 1 and CH 2 while significant difference ($P<0.05$) was observed in 10 kDa and 3 kDa fraction of CH 3 hydrolysate. Further the activity significantly differed in 1 kDa fraction of CH 1 and CH 2 while no significant difference ($P>0.05$) was observed between CH 2 and CH 3 hydrolysate.

4.3.1.5 Reducing power

The reducing power was evaluated to determine the ability of a sample to donate electrons or protons, which has been reported to be directly correlated with peptide cleavages (Cumby et al, 2008) and therefore the improved antioxidant activities of hydrolysates. In the present study, the reducing power was expressed as the ability to reduce $Fe3+$/ferricyanide complex to the $Fe2+$ form, which can be monitored for the absorbance of Perl’s Prussian blue at 700 nm. The reducing power of buffalo casein hydrolysates (CH1, CH2 and CH3) expressed as ascorbate equivalent showed maximum value in CH 1 hydrolysate (Table 4.5 & Fig 4.6). There was a linear increase in reducing power on fractionation of CH 1 hydrolysate using 10 kDa, 3 kDa and 1 kDa cutoff UF membrane. The value of reducing power increased by nearly double in the permeate of 1 kDa fraction compared to that of 3 kDa fraction of CH 1 hydrolysate. A significant difference ($P<0.05$) in reducing power of 1 kDa fraction of CH 1, CH 2 & CH 3 was observed. The enhanced reducing power of hydrolysates may be related to the increased availability of protons and electrons resulting from enzymatic hydrolysis.

Hogan *et al.* (2009) also elucidated that hydrolysis of milk protein isolate using microbial proteases resulted in the ORAC value ranging from 62.9 to 246.6 µmol TE/g and the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activities of the hydrolysate fractions ranged from 3.1 to 35.4 µmol TE/g. Cumby *et al.* (2008) prepared canola protein hydrolysates using commercial enzymes (i.e. alcalase and flavourzyme) and reported that the hydrolysates exhibited notable antioxidant activity as determined by the DPPH radical scavenging capacity and reducing power assays. The hydrolysate prepared using flavourzyme exhibited higher antioxidant activity than those prepared using a combination of the two enzymes, or alcalase alone. The authors suggested that the enzyme used for protein hydrolysis would impact antioxidant potential due in part to the mechanism of action of enzyme and subsequent presence or absence of functional groups such as hydroxyl groups on phenolic compounds (Cumby *et al.,* 2008). It could be stated that the type of enzyme employed in the hydrolysis process affects the antioxidant capacity of the hydrolysates.

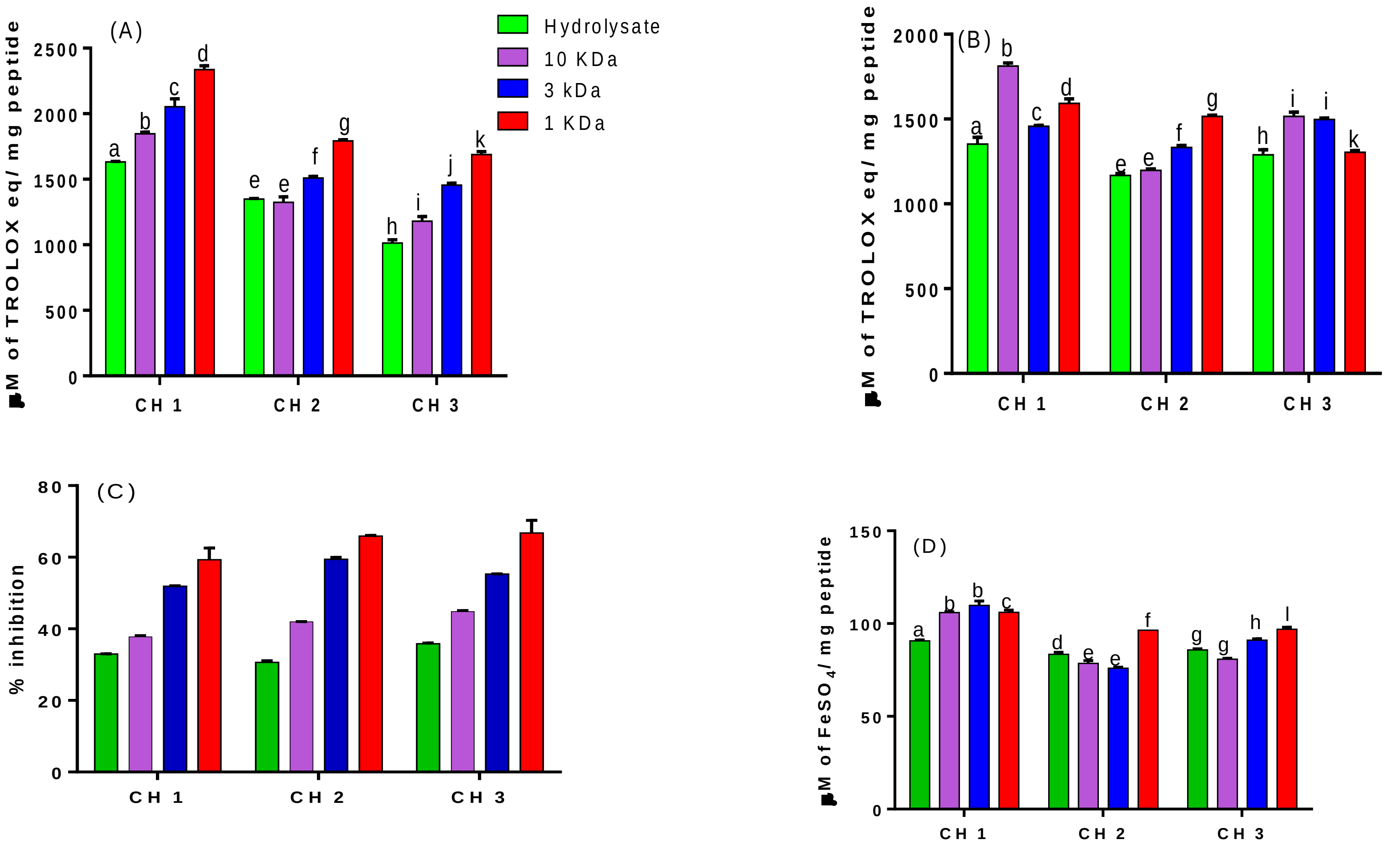


Figure 4.5: Evaluation of antioxidant activities of buffalo casein hydrolysate and their UF fraction A) ABTS assay B) ORAC assay C) DPPH D) FRAP

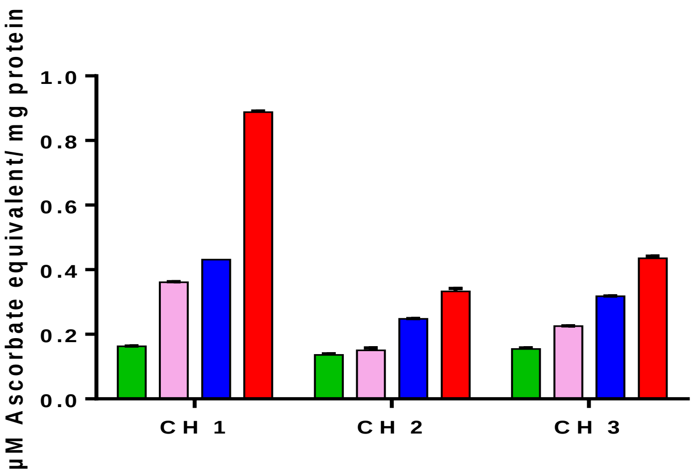


Figure 4.6 **Reducing power of buffalo casein hydrolysates and their UF fraction**

ABTS activity	Antioxidant activity µM TEAC/ mg protein			
	Hydrolysate	10 kDa fraction	3 kDa fraction	1 kDa fraction
CH 1 hydrolysate	1628.19±2.29^{aA}	1838.25±1.4^{bA}	2011.52±2.62^{cA}	2356.41±4.22^{dA}
CH 2 hydrolysate	1371.68±3.47^{aB}	1253.61±2.94^{bB}	1418.31±4.21^{cB}	1786.41±2.65^{dB}
CH 3 hydrolysate	1031.03±2.28^{aC}	1156.74±4.87^{bC}	1447.24±2.88^{cB}	1674.13±3.24^{dC}

Table 4.1: ABTS radical scavenging activity of buffalo casein hydrolysates and their UF fraction

ORAC assay	Antioxidant activity µM TEAC/ mg protein			
	Hydrolysate	10 kDa fraction	3 kDa fraction	1 kDa fraction
CH 1 hydrolysate	1324.24±1.55^{aA}	1845.49±1.07^{bA}	1454.23±3.08^{cA}	1574.66±3.20^{dA}
CH 2 hydrolysate	1158.79±2.85^{aB}	1203.59±3.58^{bB}	1140.97±2.46^{cB}	1519.93±5.02^{dA}
CH 3 hydrolysate	1269.10±2.04^{aC}	1533.05±3.62^{bC}	1503.46±3.86^{cC}	1597.32±2.61^{dA}

Table 4.2: ORAC assay of buffalo casein hydrolysates and their UF fraction

DPPH	Antioxidant activity µM TEAC/ mg protein			
	Hydrolysate	10 kDa fraction	3 kDa fraction	1 kDa fraction
CH 1 hydrolysate	2.92±0.02^{aA}	3.36±0.13^{bA}	4.74±0.11^{cA}	5.63±0.04^{dA}
CH 2 hydrolysate	2.66±0.22^{aB}	3.78±0.08^{bB}	5.50±0.31^{cB}	6.10±0.14^{dB}
CH 3 hydrolysate	3.18±0.18^{aC}	4.03±0.27^{bC}	5.50±0.13^{cB}	5.92±0.22^{dA}

Table 4.3: DPPH assay of buffalo casein hydrolysates and their UF fraction

FRAP	Antioxidant activity µM TEAC/ mg protein			
	Hydrolysate	10 kDa fraction	3 kDa fraction	1 kDa fraction
CH 1 hydrolysate	91.02 ±1.77^{aA}	105.65 ± 1.14^{bA}	111.49 ± 1.44^{bA}	105.14 ± 3.0^{cA}
CH 2 hydrolysate	84.21 ± 1.48^{aB}	77.44 ± 2.14^{bB}	75.53 ± 1.67^{bB}	96.43 ± 2.58^{cB}
CH 3 hydrolysate	85.50 ± 2.72^{aB}	81.19 ± 3.04^{aC}	90.55 ± 1.24^{bC}	96.20 ± 2.37^{cB}

Table 4.4: FRAP of buffalo casein hydrolysates and their UF fraction

Reducing Power	**µM Ascorbate equivalent/ mg protein**			
	Hydrolysate	10 kDa fraction	3 kDa fraction	1 kDa fraction
CH 1 hydrolysate	0.1633 ± 0.002^{aA}	0.36235 ± 0.004^{bA}	0.4312 ± 0.003^{cA}	0.8899 ± 0.007^{dA}
CH 2 hydrolysate	0.1339 ±0.001^{aB}	0.156 ±0.003^{bB}	0.2477 ± 0.004^{cB}	0.3394 ± 0.002^{dB}
CH 3 hydrolysate	0.1523 ±0.001^{aA}	0.2248 ± 0.003^{bC}	0.3165 ± 0.001^{cC}	0.4312 ± 0.006^{dC}

Table 4.5: Reducing power of buffalo casein bhydrolysates and their UF fraction

4.4 Fractionation of buffalo casein hydrolysates using Size Exclusion High-Performance Liquid Chromatography (SE-HPLC)

The preparations of buffalo casein hydrolysates (CH1, CH2 and CH3) generated under optimum conditions using commercial proteases alcalase, flavourzyme and their combination were characterized for their peptide profile by fractionating using size exclusion chromatography (SEC).

4.4.1 Peptide profile of hydrolysate CH 1:

Hydrolysate CH 1 from sequential hydrolysis of alcalase and flavourzyme resolved into 12 peaks as show in (Figure 4.7). On evaluating the peptide profile of hydrolysate as depicted in (Table 4.7) it was observed that peak AF-8 corresponding to 1.36 kDa represented maximum area of chromatogram (28.71%) while that of peak AF-6 represented 16.8% area corresponding to molecular weight 1.93 kDa, while the fraction of 0.59 kDa represented 14.49% chromatogram area. The peptides of size 3-1 kDa represented 70.3% and that of 1-0.5 kDa corresponded to 14.49 % area of chromatogram. On evaluating the antioxidant activity of different fraction as presented in (table 4.6), it was observed that fraction corresponding to 0.59 kDa (Peak no AF-9) showed maximum antioxidant activity based on ABTS radical scavenging activity, while peak AF-6 and AF-7 resulted in comparable antioxidant capacity whereas peak no 8 showed 1356 µM TEAC/mg peptide. Similar trend of antioxidant capacity was observed using ORAC assay.

	Peak no	Fraction No	M.Wt. (kDa)	ABTS (TEAC µM)	ABTS (TEAC µM/mg peptides)	ORAC (TEAC µM)	ORAC (TEAC µM/mg peptides)	PROTEIN content (mg/ml)
CH 1	AF-1	14	13.96	441.85	3744.46	----	--	0.11
	AF-2	20	5.98	1156.13	886.26	----	---	1.30
	AF-3	22	4.21	2166.39	1747.09	499.42	402.75	1.24
	AF-4	23	3.15	2190.07	867.70	571.63	226.47	2.52
	AF-5	25	2.77	4415.80	1451.37	2167.86	712.52	3.04
	AF-6	26+27	1.93	4616.27	2863.69	1931.57	1198.24	1.61
	AF-7	28	1.53	5124.56	2728.73	4416.63	2351.77	1.87
	AF-8	29	1.36	5277.68	1356.03	7988.41	2052.52	3.89
	AF-9	34	0.59	589.44	5457.77	2727.20	25251.84	0.10

Table 4.6: Antioxidant activitiy of hydrolysate (CH1) peak fractions separated by SEC

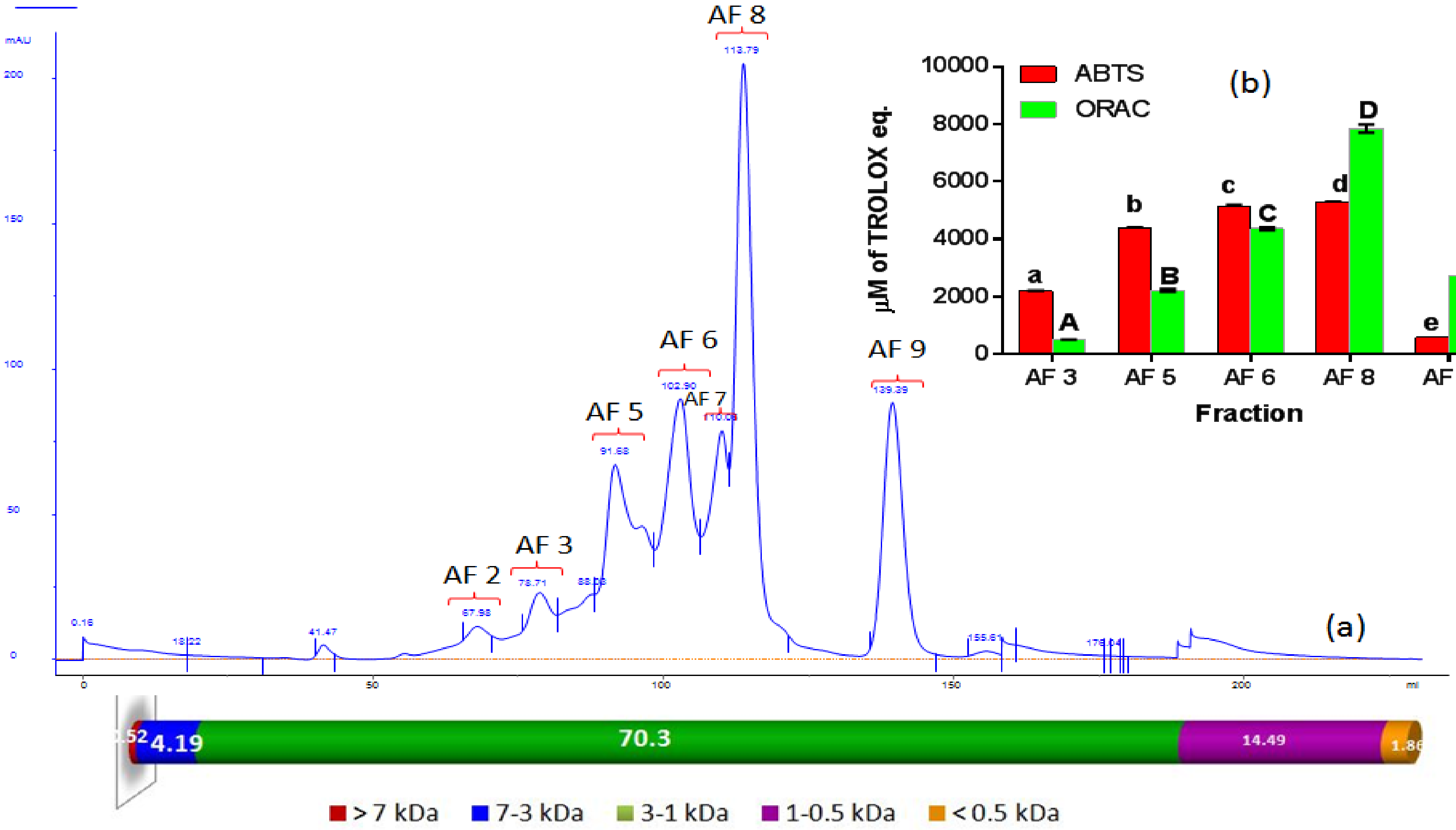


Figure 4.7 (a) SE-HPLC profile (280 nm) of CH 1 hydrolysate. (b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

Hydrolysate	Peak No	Fraction No	M.Wt. (kDa)	Kav	Retention vol. (ml)	Area (mAU*ml)	Area/Total area (v/v%)	Area/Peak area (v/v%)	> 7 kDa	7-3 kDa	3-1 kDa	1-0.5kDa	< 0.5 kDa
CH 1	AF-1	14	13.96	-0.01	41.47	12.11	0.52	0.42	0.52				
	AF-2	20	5.98	0.16	67.79	11.37	0.49	0.52		4.19			
	AF-3	22	4.21	0.23	78.63	44.57	1.93	2.05					
	AF-4	23	3.15	0.29	87.63	41.055	1.77	1.89			70.3		
	AF-5	25	2.77	0.31	91.68	337.56	14.58	15.52					
	AF-6	26+27	1.93	0.38	102.9	388..90	16.8	17.88					
	AF-7	28	1.53	0.43	110.06	236.42	10.21	10.87					
	AF-8	29	1.36	0.45	113.79	664.76	28.71	30.56				14.49	1.80
	AF-9	34	0.59	0.61	139.39	335.51	14.49	15.42					
	AF-10	37	0.35	0.72	155.69	5.95	0.26	0.27					
	AF-11	38	0.32	0.73	158.27	11.62	0.5	0.53					
	AF-12	39	0.30	0.75	160.91	24.02	1.04	1.1					

Table no 4.7: Size distribution obtained from SEC of hydrolysate CH1

4.4.2 Peptide profile of hydrolysate CH 2:

The results presented in Fig 4.8 depicts that on SEC the hydrolysate CH 2 resolved into 11 fractions labeled as A 1- A 11. Fraction no A-1 toA-11 ranged with Kav between 0.2-0.75 corresponding to molecular weight between 4.78 kDa–0.30 kDa. Fraction no A-6, corresponding to 1.90 kDa represented maximum area of chromatogram, followed by fraction no A-8 corresponding to 1.33 kDa, (17.52% of chromatogram area) and fraction A-7, 1.60 kDa representing 15.84% of chromatogram area. These fractions were analyzed for their antioxidant activity. Results as presented in (Table 4.8) shows that with decrease in size of peptide from 4.08 kDa to 0.59 kDa, the ABTS radical scavenging activity increased from 866.56 µM TEAC/mg peptide to 3871.66 µM TEAC/mg peptide. Among the shorter peptides peak 7 represent maximum antioxidant activity

4.4.3 Peptide profile of hydrolysate CH 3:

Size exclusion chromatogram (figure 4.9) clearly indicates separation of hydrolysate into ten peaks. Peptide profile as given in Table 4.9.shows that flavourzyme cleaved buffalo casein hydrolysate represents peptides of size 3.64 kDa to 0.58 kDa corresponding to peak no. F-3 to F-9. The peptides of size 0.58 kDa represented 32.28% area of chromatogram, while 1.36 kDa corresponding to 31.22% area of chromatogram. On evaluation of antioxidant activity of different fraction using ABTS and ORAC method, it was observed that peak no 10 corresponding to 0.58 kDa showed maximum antioxidant activity corresponding to 6567 µM TEAC/mg. The peak no F-6 corresponding to 2.03 kDa resulted 3298 µM TEAC/mg peptides and peak no F-7 represent major area of chromatogram showed 1256 µM TEAC/mg peptide. Based on ORAC method also the peak no F-10 being smallest peptide fraction showed maximum antioxidant activity.

It was observed that buffalo casein hydrolysed using alcalase (CH2) resulted in major antioxidant activity corresponding to peak A6, A7 and A8 corresponding to 1587, 2559 and 2626 µM TEAC/mg, respectively. The peak A9 of 590 Da although constituted minor peak showed maximum activity. However, relatively shorter peptides were obtained in flavourzyme treated hydrolysates

(CH3) corresponding to 48.19% of peak area below 3-1 kDa fraction and 36.5% area from 1-0.5kDa fraction, with maximum antioxidant activity corresponding to Peak 10 of molecular weight 580Da.Similar peptide profile was noticed in the hydrolysate on sequential treatment of alcalase and flavourzyme, resulting in 28.7 % fraction of size 1360Da (AF8) and 14.49 % as 590 Da (AF9), with antioxidant activity in AF9 as 5457 µM TEAC/mg.

It has been reported that molecular weights of peptides are related to their antioxidant activities (Li *et al.,* 2008; Fang *et al.,* 2012). The results clearly indicated that the antioxidant peptide fractions with low molecular weights exerted better residual activities. The result of relationships between molecular weights and antioxidant activities was similar to those reported in the literature (Mendis *et al.,* 2005; Rajapakse *et al.*, 2005a), which indicates that peptides with lower molecular weight generally have higher antioxidant potentials. Moure *et al.* (2006) reported that the antioxidant activity of hydrolysates is dependent on their molecular weight distribution. In the present study, results revealed that the peptide fraction with molecular weight ranging from 1600 Da -700 Da was probably associated with higher antioxidant activity. These findings are in agreement with other studies (Jeon *et al.*, 1999; Kim *et al.*, 2007) and support the fact that functional properties of antioxidant peptides are highly influenced by properties such as molecular mass.

	Peak no	Peak no	M.Wt. (kDa)	ABTS (TEAC µM)	ABTS (TEAC µM/mg peptides)	ORAC (TEAC µM)	ORAC (TEAC µM/mg peptides)	Protein content (mg/ml)
CH 2	A-2	16	4.78	**2122.98**	999.52	472.29	222.35	2.12
	A-3	17	4.08	2298.99	866.56	1932.72	5777.88	2.65
	A-4	18	3.14	4077.21	840.75	4018.90	601.89	4.85
	A-5	19	2.74	4254.79	898.68	2911.65	614.98	4.73
	A-6	20+21	1.90	5272.94	1587.52	5039.51	1517.23	3.32
	A-7	22	1.60	4988.02	2559.27	4532.13	2325.36	1.94
	A-8	23+24	1.33	2893.31	2626.70	1834.33	1665.30	1.10
	A-9	28+29	0.59	948.56	3871.66	727.20	2968.18	0.24
CH 3	F-1	9	13.93	453.75	2542.04	----	--	0.18
	--	15	---	583.47	717.67	----	--	0.81
	F-4	17	3.64	2847.86	1556.63	589.80	322.38	1.83
	F-5	19	2.71	4788.77	1187.10	1654.62	1066.09	4.03
	F-6	21	2.03	4370.00	3298.11	2448.48	1847.90	1.32
	F-7	23	1.36	5337.25	1256.86	5146.69	1211.98	4.25
	F-8	26	0.90	1092.72	2636.23	110.44	266.45	0.41
	F-9	27	0.75	1257.66	2255.90	192.47	345.23	0.56
	F-10	28	0.58	4485.30	6567.06	1424.65	2085.86	0.68

Table 4.8: Antioxidant activitiy of hydrolysates (CH1 and CH 2) peak fractions separated by SEC

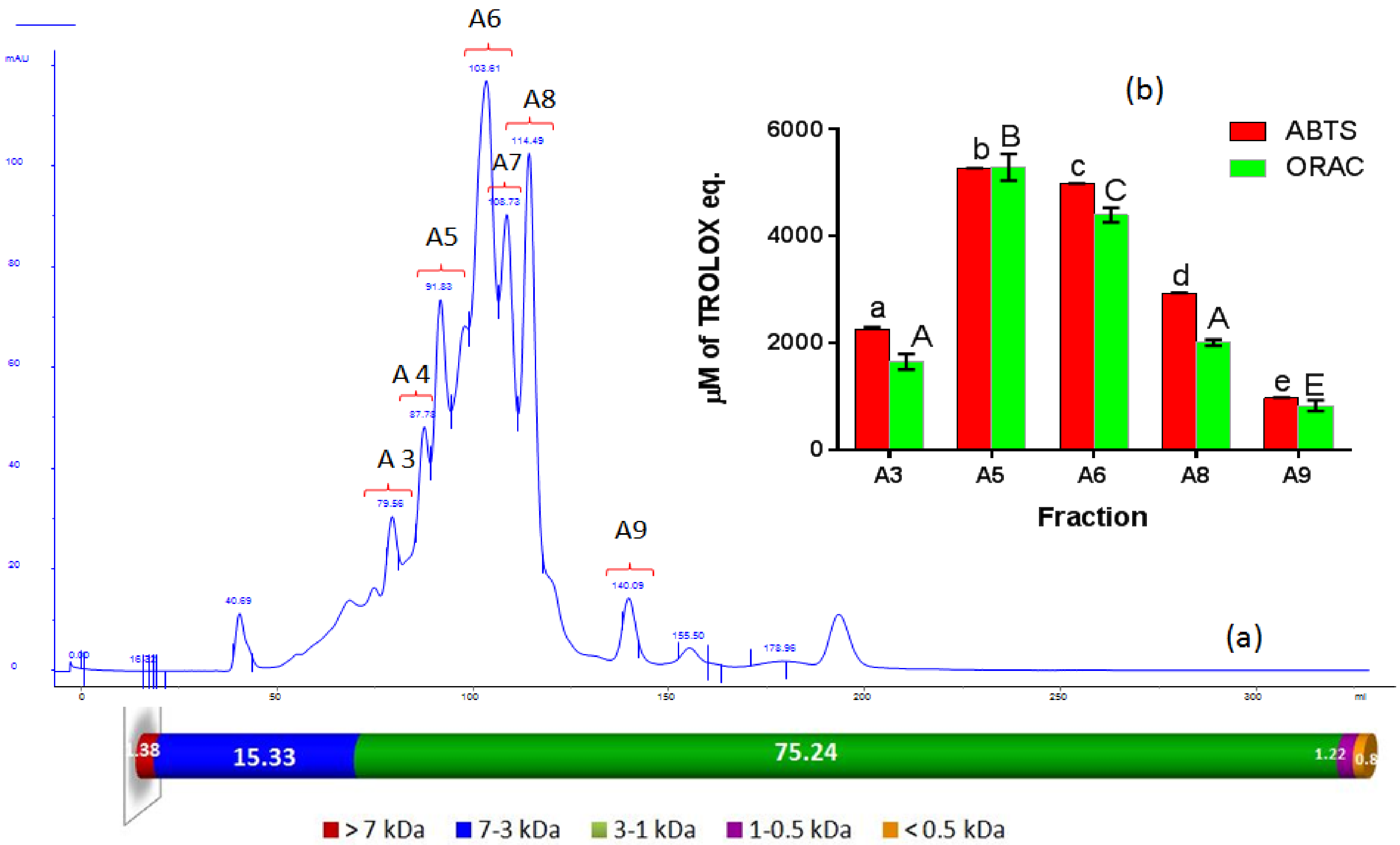


Figure 4.8: (a) SE-HPLC profile (280 nm) of CH 2 hydrolysate. (b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

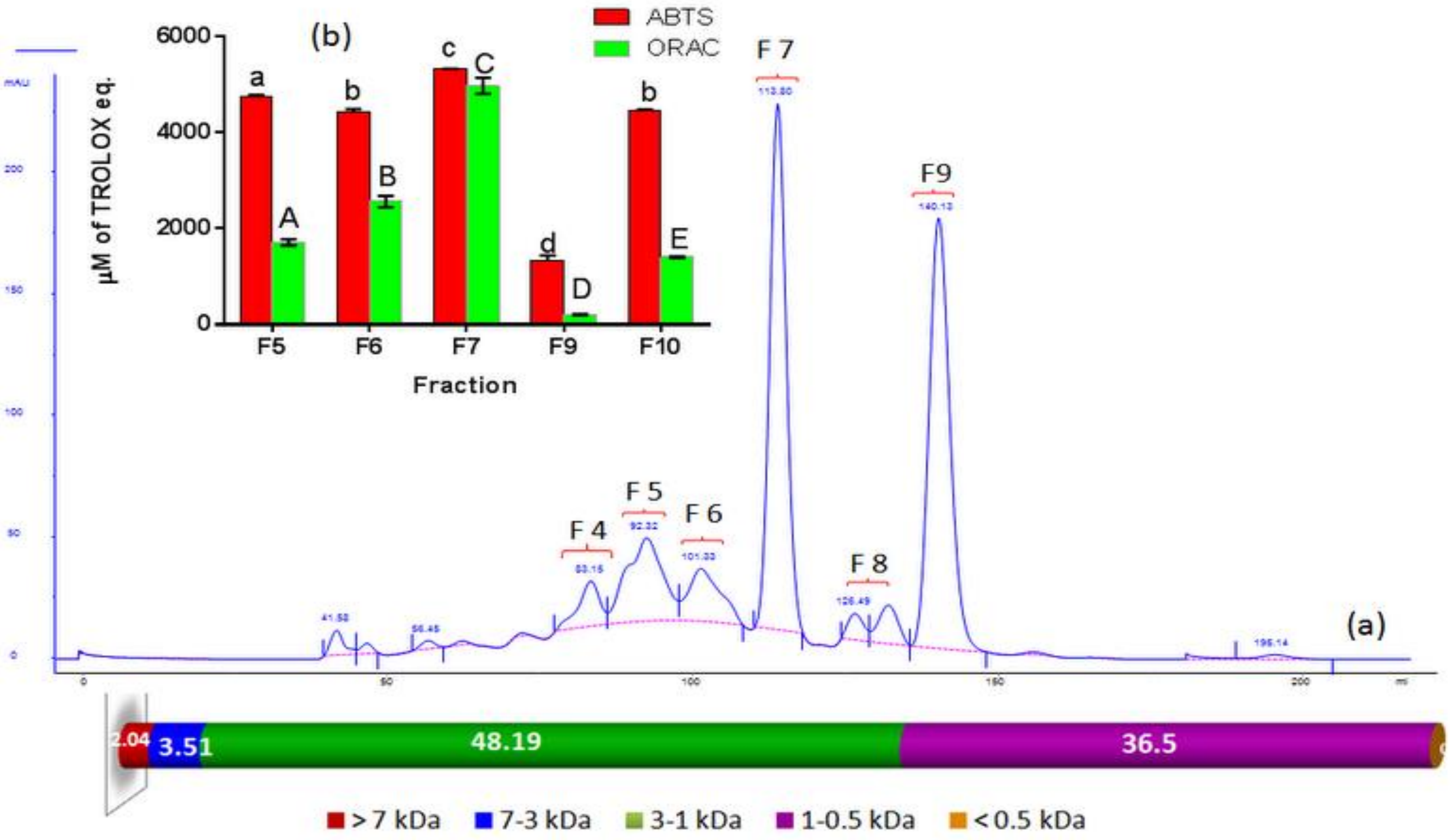


Figure 4.9: (a) SE-HPLC profile (280 nm) of CH 3 hydrolysate. (b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

hydrolysate	Peak no	Fraction No	M.Wt. (kDa)	Kav	Retention vol. (ml)	Area (mAU*ml)	Area/Total area (v/v%)	Area/Peak area (v/v%)	> 7 kDa	7-3 kDa	3-1 kDa	1-0.5kDa	< 0.5 kDa
CH 2	A-1	9	14.32	-0.01	40.69	20.77	1.38	1.38	1.38				
	A-2	16	4.78	0.20	74.75	15.69	1.04	1.04		15.33			
	A-3	17	4.08	0.23	79.63	55.76	3.71	3.71					
	A-4	18	3.14	0.29	87.77	158.79	10.58	10.58			75.24		
	A-5	19	2.74	0.31	92.00	137.38	9.15	9.15					
	A-6	20+21	1.90	0.39	103.38	491.40	32.73	32.73				1.22	
	A-7	22	1.60	0.42	108.59	237.82	15.84	15.84					
	A-8	23+24	1.33	0.46	114.45	263.09	17.52	17.52					0.81
	A-9	28+29	0.59	0.62	139.86	18.26	1.22	1.22					
	A-10	31	0.35	0.72	155.50	11.4484	0.76	0.76					
	A-11	32	0.30	0.75	160.19	0.71	0.05	0.05					
CH 3	F-1	9	13.93	-0.01	41.55	25.89	1.19	1.21	2.04				
	F-2	10	11.89	0.02	46.45	9.54	0.44	0.44					
	F-3	11	8.62	0.09	56.43	8.80	0.41	0.41					
	F-4	17	3.64	0.26	83.15	76.4428	3.51	3.55		3.51			
	F-5	19	2.71	0.32	92.32	236.66	10.9	11.03			48.19		
	F- 6	21	2.03	0.37	101.28	131.71	6.07	6.14					
	F-7	23	1.36	0.45	113.76	677.65	31.22	31.42				36.5	
	F-8	26	0.90	0.53	126.45	32.85	1.51	1.53					
	F-9	27	0.75	0.57	132.03	58.72	2.71	2.73					
	F-10	28	0.58	0.62	140.13	700.64	32.28	32.62					

Table 4.9 : Size distribution obtained from SEC of hydrolysate CH 2 and CH 3

4.5 RP-HPLC characterization of casein hydrolysates

Buffalo casein hydrolysate peptide profile was achieved by size exclusion chromatography (SEC) as well as ultrafiltration technique. hydrolysate was subjected to RP-HPLC using 0.1% TFA in water and 0.01% TFA with 10% acetonitrile in water as solvent B. Each peak was collected after several runs, pooled and freeze dried. Each peak fraction was dispersed in 200 µL distilled water for determining their antioxidant activity.

4.5.1 Identification of peptides from hydrolysate CH1

For the peptide identification, samples of CH1 hydrolysate from ultrafiltered permeate fractions both (<1 kDa) as well as (<3kDa) were assessed for their separation over RP-HPLC. Results as presented in figure 4.8 and Annexure I clearly indicates that compared to chromatogram obtained from 3kDa fraction, the corresponding peaks of 1 kDa fractions resolved with greater intensity. The chromatogram shown in figure 4.10 depicts separation into 14 peaks. Peak AF 1.11 of (1 kDa fraction) showed maximum antioxidant activity corresponding to 5693.76 µM TEAC/ mg peptide. The peak AF 1.4 being the major peak having high total antioxidant activity both by ABTS and ORAC assay was subjected to MS/MS analysis.

A total of 9 peptides were identified, of which 6 peptides corresponded to β –casein and three peptides to k- casein (Table 4.11). Peptide SKVLPVPQK , β- CN f(168-176) shares sequence similarity to β CN f(169-176) previously reported as potential antioxidant peptide with 0.99 DPPH radical scavenging activity (RSA) (Rivel *et al*., 2001) and β- CN f(170-176) with 1.05 DPPH (RSA) (Rivel *et al.,* 2001). Further Shanmughan *et al.* (2014) reported the β CN f(170-176) possessing highest antioxidant activity corresponding to 5.7 µmol TE/mg on separation from buffalo casein using pepsin-trypsin combination. The fragments β- CN f(171-176) and β- CN f(171-175) again bear the similarly to previous reported sequence β- CN f(170-176). The peptide VPYPQ, β- CN f(178-182) observed in peak 6 which possess high antioxidant activity as reported earlier (Tsopmo *et al.,* 2011) in human milk digest corresponding to ORAC value 2200 µM TEAC/mM.

The peptide RDMPIQ, β- CN f(183-188) present in peak 6 has been reported to possess antioxidant activity (Gobba *et al.,* 2014) as separation from casein digested with *A.ikkense* at 25°C for 24 h.

The peptide identified from k-casein fraction corresponding to k-CN f(98-104), k-CN (98-103) and k-CN (100-105) are part of longer antioxidant peptide ARHPHOHLSFM (Kudoh *et al* 2001) and the peptides HPHPHLSF reported to be a potent inhibitor of linoleic acid oxidation with an activity similar to that obtained from synthetic antioxidant BHT. The presence of histidine residues, which has been reported to be effective radical scavenging (Chen *et al* 1998), suggests that these peptides could be responsible for the activity.

	Peak no	**ABTS (TEAC μM)**	**ABTS (TEAC μM/mg peptides)**	**ORAC (TEAC μM)**	**ORAC (TEAC μM/mg peptides)**	**Protein content (mg/ml)**
CH 1 1kDa	AF-1.1	969.92	2953.93	460.86	1403.57	0.33
	AF-1.2	2721.93	4806.08	2138.83	3776.52	0.57
	AF-1.3	2172.94	3870.57	388.55	692.11	0.56
	AF-1.4	5523.26	1541.52	8188.85	2285.47	3.58
	AF-1.5	5241.57	2355.17	7614.00	3421.16	2.23
	AF-1.6	5156.76	2377.32	5790.74	2669.59	2.17
	AF-1.7	4465.76	5040.36	3656.23	4126.67	0.89
	AF-1.8	4054.12	4919.45	3145.36	3816.73	0.82
	AF-1.9	2865.88	4323.58	2894.51	4366.77	0.66
	AF-1.10	1653.53	3826.72	924.55	2139.67	0.43
	AF-1.11	1195.97	5693.76	49.98	237.94	0.21

Table 4.10 Antioxidant activities of hydrolysate CH 1 peak fractions separated by RP-HPLC

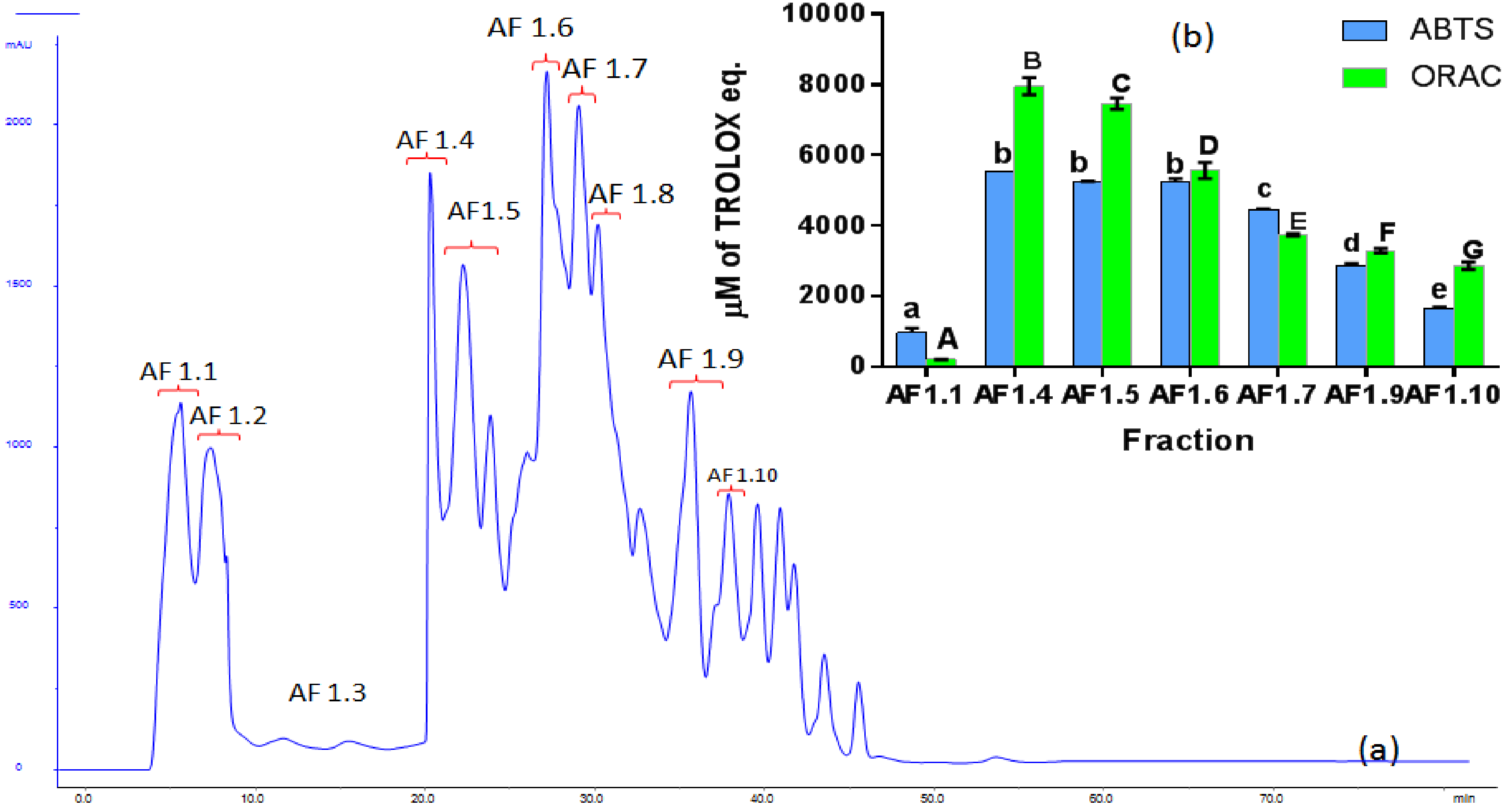


Figure 4.10: A) RP-HPLC chromatogram (214 nm) Of CH 1 (1kDa) hydrolysate. b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

Fragment	Sequence identified	Previously reported sequence	Activity	Reference
β CN f(168-176)	**SKVLPVPQK**	**KVLPVPQK** **VLPVPQK**	A.O. 0.99DPPH RSA	Rival *et al.*, 2001a
β CN f(171-176)	**LPVPQK**			
β CN f(171-175)	**LPVPQ**		A.O. 1.05 DPPH RSA	Rival et al., 2000
			A.O (buffalo Casein)	Shanmugam et al.,2014
β CN f(178-182)	**VPYPQ**	**VPYPQ**	A.O	Tsopmo et al 2011
β CN f(184-188)	**DMPIQ**			
β CN f(183-188)	**RDMPIQ**	**RDMPIQ**	A.O	Gobba et al 2014
β CN f(185-188)	**MPIQ**			
κ CN f(98-104)	**HPHPHLS**	**ARHPHPHLSFM**	A.O	Kudoh et al 2001
κ CN f(98-103)	**HPHPHL**	**HPHPHL**	A.O	Chen et al., 1996
κ CN f(100-105)	**HPHLSF**			

Table 4.11: List of peptides identified in CH 1 hydrolysate (UF 1 kDa permeate) for peak fraction AF 1.4

4.5.2 Identification of peptides from hydrolysate CH 2:

3 kDa ultrafiltration fraction of alcalase hydrolyzed buffalo casein was assessed for identification of active peptides. As shown in fig 4.11 the RPC chromatogram depicts separation of peptides into 12 peaks. Based on antioxidant activity evaluation, the peak A 3.1, A 3.3, A 3.9, A 3.11 and A 3.12 possess low protein content, thereby the specific activity of these fractions was observed to be very high. Among the major peaks A 3.5, A 3.6, A 3.7, A 3.8 the peak A 3.6 corresponded to maximum antioxidant activity i.e. 3981 µM TEAC/mg peptides. This peak no A 3.6 was selected for identification of active peptides. On MS/MS analysis the peptide KAVPYPQ, β CN f(176-182) shows homology

with AVPYPQR, a peptide found to inhibit lipid oxidation but not to scavenge radical compounds (Rival et al., 2001). The peptides SKVLPVPQK and KVLPVPQK shows homology with peptides previously reported as antioxidant i.e. KVLPVPQK (DPPH, RSA 0.99) and VLPVPQK (DPPH, RSA 1.05) (Rival *et al.,* 2001). Shanmagam *et al*. (2014) also reported VLPVPQK as potential antioxidant peptides from buffalo casein hydrolysate using pepsin-trypsin combination. The peptides RDMPIQ, β CN f(183-188) represented the antioxidative peptide earlier reported (Gobba *et al.,* 2014)

	Peak no	**ABTS (TEAC µM)**	**ABTS (TEAC µM/mg peptides)**	**ORAC (TEAC µM)**	**ORAC (TEAC µM/mg peptides)**	**Protein content (mg/ml)**
CH 2 **3 kDa**	A-3.1	984.07	6037.25	1043.89	6404.24	0.16
	A-3.2	1785.57	2340.19	1369.09	1794.34	0.76
	A-3.3	3051.55	20618.61	1534.90	10370.91	0.14
	A-3.4	2168.92	6369.80	2201.99	6466.93	0.34
	A-3.5	4495.91	3459.72	5284.25	4066.37	1.29
	A-3.6	4867.66	3981.55	5743.12	4697.65	1.22
	A-3.7	1432.38	1237.47	3213.56	2776.29	1.15
	A-3.8	2608.38	3359.15	2821.53	3633.64	0.77
	A-3.9	3328.72	9560.20	1357.52	3898.83	0.34
	A-3.10	1679.02	3272.94	1912.79	3728.62	0.51
	A-3.11	1931.19	15140.66	166.00	1301.45	0.12
	A-3.12	924.09	3271.68	1210.99	4287.44	0.28

Table 4.12: Antioxidant activities of hydrolystaes CH 2 peak fractions separated by RP-HPLC

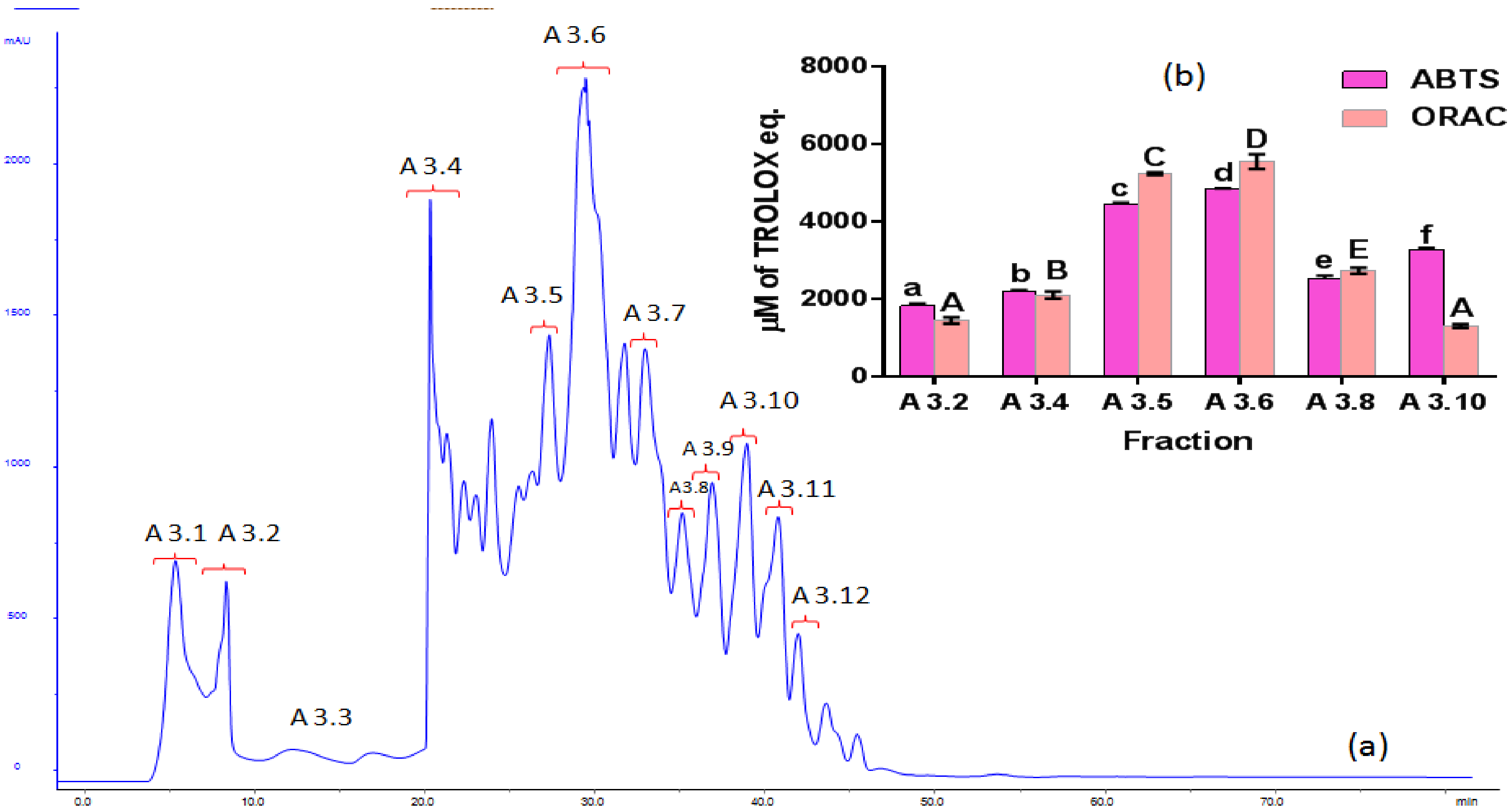


Figure 4.11: A) RP-HPLC chromatogram (214 nm) Of CH 2 (3kDa) hydrolysate. b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

Fragment	**Sequence identified**	**Previously reported sequence**	**Activity**	**Reference**
β - CN f(176-182)	KAVPYPQ	**VPYPQ**	A.O	Tsopmo *et al.,* 2011
β - CN f(169-176)	KVLPVPQK	KVLPVPQ	ACE inhibitory IC_{50}=1000µM	Maneo *et al*., 1996; ledesma *et al*., 2005
		KVLPVPQK	A.O. (0.99 DPPH RSA)	Rival *et al*., 2001a
		VLPVPQK	A.O. (1.05 DPPH RSA)	Rival *et al.,* 2000; Shanmugam *et al.,*2014
β CN f(168-176)	SKVLPVPQK	KVLPVPQK	A.O. (0.99 DPPH RSA)	Rival *et al.,* 2001a
β CN f(183-188)	RDMPIQ	RDMPIQ	A.O. (RSA)	Gobba *et al* 2014
β CN f(185-188)	MPIQ			

Table 4.13: List of peptides identified in CH 2 hydrolysate (UF 1 kDa permeate) for peak fraction A 3.6

4.5.3 Identification of peptides from hydrolysate CH 3

The 1 kDa ultrafiltration fraction of flavourzyme The RP-HPLC chromatogram depicts separation of hydrolysate into 14 peaks (figure 4.12). Result presented in table 4.13 indicate that peak F 1.1,F 1.10,F 1.11 and F 1.12 correspond to very low concentration of protein, there by express high specific antioxidant activity. However, for the purpose of identification of peptides, peak F 1.3, representing high antioxidant activity was selected.

The sequence of peptides identified in the fraction corresponded to β- CN f(193-209), β- CN f(195-209), β- CN f(194-208), β- CN f(199-209), β- CN f(203-209) and β- CN f(204-209) represented homology to β- CN f(193-209) i.e. YQEPVLGPVRGPFPIIV which has been shown to represent radical scavenging activity corresponding to IC_{50} 5.2 µM (Birkemo *et al.,* 2009). The same sequence has also been reported to possess immunomodulatory activity (Sabeena Fravin *et al*., 2010), while β- CN f(197-207) shown ACE inhibitory activity (Miguel *et al*., 2006) and β- CN f(199-208) shown to exhibit antioxidant activity (Ledsma et al., 2005). The peptides HPHPHLS, k- CN f(98-104) and HPHLS k -CN f(100-1040 are the part of sequence HPHPHLSF identified from k-CN digest of ovine casein (Chen *et al.,* 1996) which strongly inhibit lipid peroxidation.

The other peptides TTMPLW, αs_1-CN f (194-199) is reported to exhibit ACE inhibitory (IC_{50} 16 µM) and immunomodulatory property (Meisel, 1997). The peptide sequence LHLPLPLL, β- CN f(133-140) and LPLPLL, β- CN f(135-140) has been shown to possess ACE inhibitory.

	Peak no	ABTS (TEAC µM)	ABTS (TEAC µM/mg peptides)	ORAC (TEAC µM)	ORAC (TEAC µM/mg peptides)	Protein contents (mg/ml)
CH 3 1 kDa	F-1.1	1499.86	8263.68	700.22	3857.97	0.18
	F-1.2	2846.74	5335.97	1192.19	2234.66	0.53
	F-1.3	5061.03	7774.23	1641.46	2521.44	0.65
	F-1.4	3528.27	2446.79	5834.70	4046.26	1.44
	F-1.5	3960.39	5562.35	3560.36	5000.50	0.71
	F-1.6	2782.81	3097.17	4856.29	5404.89	0.90
	F-1.7	3160.47	5280.66	9263.08	15477.16	0.60
	F-1.8	2764.66	5096.14	3919.06	7224.07	0.54
	F-1.9	1841.61	4400.50	3570.49	8531.63	0.42
	F-1.10	1869.63	12547.85	3181.34	21351.30	0.15
	F-1.11	1675.47	11132.69	1159.24	7702.56	0.15
	F-1.12	1835.30	13747.54	716.91	5370.11	0.13

Table no 4.14: Antioxidant activities of hydrolysates CH 3 peak fractions separated by RP-HPLC

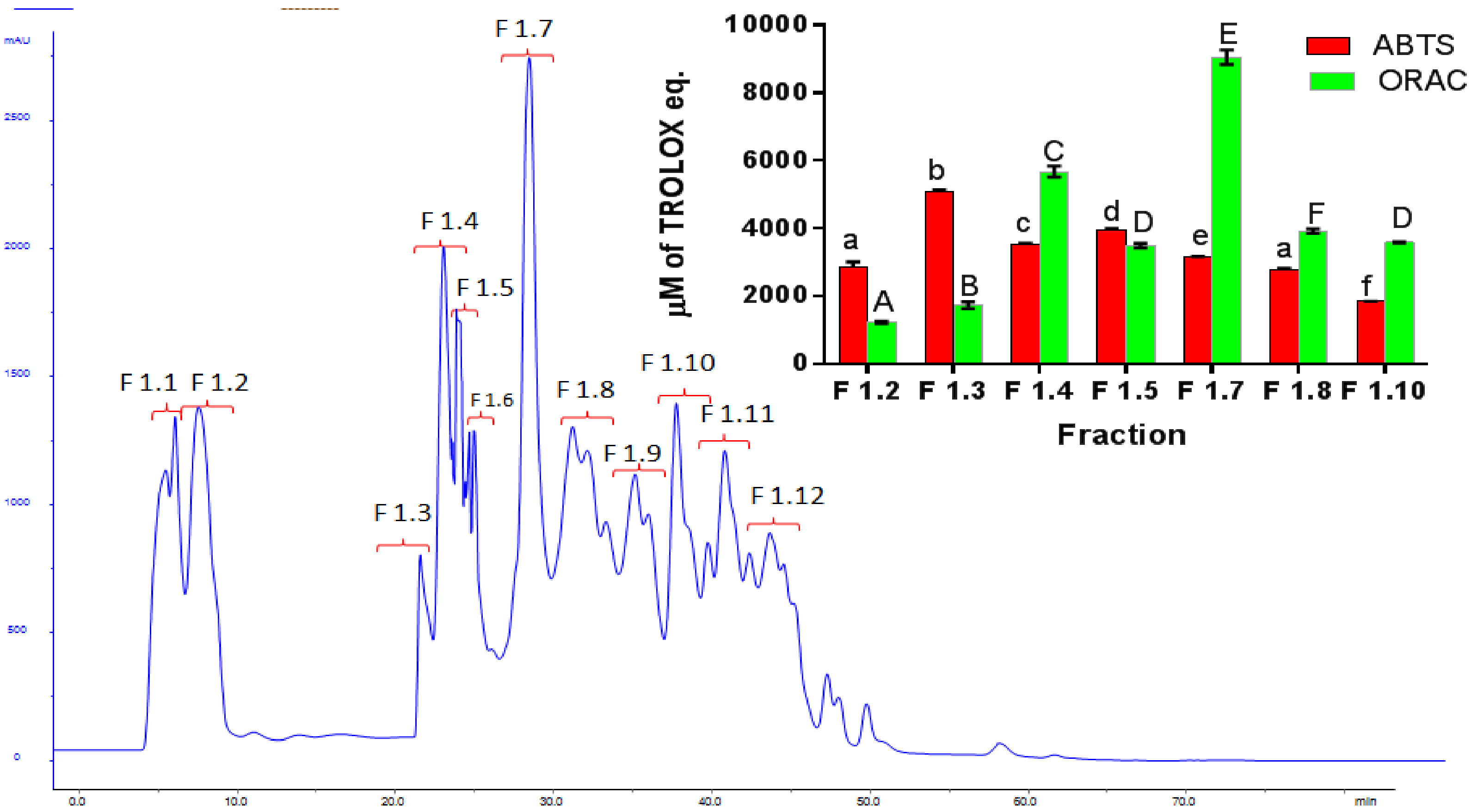


Figure 4.12: A) RP-HPLC chromatogram (214 nm) Of CH 3 (1kDa) hydrolysate. b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

Fragment	Sequence	Previously reported sequence	Activity	Reference
β-CNf(193-209)	**YQEPVLGPVRGPFPIIV**	YQEPVLGPVRGPFPIIV	Radical scavenging activity IC_{50} 5.2 µM	Birkemo *et al.,* 2009
β-CN f(194-209)	**QEPVLGPVRGPFPIIV**	YQEPVLGPVRGPFPIIV	Immunomodulatory activity	SabeenaFravin *et al.,* 2010
		VLGPVRGPFP	ACE inhibitory	Miguel *et al.,* 2006, Quiros *et al.,* 2007
		GPVRGPFPII	Antioxidant	Ledesma *et al*., 2005
β-CNf(195-209)	**EPVLGPVRGPFPIIV**	YQEPVLGPVRGPFPIIV	Immunomodulatory	SabeenaFravin *et al.,* 2010
		VLGPVRGPFP	ACE inhibitory	Miguel *et al.,* 2006, Quiros *et al.,* 2007
		GPVRGPFPII	Antioxidant	Ledesma *et al.,* 2005
β-CN f(194-208)	**QEPVLGPVRGPFPII**	YQEPVLGPVRGPFPIIV	Immunomodulatory	SabeenaFravin *et al.,* 2010
		VLGPVRGPFP	ACE inhibitory	Miguel *et al.,* 2006, Quiros *et al.,* 2007
		GPVRGPFPII	Antioxidant	Ledesma *et al.,* 2005
	GPVRGPFPIIV β-CN f(199-209)	YQEPVLGPVRGPFPIIV	Immunomodulatory	Sabeena Fravin *et al.,* 2010
		VLGPVRGPFP	ACE inhibitory	Miguel *et al.,* 2006, Quiros *et al.,* 2007
		GPVRGPFPII	Antioxidant	Ledesma *et al.,* 2005
	GPFPIIV β-CN f(203-209)	GPVRGPFPII	Antioxidant	Ledesma *et al.,* 2005

	PFPIIV β-CN f(204-209)	GPVRGPFPII	Antioxidant	Ledesma *et al.,* 2005
β-CN f(133-140)	**LHLPLPLL**	**HLPLP**	Antihypertensive	(Hernández-Ledesma *et al.* 2007
β-CN f(135-140)	**LPLPLL**	**HLPLP**	Antihypertensive	(Hernández-Ledesma *et al.* 2007
α_{s1}-CN f(194-199)	**TTMPLW**	**TTMPLW**	ACE inhibitory (IC 50_{50}=16 μM); Immunomodulatory (+162)	Meisel, 1997
α_{s1}-CN f(196-199)	**MPLW**	**TTMPLW**	ACE inhibitory (IC 50_{50}=16 μM); Immunomodulatory (+162)	Meisel, 1997
κ - CN f(98-104)	**HPHPHLS**	**HPHPHLSF**	Inhibit lipid peroxidation	Chen *et al.,* 1996
κ - CN f(100-104)	**HPHLS**	**HPHPHLSF**	Inhibit lipid peroxidation	Chen *et al.,* 1996

Table 4.15: List of peptides identified in CH 3 hydrolysate (UF 1 kDa permeate) for peak fraction F1.3

4.6 Techno functional property

4.6.1 Solubility-

Solubility of hydrolysate at different pH level was estimated by the method of (kasran *et al*., 2012) with slight modification. Solubility is dependent on the structure, temperature and pH of an environment, concentration and charge of ions, and interaction with other molecules (Pearce, 1995).The solubility of hydrolysates CH1 was about 95% in the pH range 6-9, 92% between pH 4–5 and 88% at pH 3.In case of CH2 and CH3 hydrolysate, relatively lower solubility was observed. Luo *et al.* (2013) reported similar results on solubility of papain, pancreatin and trypsin digest of sodium caseinate and reported that the lowest solubility at pH 4.0, which is near the pI of caseins, and the solubility above pH 4.0 increased with an increase in pH.

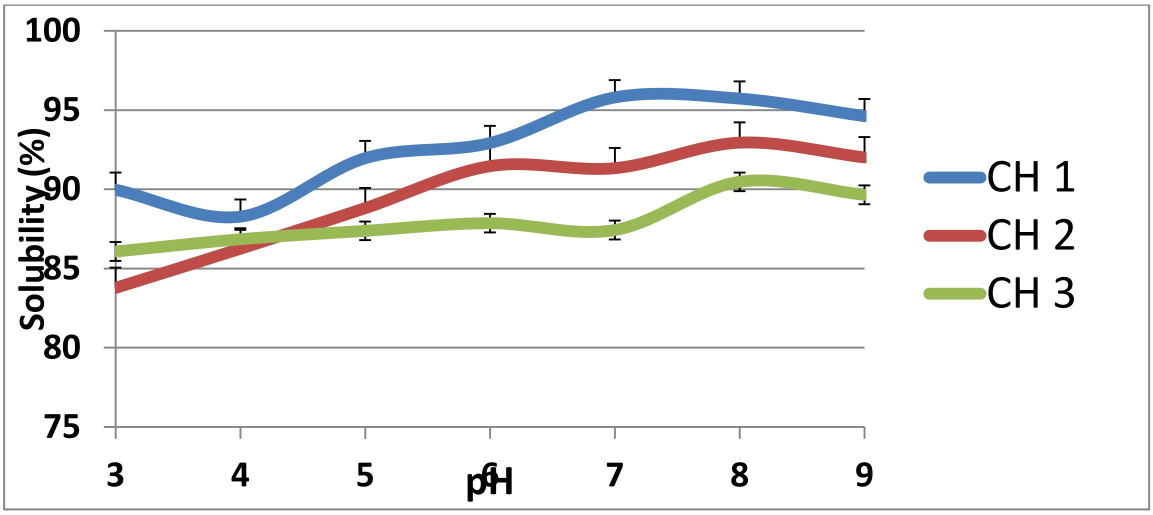


Figure 4.13: Solubility of buffalo casein hydrolysate

4.6.2 Effect of enzymatic treatment on foaming of buffalo casein hydrolysate

Foaming properties of undigested caseinate and hydrolysate were determined by the method of De Wit *et al*., (1988) using a household mixer by whipping 50ml of 3 percent (w/v) protein dispersions at room temperature. The foaming capacity or foaming stability was expressed in terms of % overrun, the foam was poured in measuring cylinder and the volume of foam was measured and results shown in histogram (Figure 4.14). Proteins in dispersions cause a lowering of the surface tension at the water–air interface, thus creating foaming capacity (Surowka & Fik, 1992), thus buffalo casein hydrolysates showed an increase in the overrun as compared to that of undigested caseinate. Kong *et al.* (2007) suggested an increase in surface activity, probably due to the initially greater number of polypeptide chains that arose from partial proteolysis, which allowed more air to be incorporated. An improvement in foaming capacity for enzymatically modified food proteins was reported by Adler-Nissen (1986).The foam stability was measured by decrease in the height of foam volume after 1 hr of standing and Foam stability of casein hydrolysate was inferior to that of undigested caseinate (Figurure 4.14); the differences may be due to the reason that our samples had lower molecular weight peptides as compared to undigested caseinate. Similar results were indicated by Rhicha *et al*., (2007) the foam volume of hydrolysates increased or remained same as that of whey protein concentrates, but the stability of foam decreased

4.6.3 Emulsifying property

Emulsifying activity (EAI) and emulsion stability indexes (ESI) of hydrolysates were determined by the turbidimetric method (Pearce & Kinsella, 1978), with slight modifications. EAI and ESI of three hydrolysate (CH 1, CH 2 & CH 3) was increased as compared to the non-digested caseinate (figure 4.15. EAI and ESI of casein hydrolysate treated with different proteases were investigated by Luo *et al.,* (2012). They reported that hydrolysate had smaller casein mass as a result it showed higher EAI.

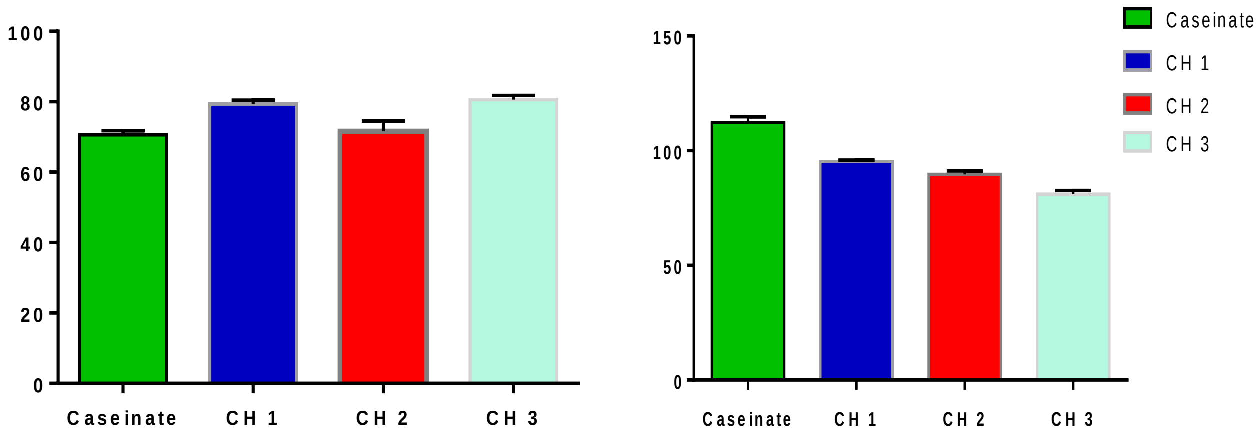


Figure 4.14 Foaming property of buffalo casein hydrolysate A) Foaming capacity B) Foaming stability

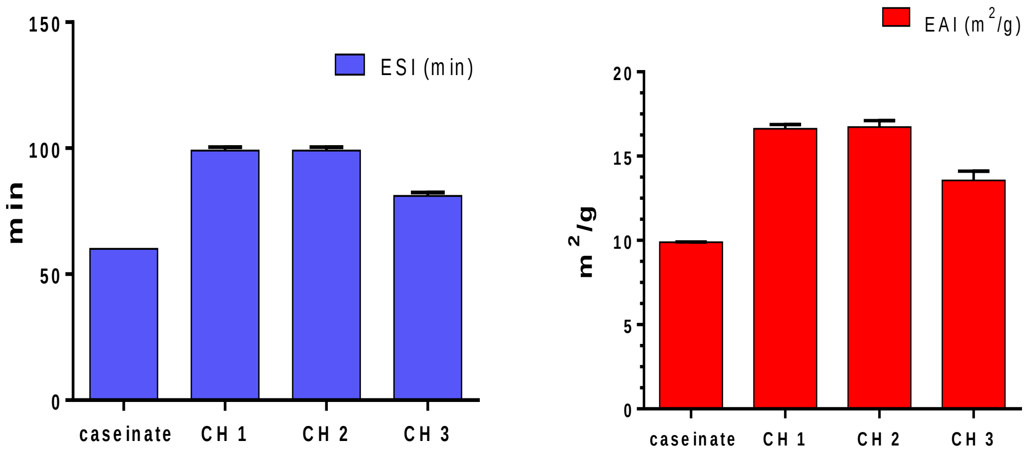


Figure 4.15 Emulsifying property of buffalo casein hydrolysate A) emulsifying stability index B) emulsifying activity index

4.7 Preparation of whey permeate beverage

The presences of bitter peptides in casein and whey protein hydrolysate possess a challenge for their utilization in functional beverages due to poor consumer acceptance. There have been several investigation into developing whey- based fruit juice mixes with various formulation to select the optimal blend based on sensory perception (Djuric *et al.,* 2004). In order to utilize casein hydrolysate preparation enriched in antioxidant peptides, whey permeate was used as a matrix for formulation and aspartame was added as sweetning agent, together with synthetic flavor to improve consumer appeal. Based on preliminary trials casein hydrolysate was added @ 0.1%, 0.5%, and 2% level. The sensory properties of preparation were assessed (figure 4.16).

4.7.1 Utilization of buffalo casein hydrolysates in food system

The biofunctional buffalo casein hydrolysate was used in whey permeate drink to assess the suitability of this product as the delivery medium for the bioactive peptides. The product was prepared and evaluated for their sensory and biological properties.

4.7.2 Preparation of whey permeate drink

Five types of whey permeate drink were prepared i.e. without casein hydrolysate (control), with 0.1%, 0.5%, 1% and 2% casein hydrolysate. The sensory properties of these whey drink samples were studied and results are presented in (Figure 4.16). Sweetener and flavour was used for masking the bitter taste of casein hydrolysate.

According to sensory it is clear that 1% and 2% casein hydrolysate fortified whey drink was prepared with the addition of sweetner and flavour had more bitterness than the 0.5 % casein hydrolysate fortified product. Antioxidant activity of 0.5 % hydrolysate fortified product was studied**.**

4.7.3 Sensory evaluation of whey drink

The sensory evaluation of the whey drink was done using 9-point Hedonic scale for different parameters like color and appearance, mouth feel, sweetness, flavor and overall acceptability. From the Figure 4.16, it is clear the flavor score for 0.1%. 0.5%, 1% and 2% casein hydrolysate supplemented whey drinks were lower than the control whey drink. This might be due to the bitter taste of the hydrolysate, which was more pronounced in whey drink supplemented with 2% casein hydrolysate. For the mouth feel, all the whey drinks got the same score. In the overall acceptability criteria control and 0.5% casein hydrolysate supplemented whey drink got the same score compared with 0.1%, 1% and 2% casein hydrolysate supplemented whey drink got lesser consumer acceptability.

4.7.4 Antioxidant activity whey drink

The antioxidant activity is shown in Table 4.15. The antioxidant activity of whey permeate fraction corresponding to 4.54 mM TEAC might be due to presence of low molecular weight non-protein nitrogen components such as urate which is responsible for antioxidant activity of whey using ABTS radical scavenging assay. From the values it is clear that after the addition of casein hydrolysate there was a highly significant increase in the antioxidant property of the whey drink. The incorporation of casein hydrolysate in whey drink (0.5%) raised its antioxidant activity from 3.81 fold compared to control at 0 day. The antioxidant activity was significantly decreased during storage of whey beverage as shown in figure

The antioxidant activity (TEAC) of the whey drink added with hydrolysate was very high than that of apricot juice (2.48 mM of trolox/l), peach juice (2.51 mM of trolox/l) lemon juice (2.21 mM of trolox/l), apple juice (1.83 mM of trolox/l) and pineapple juice (1.50 mM of trolox/l) with addition 0.5% casein hydrolysate (Pellagrani *et al.,* 2003). During storage of product at 4°C, about 85% retain of antioxidant activity was observed after three weeks period.

Product	**TEAC value (mM TEAC)**
Control	4.541± 0.04
0.5% casein hydrolysate	14.32± 0.02

Table no 4.15: Antioxidant activity of casein hydrolysate added Whey Drink

Product (0.5 % hydrolysate)	**TEAC value (mM TEAC)**
O days	**14.32 ± 0.015**
7 days	**14.08 ± 0.029**
14 days	**13.43± 0.045**
21 days	**11.49± 0.057**

Table no 4.16: Antioxidant activity of casein hydrolysate added Whey Drink during storage

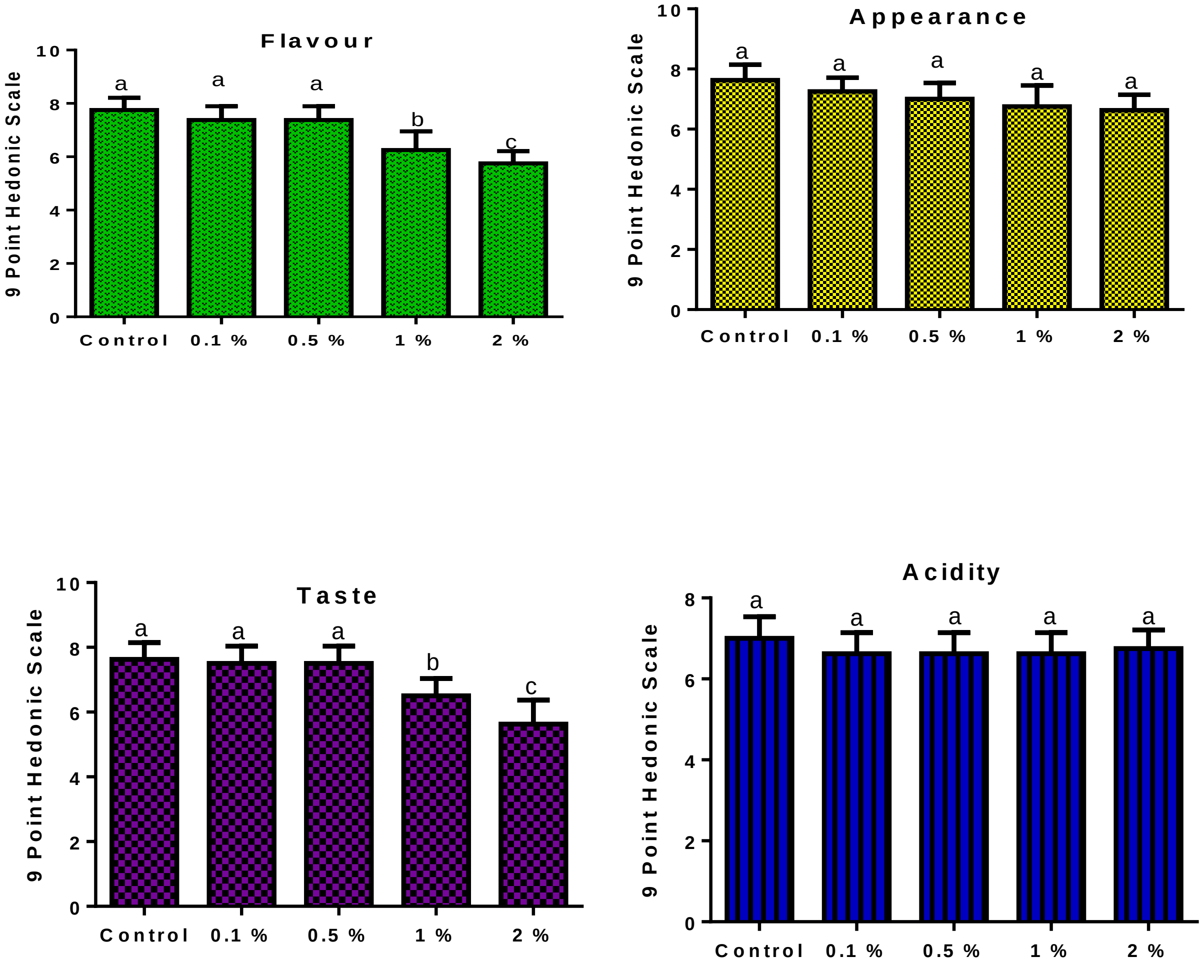


Figure 4.16: Sensory evaluation of whey drink enriched with casein hydrolysate

SUMMARY AND CONCLUSIONS

- Comparative evaluation of pH-stat and OPA method for the measurement of degree of hydrolysis of buffalo sodium caseinate using alcalase and flavourzyme showed that comparable values of hydrolysis in case of alcalase hydrolysis only.
- Antioxidant activity of CH1 was observed to be 1.57 fold greater than CH2 and 1.33 fold greater than CH3.
- Ultrafiltration of whole hydrolysate resulted in maximum antioxidant activity in 1kDa fraction as evaluated using different antioxidant assays based on ABTS, FRAP , DPPH, ORAC and reducing power.
- The characteristic peptide profile of CH1 indicated the peptides of size 3-1 kDa represented 70.3% and that of 1-0.5 kDa corresponded to 14.49 % area of chromatogram. Fraction corresponding to 0.59 kDa (Peak no 9) showed maximum antioxidant activity based on ABTS radical scavenging activity.
- The characteristic peptide profile of CH2 showed that the Fraction no 6, corresponding to 1.90 kDa r epresented maximum area of chromatogram, followed by fraction no 8 corresponding to 1.33 kDa, (17.52% of chromatogram area) and fraction 7, 1.60 kDa representing 15.84% of chromatogram area.
- The characteristic peptide profile of CH3 indicated the peptides of size 0.58 kDa represented 32.28% area of chromatogram, while 1.36 kDa corresponding to 31.22% area of chromatogram.
- Reverse phase chromatography of 1kDa and 3kda fractions of CH1, CH2 and CH3 hydrolysates were subjected to separation based on hydrophobicity of peptides.
- On subjecting to HPLC-TOF-ESI separation four potential antioxidant peptides were identified in CH1 hydrolysate (SKVLPVPQK_1, VPYPQ_2, RDMPIQ_3, HPHPHLS_4) along with two potential fragments each of peptide 1, 3 and 4. The peptide sequence of active antioxidant fractions from CH2 and CH3 hydrolysates were also analysed.

- The solubility of hydrolysates CH1 was about 95% in the pH range 6-9, 92% between pH 4 – 5 and 88% at pH 3, in case of CH2 and CH3, relatively lower solubility was observed.
- Emulsifying and foaming capacity of casein hydrolysates increased compared to undigested casein.
- The whey permeate based beverage containing CH1 hydrolysate was formulated at pH 4.6 after subjecting to heating at sterilization temperature. The product was rated acceptable based on sensory evaluation, with 3.18 fold enhancement of antioxidant activity as compared to control. The product retained over 85% level of initial antioxidant activity after storage for 3 weeks at 4^{o}C.

Conclusions:

- **Combined action of proteases resulted higher antioxidant peptides from buffalo casein**
- **Antioxidant peptide enriched buffalo casein hydrolysate can be used as nutraceutical ingredient**

BIBLIOGRAPHY

Achouri, A., Zhang, W., & Shiying, X. (1999). Enzymatic hydrolysis of soy protein isolate and effect of succinylation on the functional properties of resulting protein hydrolysates. Food Research International, 31(9): 617-623.

Adler-Nissen J. (1986). Enzymic hydrolysis of food proteins.New York: Elsevier Applied Science Publishers. p 110-169.

Adler-Nissen, J.,(1993). Protease.InEnzyme in Food Processing, 3rd Ed. Nagodawithana, T., Reed, G., Academic Press. San Diego, CA, 159-203.

Agboola, S. O. and Dalgleish, D. G.(1996).Enzymatic hydrolysis of milk proteins used for emulsion formation. 1. Kinetics of protein breakdown and storage stability of the emulsions. Journal Agriculture and Food Chemistry., 44**:** 3631-3636.

Ajibola, C. F., Fashakin, J. B., Fagbemi, T. N., and Aluko, R. E. (2011).Effect of peptide size on antioxidant properties of African yam bean seed (Sphenostylisstenocarpa) protein hydrolysate fractions. International Journal of Molecular Sciences, 12(10): 6685-6702.

Althouse, P.J., Dinakar, P. and Kilara, A.(1995).Screening of proteolytic enzymes to enhance foaming of whey protein isolates. Journal of Food Science., 60: 1110-1112.

Alvarez, V.B., Wolters, C.L.,Vodovotz, Y. and Ji, T. (2005). Physical properties of ice cream containing milk protein concentrate. Journal of Dairy Science, 88(3): 862-71.

Apak, R., Gorinstein, S., Böhm, V., Schaich, K. M., Özyürek, M., and Güçlü, K. (2013).Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). Pure and Applied Chemistry, 85(5): 957-998.

Augustin, M. A., Cheng, L. J., Glagovskaia, O., Clarke, P. T. and Lawrence, A.(2003). Use of blends of skim milk and sweet whey protein concentrates in reconstituted yogurt. Australian Journal of Dairy Technology, *58*(1): 30-35.

Aziznia, S., Khosrowshahi, A., Madadlou, A. and Rahimi, J. (2008). Whey protein concentrate and gum tragacanth as a fat replacers in nonfat yogurt: Chemical, physical and microstructural properties. Journal of Dairy Science, 91(7): 2545-2552

Bhagya, S., and Srinivasan, K. S. (1999). Effect of different methods of drying on functional properties of enzyme treated ground flour. Journal of Food Science and Technology, 22: 329.

Becker, E. M., Nissen, L. R., and Skibsted, L. H. (2004). Antioxidant evaluation protocols: Food quality or health effects. European Food Research and Technology, 219(6): 561-571.

Bennett, K. L., Stensballe, A., Podtelejnikov, A. V., Moniatte, M., and Jensen, O. N. (2002).Phosphopeptide detection and sequencing by matrix-assisted laser desorption/ionization quadrupole time-of-flight tandem mass spectrometry Journal of Mass Spectrometry, 37(2): 179-190.

Benzie, I. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. Analytical biochemistry, 239(1): 70-76.

Bernardini,Di., Mullen, R., , Bolton A. M., Kerry, D., O'Neill,J.E., and Hayes, M. (2012). Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions. Meat science, 90(1): 226-235.

Bezerra, V. S., Campos, J. F., Silva, R. A. D., Porto, T. S., Lima Filho, J. L. D., and Porto, A. L. F. (2013). Biotechnological richness of the northeastern semi-arid region: antioxidant activity of casein hydrolysates from Moxotó goat milk (Capra hircus Linnaeus, 1758) obtained by papain action. Food Science and Technology (Campinas), 33(3): 513-520.

Britten, M., Giroux, H. J. and Gaudin, V.(1994). Effect of pH during heat processing of partially hydrolyzed whey protein. Journal of Dairy Science., 77: 676-684.

Caessens, P. W. J. R., Daamen, W. F., Gruppen, H., Visser, S. and Voragen, A. G. J. (1999) b.β -Lactoglobulin hydrolysis. II. Peptide identification, SH/SS exchange, and functional properties of hydrolysate fractions formed by the action of plasmin. Journal of Agricultural and Food Chemistry*,* 47**:** 2980-2990.

Caessens, P. W. J. R.(1999). Enzymatic hydrolysis of β--casein and β--lactoglobulin foam and emulsion properties of peptides in relation to their molecular structure.Ph.D. Thesis Wageningen Agricultural University, Netherland.

Calderon de la Barca, A. M., Ruiz-Salazar, R. A., and Jara-Marini, M. E. (2000).Enzymatic hydrolysis and synthesis of soy protein to improve its amino acid composition and functional properties. Journal of Food Science, 65: 246–253.

Cao, G., Alessio, H. M., and Cutler, R. G. (1993).Oxygen-radical absorbance capacity assay for antioxidants. Free radical biology and medicine, 14(3): 303-311.

Castro,De., R. J. S., and Sato, H. H. (2014). Production and biochemical characterization of protease from *Aspergillus oryzae*: An evaluation of the physical–chemical parameters using agroindustrial wastes as supports. Biocatalysis and Agricultural Biotechnology, 3(3): 20-25.

Castro De., R. J. S., and Sato, H. H. (2015). Biologically active peptides: Processes for their generation, purification and identification and applications as natural additives in the food and pharmaceutical industries. Food Research International, 74: 185-198.

Castimopoolas, N., Funk, S. K., and Meyer, E. W. (1970).Thermal aggregation of glycinin subunits.Cereal Chemistry, 47: 331.

Celus, I., Brijs, K., and Delcour, J. A.(2006) The effects of malting and mashing on barley protein extractability. Journal of Cereal Science, 44: 203–211

Celus, I.; Brijs, K.and Delcour, J.A. (2007).Enzymatic hydrolysis of brewers' spent grain proteins and technofunctional properties of the resulting hydrolysates. Journal of Agricultural and Food Chemistry., 55: 8703–8710

Chan, W. M.,and Ma, C. Y. (1999). Acid modification of proteins from soymilk residue (okara). Food Research International, 32(2): 119-127.

Chang, O. K,.Seol, K. H., Jeong S. G, Oh, M.H., Park B.Y., Perrin C and Ham J.S. (2013). Casein hydrolysis by *Bifidobacterium longum* KACC91563 and antioxidant activities of peptides derived therefrom. Journal of Dairy Science. 96: 5544–5555.

Cheeseman, K.H., and Slater, T.F. (1993).An introduction to free radical biochemistry. British Medical Bulletin, 49(3): 481-493.

Chen, H.-M., Muramoto, K., Yamauchi, F., Fujimoto, K., and Nokihara, K. (1998).Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. Journal of Agricultural and Food Chemistry, 46(1): 49-53.

Chen, J., Lindmark-Månsson, H., Gorton, L., and Åkesson, B. (2003).Antioxidant capacity of bovine milk as assayed by spectrophotometric and amperometric methods.International Dairy Journal, *13*(12): 927-935.

Chen, H., and Li, J. (2012).Antioxidant activities of polysaccharides from Lentinusedodes and their significance for disease prevention. International Journal of Biological Macromolecules, 50(1): 214-218.

Chobert, J. M., Bertrand-Harb, C., Nicolas, M. G.(1988)a. Solubility and emulsifying properties of caseins and whey proteins modified enzymatically by trypsin. Journal of Agricultural and Food Chemistry., 36: 883-892.

Chobert, J. M., Sitohy, M. Z., Whitaker, J. R.(1988)b. Solubility and emulsifying properties of caseins modified enzymatically by *Staphylococcus aureus*V8 protease Journal of Agricultural and Food Chemistry., 36**:** 220-224.

Clarkson, P.M., and Thompson, H.S. (2000). Antioxidants: what role do they play in physical activity and health? American Journal of Clinical Nutrition, 72: 637S-647S.

Clausen, M. R., Skibsted, L. H., and Stagsted, J. (2009).Characterization of major radical scavenger species in bovine milk through size exclusion chromatography and functional assays.Journal of Agricultural and Food Chemistry, 57(7): 2912 2919.

Clemente, A.,(2000). Enzymatic protein hydrolysates in human nutrition. Trends in Food Science and Technology, 7: 254-26

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. Trends Food Science and Technology., 11: 254–262.

Contreras. D. M. M., Hernandez-Ledesma, B., Amigo, L., Martin-Alvarez, P.J. and Recio, I. (2011). Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. LWT-Food Science and.Technol.44: 9-15.

Cornelly, v. d. V., Gruppen, H., de Bont, D., and Voragen, A. G. (2002).Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. International Dairy Journal, 12(10): 813-820.

Corrêa, A. P. F., Daroit, D. J., Coelho, J., Meira, S. M., Lopes, F. C., Segalin, J., , A. (2011). Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. Journal of the Science of Food and Agriculture, 91(12): 2247-2254.

Cumby, N., Zhong, Y., Naczk, M., andShahidi, F. (2008).Antioxidant activity and water-holding capacity of canola protein hydrolysates. Food Chemistry, 109(1): 144-148.

Dąbrowska, A., Szołtysik, M., Babij, K., Pokora, M., Zambrowicz, A., and Chrzanowska, J. (2013).Application of Asian pumpkin (Cucurbitaficifolia) serine proteinase for production of biologically active peptides from casein. Acta Biochimica Polonica, 60(1): 117-122.

Dany, D., Don, E. O., Sylvie, F., Gauthier and Foegeding, E. A. (2003). Enzyme-Induced gelation of extensively hydrolyzed whey proteins by alcalase: peptide identification and determination of enzyme specificity. Journal of Agriculture and Food Chemistry, 51: 6300-6308.

Davalos, A., Miguel, M., Bartolome, B., and Lopez-Fandino, R. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. Journal of Food Proteins, 67: 1939-1944.

Davies, M. J., and Dean, R. T. (1997). Radical-mediated protein oxidation: from chemistry to medicine. Oxford University Press: Oxford, New York.

De Wit, J. N., Klarenbeek, G. and Adamse, M. 1988. Evaluation of functional properties of whey protein concentrates and whey protein isolates. 2. Effect of processing history and composition. Neth Milk Dairy Journal., 40: 41-56.

Díaz, M., Dunn, C. M., McClements, D. J., and Decker, E. A. (2003).Use of caseinophosphopeptides as natural antioxidants in oil-in-water emulsions.Journal of Agricultural and Food Chemistry, 51(8): 2365-2370.

Díaz, M., and Decker, E. A.(2004). Antioxidant mechanisms of caseinophosphopeptides and casein hydrolysates and their application in ground beef. Journal of Agricultural and Food Chemistry, *52*(26): 8208-8213.

Dickinson, E., Murray A., Murray, B. S. and Stainsby, G.(1987). Food Emulsions and Foams, ed. E. Dickinson, Royal Society of.Chemistry, London.86-89.

Doucet, D., Otter, D. E., Gauthier, S. F., and Foegeding, E. A. (2003). Enzyme-induced gelation of extensively hydrolyzed whey proteins by Alcalase: peptide identification and determination of enzyme specificity. Journal of Agricultural and Food Chemistry, 51(21): 6300-6308.

Farvin, S., K.H, Baron, C. P., Nielsen, N. S., Otte, J., and Jacobsen, C. (2010). Antioxidant activity of yoghurt peptides: Part 2–Characterisation of peptide fractions. Food Chemistry, 123(4): 1090-1097.

Fang, X., Xie, N., Chen, X., Yu, H., and Chen, J. (2012).Optimization of antioxidant hydrolysate production from flying squid muscle protein using response surface methodology. Food and Bioproducts Processing, 90(4): 676-682.

FitzGerald, R. J., and Meisel, H. (2000).Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. British Journal of Nutrition, 84(S1): 33-37.

Gibbs, B. F., Zougman, A., Masse, R., and Mulligan, C. (2004).Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food.Food Research International, 37(2): 123-131.

Gliszczyńska-Świgło, A. (2006). Antioxidant activity of water soluble vitamins in the TEAC (trolox equivalent antioxidant capacity) and the FRAP (ferric reducing antioxidant power) assays. Food Chemistry, 96(1): 131-136.

Gobba, De., C., Espejo-Carpio, F. J., Skibsted, L. H., and Otte, J. (2014). Antioxidant peptides from goat milk protein fractions hydrolysed by two commercial proteases. International Dairy Journal, 39(1): 28-40.

Guo, H., Kouzuma, Y,and Yonekura, M.(2009). Structures and properties of antioxidative peptides derived from royal jelly protein. Food Chemistry 113: 238 245

Gupta, A., Mann, B., Kumar, R., and Sangwan, R. B. (2009). Antioxidant activity of Cheddar cheeses at different stages of ripening. International Journal of Dairy Technology, 62(3): 339-3

Gupta, A., Mann, B., Kumar, R., and Sangwan, R.B. (2010). Identification of antioxidant peptides in cheddar cheese made with adjunct culture *Lactobacillus casei*ssp*casei* 300, *Milchwissenschaft*. 65: 396-399.

Guzman-Gonzalez, M., Morais, F., Ramos, M. and Amigo, L. (1999).Influence of skimmed milk concentrate replacement by dry dairy producсts in a low fat set-type yoghurt model system. 1: Use of whey protein concentrates and skimmed milk powder. Journal of the Science of Food and Agriculture, 79: 1117-1122.

Haque, Z. U. and Mozaffar, Z.(1992).Casein hydrolysate.II.Functional properties of peptides.Food Hydrocolloids, 5**:** 559-571.

Haque, E., Chand, R., and Kapila, S. (2008). Biofunctional properties of bioactive peptides of milk origin. Food Reviews International, 25(1): 28-43.

Hernandez-Ledesma, B., Davalos, A., Bartolome, B., and Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from alphalactalbumin and beta-lactoglobulin- Identification of active peptides by HPLC-MS/MS. Journal of Agricultural and Food Chemistry, 53: 588-593.

Hernández-Ledesma B, Quirós A, Amigo L and Recio I.(2007) Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. International Dairy Journal;17:42–9.

Hidalgo, J.and Camper, E. (1977). Solubility and heat stability of whey Protein Concentrate.Journal of Dairy Science, 60**:** 1515-1518

Hidalgo, M. E., Daroit, D. J., Folmer Correa, A. P., Pieniz, S., Brandelli, A., & Risso, P. H. (2012). Physicochemical and antioxidant properties of bovine caseinate hydrolysates obtained through microbial protease treatment. International journal of dairy technology, 65(3): 342-352.

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 Chemistry, 117(3): 438-443.

Hu, W., Zhang, C., Wu, R., Sun, Y., Levine, A., and Feng, Z. (2010).Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. Proceedings of the National Academy of Sciences, 107(16): 7455-7460.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A., andDeemer, E. K. (2002). Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. Journal of Agricultural and Food Chemistry, 50(7): 1815-1821.

Huang, D., Ou, B., and Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. Journal of agricultural and food chemistry, 53(6): 1841-1856.

Intarasirisawat, R., Benjakul, S., Visessanguan, W., Wu, J.,(2012). Antioxidativeandfunctional properties of protein hydrolysate from defatted skipjack (Katsuwonouspelamis)roe. Food Chemistry., 135: 3039–3048

Irshad, M., Zafaryab, M., Singh, M., andRizvi, M. (2012). Comparative analysis of the antioxidant activity of Cassia fistula extracts. International Journal of Medicinal Chemistry, 2012.

Je, J. -Y., Park, P. -J., and Kim, S. -K. (2005). Antioxidant activity of a peptide isolated from Alaska pollak (Theragrachalcogramma) frame protein hydrolysate. Food Research International, 38(1): 45–50.

Jeon, Y. J., Byun, H. G., and Kim, S. K. (1999). Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. Process Biochemistry, 35(5): 471-478.

Johansen, J. S., Harris, A. K., Rychly, D. J., andErgul, A. (2005). Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. British Journal of Diabetes & Vascular Disease, 4(1): 5.

Kansci, G., Genot, C., Meynier, A., Gaucheron, F., andChobert, J.-M. (2004). β-caseinophosphopeptide (f1-25) confers on β-casein tryptic hydrolysate an antioxidant activity during iron/ascorbate-induced oxidation of liposomes. Le Lait, 84(5): 449-462.

Kasran, M., Cui, S. W., and Goff, H. D. (2013). Emulsifying properties of soy whey protein isolate fenugreek gum conjugates in oil-in-water emulsion model system. Food Hydrocolloids, 30(2): 691-697.

Kim, G. N., Jang, H. D., and Kim, C. I. (2007). Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using Alcalase. Food Chemistry, 104(4): 1359-1365.

Kishore, P. and Mann, B.(2009). Antioxidant activity of whey protein hydrolysates.NDRI.*News Letter* pp.3.

Kitts, D. D., andWeiler, K. (2003).Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. Current Pharmaceutical Design, 9(16): 1309-1323.

Kitts, D. (2005). Antioxidant properties of casein-phosphopeptides. Trends in Food Science and Technology, 16(12): 549-554.

Kong, B. and Xiong, X. L. (2006).Antioxidant activity of zeinhydrolysates in a liposome system and the possible mode of action.Journal of Agriculture and Food Chemistry. 54: 6059–6068

Kong, X.; Zhou, H.; Qian, H.(2007) Enzymatic preparation and functional properties of wheat gluten hydrolysates. Food Chemistry., 101: 615–620

Korhonen, H., and Pihlanto, A. (2003).Food-derived bioactive peptides-opportunities for designing future foods. Current Pharmaceutical Design, 9(16): 1297-1308.

Korhonen, H., and Pihlanto, A. (2006). Bioactive peptides: production and functionality. International Dairy Journal, *16*(9): 945-960.

Kuba, M., Tanaka, K., Tawata, S., Takeda, Y., and Yasuda, M. (2003). Angiotensin I-converting enzyme inhibitory peptides isolated from tofuyo fermented soybean food. Bioscience, Biotechnology and Biochemistry, *67*(6): 1278-1283.

Kudoh, Y., Matsuda, S., Igoshi, K. and Oki, T. (2001). Antioxidative peptide from milk fermented with Lactobacillus delbrueckii ssp. bulgaricus IFO13953. J. Japanese Society and Food Science. 48: 44–50.

Kumar, R., Singhal, V., Bhagat, S. R., and Mann, B. (2010). Comparative studies on antioxidant activity of buffalo and cow milk casein and their hydrolysates. Milchwissenschaft, 65(3): 287-290.

Kullisaar T. Zilmer M, Mikelsaar M,Vihalemm T, Annuk H, Kairane C, Kilk A. 2002. Two antioxidativelactobacilli strains as promising probiotics. International Journal of Food Microbiology 72(3): 215-224.

Laakso, S. (1984).Inhibition of lipid peroxidation by casein Evidence of molecular encapsulation of 1, 4-pentadiene fatty acids.Biochimicaet BiophysicaActa (BBA)-Lipids and Lipid Metabolism, 792(1): 11-15.

Laguerre, M., Lecomte, J., and Villeneuve, P. (2007). Evaluation of the ability of antioxidants to counteract lipid oxidation: Existing methods, new trends and challenges. Progress in Lipid Research, 46(5): 244-282.

Lahl, W.J.; Grindstaff, D.A. Spices and seasonings: Hydrolyzed proteins. In Proceedings of the 6th SIFST Symposium on Food Ingredients—Applications, Status and Safety, Singapore Institute of Food Science and Technology, Singapore, 27–29 April 1989; pp. 51–65.

Lahl, W.J., Braun, S.D., (1994).Enzymatic production of protein hydrolysate for food use. Journal of Food Technology, 48: 68-71.

Li, C. H., Matsui, T., Matsumoto, K., Yamasaki, R., and Kawasaki, T. (2002).Latent production of angiotensin I-converting enzyme inhibitors from buckwheat protein. Journal of Peptide Science, 8(6): 267-274.

Li, B., Chen, F., Wang, X., Ji, B. P., and Wu, Y. (2007).Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. Food Chemistry. 102: 1135–1143.

Li, Y., Jiang, B., Zhang, T., Mu, W., and Liu, J. (2008). Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). Food Chemistry, 106(2): 444-450.

Li, Y. W., Li, B., He, J., and Qian, P. (2011). Structure–activity relationship study ofnantioxidative peptides by QSAR modeling: the amino acid next to C-terminus affects the activity. Journal of Peptide Science, 17(6): 454-462.

Li, Z., Jiang, A., Yue, T., Wang, J., Wang, Y., and Su, J. (2013). Purification and identification of five novel antioxidant peptides from goat milk casein hydrolysates. Journal of Dairy Science, 96(7): 4242-4251.

Lieske, B. and Konrad, G.(1996). Physico-chemical and functional properties of whey protein as affected by limited papain proteolysis and selective ultrafiltration. International Dairy Journal.,6: 13-31.

Limón, R. I., Peñas, E., Torino, M. I., Martínez-Villaluenga, C., Dueñas, M., and Frias, J. (2015). Fermentation enhances the content of bioactive compounds in kidney bean extracts. Food chemistry, 172: 343-352.

Lin, Y., Le, G., Wang, J., Li, Y., Shi, Y., and Sun, J. (2010).Antioxidative peptides derived from enzyme hydrolysis of bone collagen after microwave assisted acid pretreatment and nitrogen protection. International Journal of Molecular Science. 11: 4297–4308.

Liu, Q., Kong, B., Xiong, Y. L., and Xia, X.(2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. Food Chemistry, 462 118(2): 403-410.

López-Expósito, I., Quirós, A., Amigo, L., andRecio, I. (2007). Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. Le Lait87(4-5): 241-249.

.López-Expósito, I., Quirós, A., Amigo, L., andRecio, I. (2007). Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. Le Lait, 87(4-5): 241-249.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin’s phenol reagent. Journal of Biological Chemistry, 193(1): 265-275.

Ludwig, I., Krause, W. and Hajos, G.(1995). Functional properties of enzymatically modified milk proteins.Acta Aliment., 24: 289-296.

Luo, Y., Teng, Z., Wang, T. T. Y., and Wang,Q.(2013). Cellular Uptake and Transport of Zein Nanoparticles: Effects of Sodium Caseinate. Journal of Agricultural and Food Chemistry, 61(31): 7621-7629

Luo, Y., Pan, K., & Zhong, Q. (2014). Physical, chemical and biochemical properties of casein hydrolyzed by three proteases: partial characterizations. Food chemistry, 155: 146-154.

Maeno, M., Yamamoto, N., and Takano, T. (1996). Identification of an Antihypertensive Peptide from Casein Hydrolysate Produced by a Proteinase from Lactobacillus helveticus CP790. Journal of Dairy Science, 79(8): 1316-1321.

Mann, B., Kumari, A., Kumar, R., Sharma, R., Prajapati, K., Mahboob, S., and Athira, S. (2014). Antioxidant activity of whey protein hydrolysates in milk beverage system. Journal of Food Science and Technology, 52(6): 3235-3241.

Martinez, C., Becerra, M., Chafer, M., Albors, A., Carot, J. M. and Chiralt, A.(2002). Influence of substituting milk powder for whey powder on yoghurt quality. Trends in Food Science and Technology, 13: 334-340.

McClements, D., and Decker, E. (2000). Lipid oxidation in Oil-in-Water emulsions: Impact of molecular environment on chemical reactions in heterogeneous food systems. Journal of Food Science, 65(8): 1270-1282.

Medina Hernandez, M. J., Bonet Domingo, E., Villanueva Camanas, R. M., and Garcia Alvarez-Coque, M. C. (1990b). Evaluation of the proteolysis degree with the o-phthaldialdehyde/N-acetyl-l-cysteine reagent.Frenesius Journal of Analytical Chemistry, 338: 62–65.

Megías, C., Yust, M. d. M., Pedroche, J., Lquari, H., Girón-Calle, J., Alaiz, M., Millán, F., andVioque, J. (2004). Purification of an ACE inhibitory peptide after hydrolysis of sunflower (Helianthus annuus L.) protein isolates. Journal of Agricultural and Food Chemistry, *52*(7): 1928-1932.

Meisel, H. (1997). Biochemical properties of regulatory peptides derived from milk proteins. Peptide Science, 43(2): 119-128.

Meisel, H. (2001). Bioactive peptides from milk proteins: a perspective for consumers and producers. Australian Journal of Dairy Technology, 56(2): 83-92.

Meisel, H. (2004). Multifunctional peptides encrypted in milk proteins. Biofactors, 21(1): 55-61.

Mendis, E., Rajapakse, N., and Kim, S. K. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. Journal of Agricultural and Food Chemistry., 53, 581-587.

Miquel, E., Alegría, A., Barberá, R., and Farré, R. (2006). Casein phosphopeptides released by simulated gastrointestinal digestion of infant formulas and their potential role in mineral binding. International Dairy Journal, 16(9): 992-1000.

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., and Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates.Clinical science, 84: 407-407.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin Journal of Science Technology, 26(2): 211-219.

Mota, M. V. T., Ferreira, I. M. P. L. V. O., Oliveira, M. B. P., Rocha, C., Teixeira, J. A., Torres, D., and Gonçalves, M. P. (2006). Trypsin hydrolysis of whey protein concentrates: Characterization using multivariate data analysis. Food chemistry, 94(2): 278-286.

Motoi, H., and Kodama, T. (2003). Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides from wheat gliadin hydrolysate.Food/Nahrung, *47*(5): 354-358.

Moure, A., Domínguez, H., and Parajó, J. C. (2006).Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. Process Biochemistry, 41(2): 447-456.

Mutilangi, W. A. M., Panyam, D. and Kilara, A.(1996). Functional properties of hydrolysates from proteolysis of heat-denatured whey protein isolate. Journal of Food Science., 61**:** 270-274.

Nidhi Y.(2010).Evaluation of bioactivity and functionality of whey protein hydrolysates. M.Sc. Thesis NDRI, Karnal

Nielsen, P. M.(1997). Functionality of protein hydrolysates.In Food proteins and their applications; (Damadoran, S., Paraf, A., ed.); Marcel Dekker: New York, pp. 443-472.

Nielsen, P., Petersen, D., and Dambmann, C. (2001).Improved method for determining food protein degree of hydrolysis. Journal of Food Science, 66(5): 642-646.

Nordberg, J., and Arner, E. S. (2001).Reactive oxygen species, antioxidants, and the mammalian thioredoxin system.Free radical biology and medicine, 31(11): 1287-1312.

Ou, B., Hampsch-Woodill, M., and Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. Journal of Agricultural and Food chemistry, 49(10): 4619-4626.

Oxman, J. D., Bui, H. T., and Jacobs, D. W. (2000). U.S. Patent No. 6,159,012. Washington, DC: U.S. Patent and Trademark Office.

Pan, M.; Jiang, T.S.; Pan, J.L. (2011). Antioxidant activities of rapeseed protein hydrolysates.Food Bioprocess Technology., 4: 1144–1152.

Pan, K., Zhong, Q., and Baek, S. J. (2013).Enhanced dispersibility and bioactivity of curcumin by encapsulation in casein nanocapsules.Journal of Agricultural and Food Chemistry*, 61*(25): 6036- 6043

Panyam, D. and Kilara, A.(1996).Enhancing the functionality of food proteins by enzymatic modification.Trends Food Science and Technology., *7*: 120-125.

Peng, X., Kong, B., Xia, X., and Liu, Q. (2010). Reducing and radical-scavenging activities of whey protein hydrolysaye prepared with Alcalase. International Dairy Journal, 20: 360-365.

Pearce, K. N., and Kinsella, J. E. (1978).Emulsifying properties of proteins: evaluation of a turbidimetric technique. Journal of Agricultural and Food Chemistry, 26(3): 716-723.

Pearce, R. J. and Regester, G. O. 1996. New opportunities from the isolation and utilization of whey proteins. Journal of Dairy Science, 79: 1454-1459

Pedersen, B. (1994). Removing bitterness from protein hydrolysates. Food technology (USA).

Pedroche, J., Yust, M. M., Lqari, H., Girón-Calle, J., Vioque, J., Alaiz, M., andMillán, F. (2004). Production and characterization of casein hydrolysates with a high amino acid Fischer's ratio using immobilized proteases. International Dairy Journal, 14(6): 527-533.

Perez-Campo, R., Lopez-Torres, M., Rojas, C., Cadenas, S., and Barja, G. (1994). Longevity and antioxidant enzymes, non-enzymatic antioxidants and oxidative stress in the vertebrate lung: a comparative study. Journal of Comparative Physiology B, 163(8): 682-689.

Pellagrani, N., Serafini, M., Colombi, B., Delrio, D. and Brighenti, F. 2003. Total antioxidant capacity of plant foods, beverages and oils consumed in italy assayed by three different methods. Journal of Nutrition, 133: 2812-2819.

Petrilli, p., Pucci, p., Pelissier, j. p., and Addeo, f. (1987). Digestion by pancreatic juice of a beta-casomorphin-containing fragment of buffalo beta-casein. International journal of peptide and protein research, 29(4): 504-508.

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. International Dairy Journal, 19(11): 643-654.

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. International Dairy Journal, 16(11): 1306-1314.

Power, O., Jakeman, P., and FitzGerald, R. (2013). Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides. Amino Acids, 44(3): 797-820.

Prior, R. L., and Cao, G. (1999). In vivo total antioxidant capacity: comparison of different analytical methods 1. Free Radical Biology and Medicine, 27(11): 1173-1181.

Prior, R. L., Wu, X., and Schaich, K. (2005).Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agriculture and Food Chemistry. 53: 4290-4302

Quirós, A., Ramos, M., Muguerza, B., Delgado, M. A., Miguel, M., Aleixandre, A., and Recio, I. (2007). Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. International Dairy Journal, 17(1): 33-41.

Rahma, E. H., and Mostafa, M. M. (1998).Functional properties of peanutBfruit as affected by different heat treatment. Journal of Food Science and Technology, 25: 11–15.

Rajapakse, N., Mendis, E., Byun, H. G., and Kim, S. K. (2005a). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. The Journal of Nutritional Biochemistry, 16(9): 562-569.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cationdecolorization assay. Free radical biology and medicine, 26(9): 1231-1237.

Rejtar, T., Chen, H. S., Andreev, V., Moskovets, E., and Karger, B. L. (2004).Increased identification of peptides by enhanced data processing of high-resolution MALDITOF/TOF mass spectra prior to database searching. Analytical Chemistry,*76*(20): 6017-6028.

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., Kakuda, Y., and Xue, S. J. (2008). Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization–mass spectrometry. Food Chemistry, 108: 727–736.

Rice-Evans, C. A., Miller, N. J., and Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free radical biology and medicine, 20(7): 933-956.

Rival, S. G., Fornaroli, S., Boeriu, C. G., and Wichers, H. J. (2001). Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. Journal of Agricultural and Food Chemistry, 49(1): 287-294.

Rival, S. G., Boeriu, C. G., and Wichers, H. J. (2001a).Caseins and casein hydrolysates.Antioxidative properties and relevance to lipoxygenase inhibition. Journal of Agricultural and Food Chemistry, *49*(1): 295-302.

Rival, S. G., Fornaroli, S., Boeriu, C. G., and Wichers, H. J. (2000). Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. Journal of Agricultural and Food Chemistry, 49(1): 287-294.

Sadat, L., Cakir-Kiefer, C., N'Negue, M. A., Gaillard, J. L., Girardet, J. M., and Miclo, L. (2011).Isolation and identification of antioxidative peptides from bovine α-lactalbumin.International dairy journal, 21(4): 214-221.

Sánchez, C. C., and Patino, J. M. R. (2005).Interfacial, foaming and emulsifying characteristics of sodiumcaseinate as influenced by protein concentration in solution. Food Hydrocolloids*, 19*(3): 407-416.

Sarmadi, B. H., and Ismail, A. (2010).Antioxidative peptides from food proteins: a review. Peptides, 31(10): 1949-1956

Schaafsma, G. (2009). Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. European Journal of Clinical and Nutrition., 63: 1161–1168.

Segura-Campos, M. R., Salazar-Vega, I. M., Chel-Guerrero, L. A., and Betancur-Ancona, D. A. (2013). Biological potential of chia (Salvia hispanica L.)

protein hydrolysates and their incorporation into functional foods. LWT-Food Science and Technology, 50(2): 723-731.

Sekul, A. A., Vinnett, C. H., and Ory, R. L. (1978). Some functional properties of peanut proteins partially hydrolyzed with papain. Journal of Agricultural and Food Chemistry, 26: 855–858.

Serafini, M. (2006).The role of antioxidants in disease prevention. Medicine, 34(12): 533-535.

Shanmugam, V. P., Kapila, S., Sonfack, T. K., and Kapila, R. (2015). Antioxidative peptide derived from enzymatic digestion of buffalo casein. International Dairy Journal, 42: 1-5.

Sharp, J. S., Becker, J. M., and Hettich, R. L. (2004).Analysis of protein solvent accessible surfaces by photochemical oxidation and mass spectrometry. Analytical Chemistry, 76(3): 672-683.

Shenana, M. E., Abdou, S. M., Hassan, H. M., EI-AI-fy, M. B and Nasr, W. I. (2007).Utilization of UF and RO-retentates in ice cream making.Egyptian Journal of Dairy science, 35: 243-251

Sinha, R., Radha, C., Prakash, J., and Kaul, P. (2007). Whey protein hydrolysate: Functional properties, nutritional quality and utilization in beverage formulation. Food Chemistry, 101(4): 1484-1491.

Slattery, H., Fitzgerald, R. J.(1998). Functional properties and bitterness of sodium caseinatehydrolysates prepared with a *Bacillus* proteinase. Journal of Food Science., 63: 418-422

Sodini, I., Remeuf, F., Haddad, S., and Corrieu, G. (2004). The relative effect of milk base, starter, and process on yogurt texture: a review. Critical reviews in food science and nutrition, 44(2): 113-137.

Spellman, D., McEvoy, E., O'cuinn, G., and FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. International Dairy Journal, 13(6): 447-453.

Su, R., Liang, M., Qi, W., Liu, R., Yuan, S., and He, Z. (2012). Pancreatic hydrolysis of bovine casein: Peptide release and time-dependent reaction behaviour. Food Chemistry, 133(3): 851-858

Suetsuna, K., Ukeda, H., and Ochi, H. (2000). Isolation and characterization of free radical scavenging peptides derived from casein. Journal of Nutritional Biochemistry, 11: 128-131.

Svenning, C., Molland, T., Langsrud, T., Vegarud, G .E.(1993). Characterization study of peptides derived from casein proteolysis. In Protein and fat globule modifications by heat treatment, homogenisationand other technological means for high quality dairy products: Proceedings of the IDF seminar held in Munich; IDF, Ed.; International Dairy Federation: Sonthofen, Gemany, pp. 96-105

Tauzin, J., Miclo, L., Roth, S., Mollé, D., and Gaillard, J. L. (2003). Tryptic hydrolysis of bovine αS2-casein: identification and release kinetics of peptides. International Dairy Journal, 13(1): 15-27.

Terahara, M., Kurama, S., and Takemoto, N. (2001).Prevention by lactic acid bacteria of the oxidation of human LDL.Bioscience, biotechnology, and biochemistry, 65(8): 1864-1868.

Thannickal, V. J., and Fanburg, B. L. (2000).Reactive oxygen species in cell signaling.American Journal of Physiology-Lung Cellular and Molecular Physiology, 279(6): L1005-L1028.

Tsopmo A., RomanowskiA., L. Banda, J.C. Lavoie, H. Jenssen, J. K.Friel, (2011). Novel anti-oxidative peptides from enzymatic digestion of human milk Food Chemistry, 126: 1138–1143

Turgeon, S. L., Gauthier, S. F., & Paquin, P. (1991). Interfacial and emulsifying properties of whey peptide fractions obtained with a two-step ultrafiltration process. Journal of Agricultural and Food chemistry, 39(4): 673-676.

Uhlig, H.1998. Industrial enzymes and their applications; Wiley: New York.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. M., and Mazur, M. (2006).Free radicals, metals and antioxidants in oxidative stress-induced cancer.Chemico-biological interactions, 160(1): 1-40.

Ven, V. D. V., Gruppen, H., Dries, B. A., de Bont., Alphons ,G. J. and Voragen. (2002). Optimization of ACE inhibition by Whey protein hydrolysates using response surface methodology. International Dairy Journal, 12**:** 813-820

Vioque, J., Sánchez-Vioque, R., Clemente, A., Pedroche, J., & Millán, F. (2000). Partially hydrolyzed rapeseed protein isolates with improved functional properties. Journal of the American Oil Chemists' Society, 77(4): 447-450.

Vojdani, F. and Whitaker, J. R. (1994).Chemical and enzymatic modification of proteins for improved functionality. In Protein functionality in food systems; Marcel Dekker: New York, pp. 261-310.

Wade, A. M., and Tucker, H. N. (1998).Antioxidant characteristics of L-histidine. Journal of Nutritional Biochemistry., 9: 308-315.

Welch, K. D., Davis, T. Z., Van Eden, M. E., and Aust, S. D. (2002). Deleterious iron-mediated oxidation of biomolecules. Free radical biology and medicine, 32(7): 577-583.

Whitaker, J. R. (1995). Polyphenol oxidase. In Food Enzymes (pp. 271-307). Springer US.

Wu, H. C., Chen, H. M., and Shiau, C. Y. (2003).Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomberaustriasicus). Food research international, 36(9): 949-957.

Xiong, Y. L. (2010). Antioxidant peptides. Ames, Iowa, USA: Wiley-Blackwell.

Xue- Ying, M., Xue, C., Xu, W. and Si-Jia, W. (2011).Free radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis. Food chemistry., 126: 484-490.

Yoshie-Stark, Y.; Wada, Y. and Wäsche, A.(2008) Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. Food Chemistry, 107: 32–39.

Zhang, Y., Niu, Y., Luo, Y., Ge, M., Yang, T., Yu, L., and Wang, Q.(2014).Fabrication, characterization and antimicrobial activities of thymol-loaded zein nanoparticles stabilized by sodium caseinate–chitosan hydrochloride double layers. Food Chemistry, *142:* 269-275.

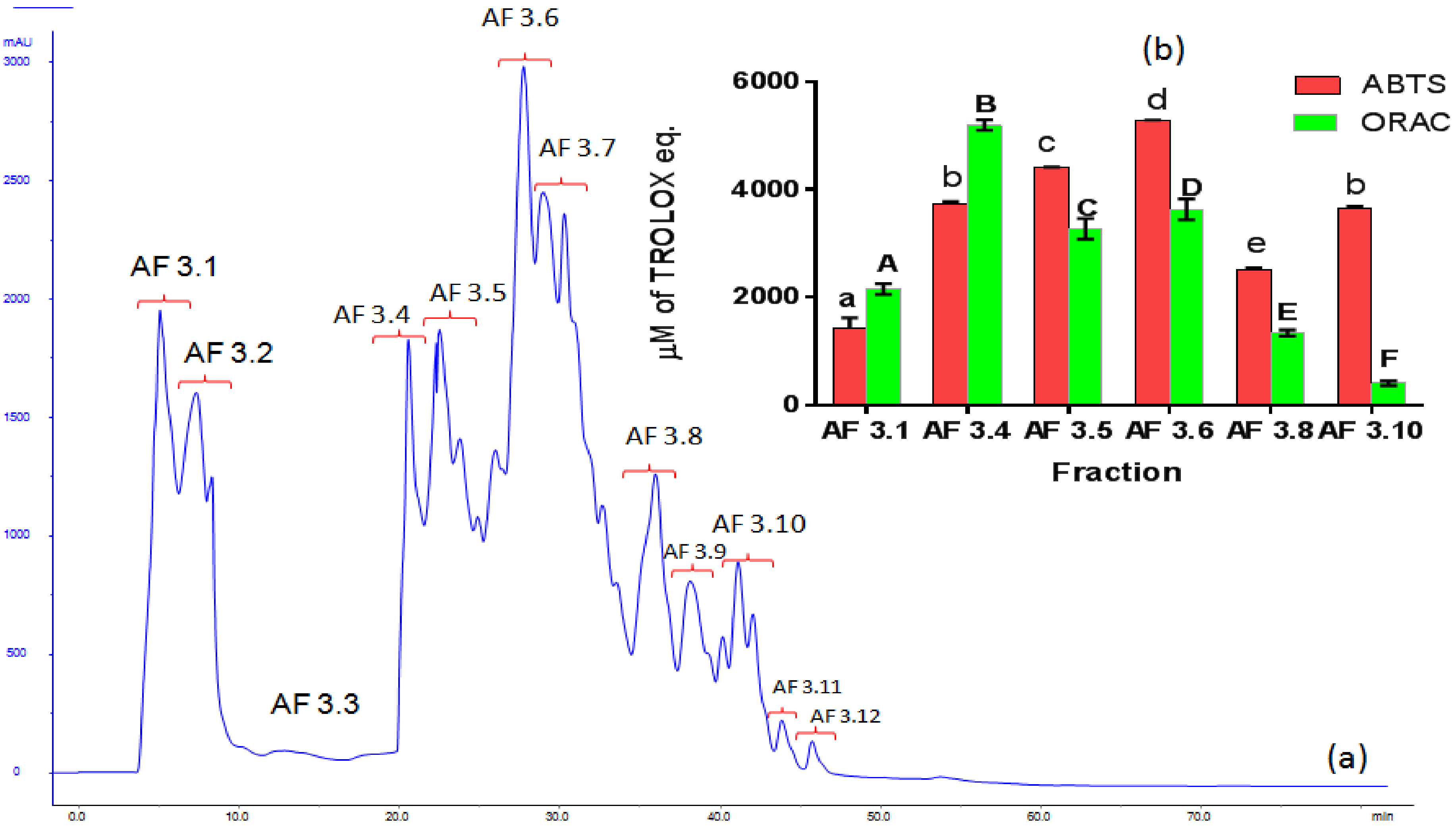
Zhao, X. H., Wu, D., and Li, T. J. (2010).Preparation and radical scavenging activity of papain-catalysed casein plasteins. Dairy Science and Technology, 90(5): 521-535.

Zhidong. L., Benheng. G., Xuezhong. C., Zhenmin L, Yun. D, Hongliang H, and Wen. R, (2013) Optimization of hydrolysis conditions for antioxidant hydrolysate production from whey protein isolates using response surface methodology, Irish journal of Agriculture and Food Research 52: 53-65

Zhu, L., Chen, J., Tang, X., and Xiong, Y. L. (2008).Reducing, radical scavenging, and chelation properties of in vitro digests of alcalase-treated zein hydrolysate. Journal of Agricultural and Food Chemistry, 56(8): 2714-2721.

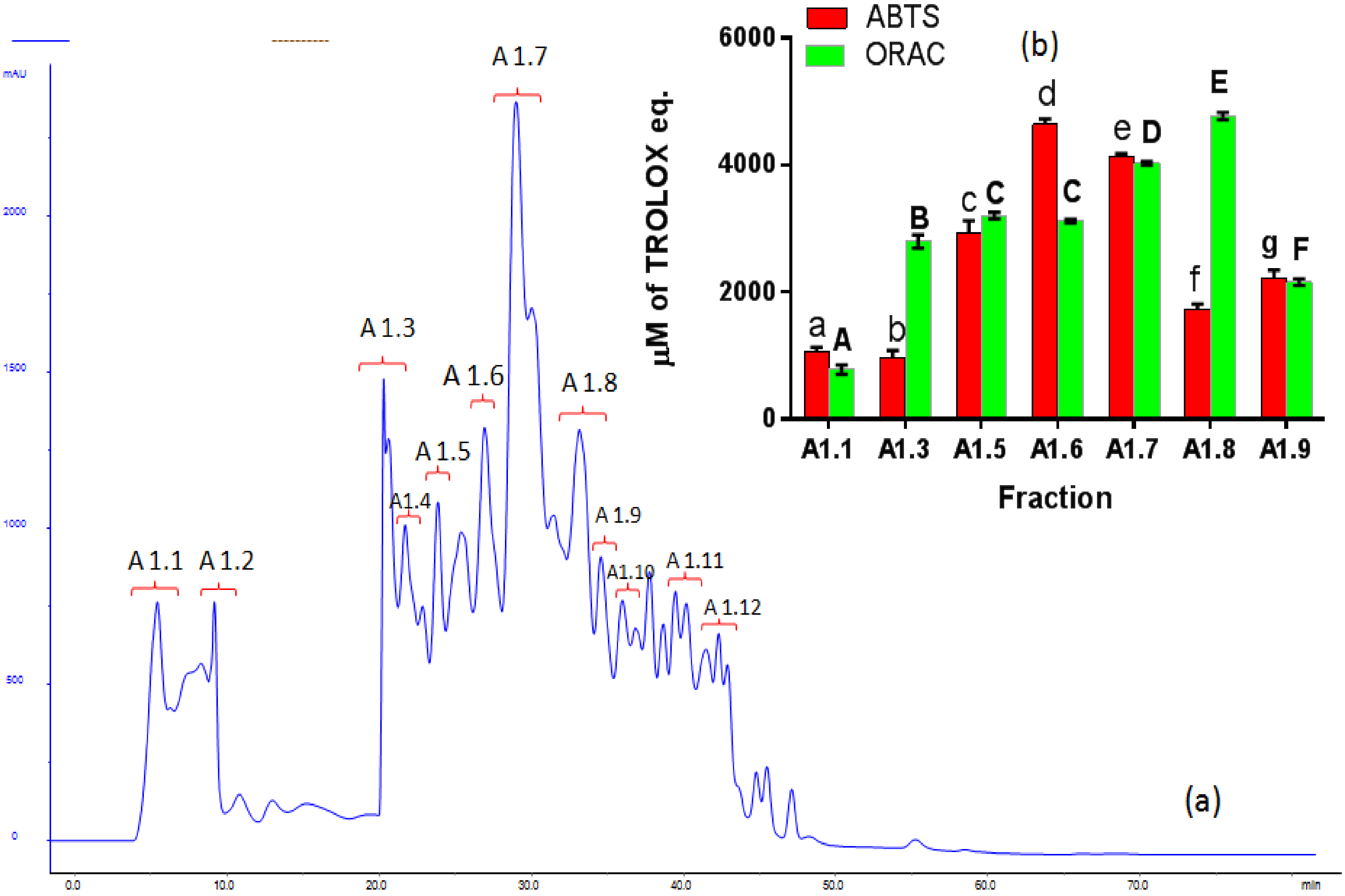
Zommara M, Tachibana N, Sakono M, Suzuki Y, Odo T, Hashiba H and Imaizami K. (1996). Whey from cultured skim milk decrease serumcholesterol and increase antioxidant enzymes in liver and red blood cells in rats. Nutritional Research 16(2): 293-302.

ANNEXTURE I



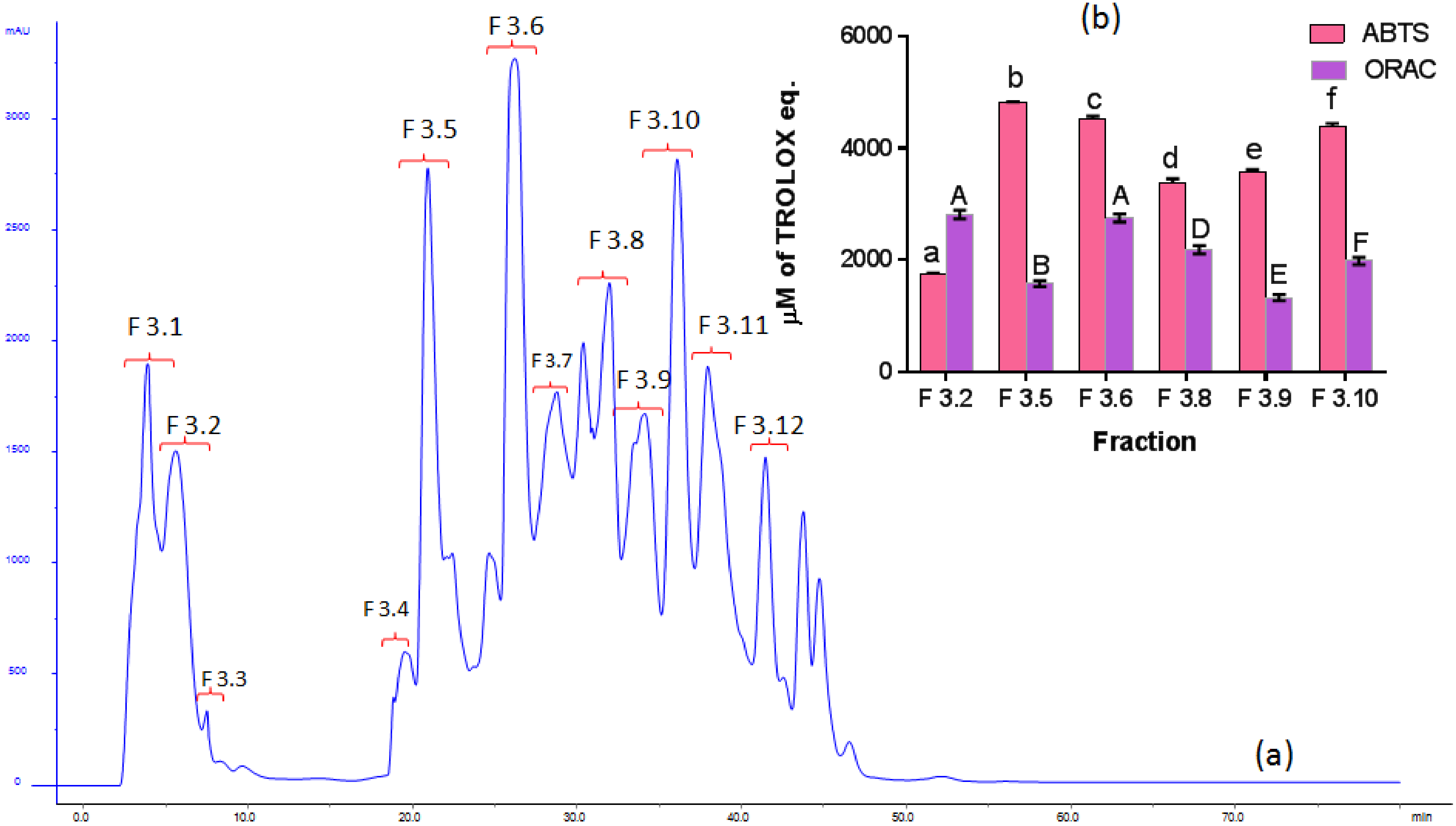
A) RP-HPLC chromatogram (214 nm) Of CH 1 (3kDa) hydrolysate. b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

ANNEXTURE II



A) RP-HPLC chromatogram (214 nm) Of CH 2 (1kDa) hydrolysate. b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

ANNEXTURE III

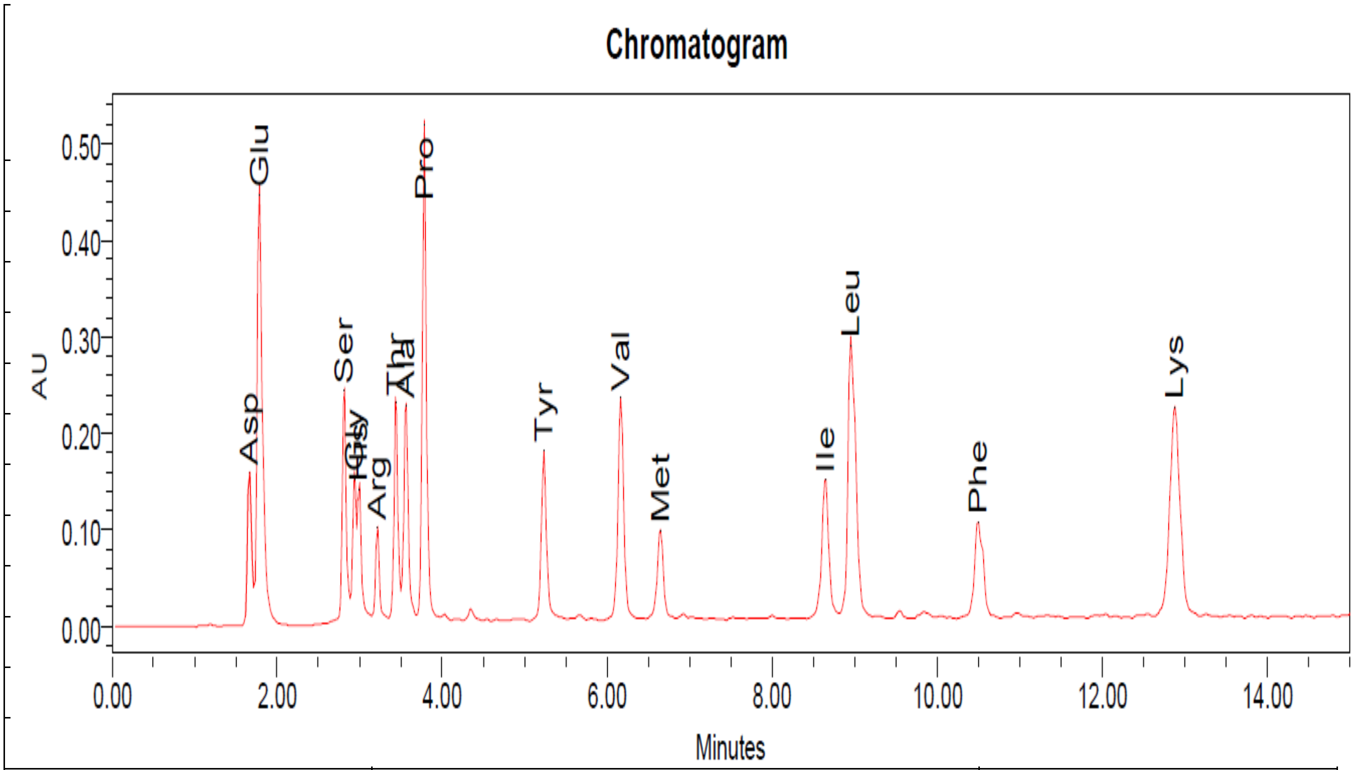


A) RP-HPLC chromatogram (214 nm) Of CH 3 (3kDa) hydrolysate. b) Insert graph represents the antioxidant activity of corresponding fractions. Result are mean values ± SD. Error bars are SD from 3 replicate. Different superscripts above bars indicate significant difference between fractions ($p < 0.05$)

	Peak no	ABTS (TEAC µM)	ABTS (TEAC µM/mg peptides)	ORAC (TEAC µM)	ORAC (TEAC µM/mg peptides)	LOWERY (mg/ml)
CH1 3 kDa	AF-3.1	1216.12	1558.12	2050.52	2627.18	0.78
	AF-3.2	1675.86	768.21	1363.20	624.89	2.18
	AF-3.3	1250.06	1503.37	1424.58	1713.26	0.83
	AF-3.4	3776.10	1705.94	5290.41	2390.06	2.21
	AF-3.5	4417.77	1304.71	3460.21	1021.91	3.38
	AF-3.6	5285.97	1491.73	3829.81	1080.79	3.54
	AF-3.7	5184.94	1516.06	797.15	233.08	3.42
	AF-3.8	2553.53	4085.64	1283.19	2053.09	0.62
	AF-3.9	3689.68	3235.13	360.64	316.20	1.14
	AF-3.10	1870.81	5182.30	514.33	1424.73	0.36
	AF-3.11	1092.60	5842.76	1595.87	8534.06	0.18
	AF-3.12	564.97	2597.57	819.71	3768.78	0.21
	AF-3.13	1216.12	855.17	2050.52	1255.18	0.25
CH 2 1kDa	A-1.1	1056.37	6837.32	700.90	4536.58	0.15
	A-1.2	470.00	550.03	1039.16	1216.10	0.85
	A-1.3	951.93	10636.06	2687.37	30026.51	0.09
	A-1.4	2547.20	5896.30	2568.40	5945.38	0.43
	A-1.5	2917.52	3289.20	3153.28	3554.99	0.89
	A-1.6	4644.34	4290.38	3148.53	2908.57	1.08
	A-1.7	4128.13	3177.93	4059.72	3125.27	1.30
	A-1.8	1726.05	1426.48	4829.88	3991.64	1.21
	A-1.9	2207.58	5824.75	2104.24	5552.07	0.38
	A-1.10	1642.34	3929.03	1014.70	2427.51	0.42
	A-1.11	1633.17	10815.68	657.59	4354.91	0.15
	A-1.12	1794.61	11503.91	851.49	5458.26	0.16
	A-1.13	2146.99	6870.36	1075.98	3443.15	0.31
	A-1.14	875.79	2090.20	1584.20	3780.90	0.42
	F-1.12	1835.30	13747.54	716.91	5370.11	0.13
CH 3 3 kDa	F-3.1	984.07	606.89	1472.02	907.81	1.62
	F-3.2	1730.32	817.34	2737.40	1293.05	2.11
	F-3.3	3051.55	1235.44	4238.16	1715.85	2.47
	F-3.4	2455.26	1147.31	2737.40	1279.15	2.14
	F-3.5	4827.40	1373.37	1527.03	434.43	3.51
	F-3.6	4480.92	1280.26	2821.15	806.04	2.5
	F-3.7	1432.38	1063.77	3722.67	2764.70	1.34
	F-3.8	3326.61	1113.88	2202.63	737.52	2.98
	F-3.9	3619.43	1151.76	1270.59	404.32	3.14
	F-3.10	4441.45	1645.28	1919.32	710.99	2.69
	F-3.11	1931.19	1158.13	1082.48	649.16	1.66
	F-3.12	924.09	783.45	616.49	522.66	1.17

Antioxidant activity of hydrolysates peak fractions separated by RP-HPLC

ANNEXTURE V



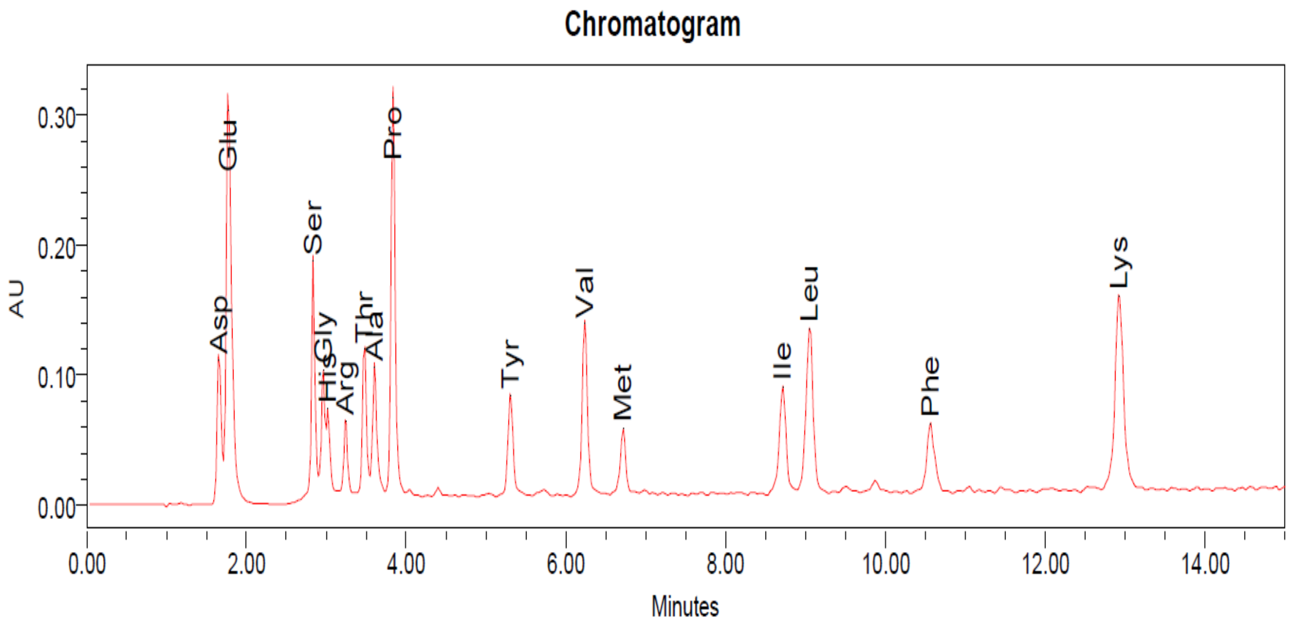
Ile	5.4	**5.6**
Leu	8.6	**11.8**
Phe	4.1	**4.5**
Lys	6.8	6.2

Amino acid composition of casein hydrolysate

Note: Asparagine, glutamine are deamidated and converted to aspartic acid and glutamic acid respectively .

Tryptophan is not detected as it is completely destroyed in acid hydrolysis

Chromatogram of Amino acid profiling CH 1 (1 kDa) hydrolysate



Chromatogram of Amino acid profiling CH 1 (10 kDa) hydrolysate

ANNEXTURE VI

Sensory evaluation score card for whey beverage enriched with buffalo casein hydrolysate

Kindly evaluate the given sample of whey beverage for following quality attributes according to 9 point hedonic scale:

Like Extremely	9
Like Very Much	8
Like Moderately	7
Like Slightly	6
Neither Like nor Dislike	5
Dislike Slightly	4
Dislike Moderately	3
Dislike Very Much	2
Dislike Extremely	1

Attributes	**Sample Code**	
	1	**2**
Flavour		
Consistency/ Viscosity		
Appearance		
Product Acidity		

Remarks, if any --
-

Date:

Signature:

Name: