ANTIOXIDANT EFFECTS OF CASEINOPHOSPHOPEPTIDES (CPP) AND ASSOCIATED CONJUGATES IN CHEMICAL MODELS AND CELL CULTURE SYSTEMS IN VITRO.

By

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ABSTRACT

Caseinophosphopeptides (CPP) derived from tryptic digests of bovine milk casein were evaluated for potential antioxidant activity. Two different commercially available (Meiji Seika Kaisha Ltd. Japan) CPP fractions, CPP-I and III, were shown to interact with ferrous iron, ferric iron and calcium ions. CPP-I and III exhibited significant (p<0.01) inhibition of both site-specific and non site-specific degradation of deoxyribose in a Fenton reaction oxidation test and protected liposomes from oxidation induced by ferrous iron and free radical AAPH. CPP-I was more effective (p<0.01) at quenching ABTS radicals than CPP-III. Laboratory synthesized CPP and CPP-saccharide conjugates were also analyzed for antioxidant activity. Different preparation techniques yield CPP of different calcium concentrations and peptide sizes. It appears that all CPP peptides carry the required polar, acidic Ser-P-Ser-P-Ser-P-Glu-Glu domain for ideal conformation for metal binding. CPP III was shown to be the most effective (p<0.01) at binding iron and lowering deoxyribose oxidation, while CPP-II was more effective (p<0.01) at sequestering ABTS radicals. All of the CPP preparations were equally effective (p>0.05) at quenching peroxyl radicals in the liposome peroxidation model. Conjugation of CPP with polysaccharide galactomannan and oligosaccharide xyloglucan did not enhance the bioactivity of CPP as predicted. Hence, both commercially available and laboratory synthesized CPP can act as a primary and also a secondary antioxidant by displaying both metal sequestering and free radical quenching activity in aqueous and emulsion models. Using a reduced serum culture system, laboratory synthesized CPP-III was also shown to be relatively non-toxic to intestinal cells and to successfully protect human intestinal embryonic Int-407 and colon carcinoma Caco-2 cells from ferrous
sulphate-induced cytotoxicity. With a selective affinity to sequester free metal ions, such as iron, and a low toxicity in intestinal cells, bioactive casein derived peptides can act as a potential antioxidant co-nutrient that serves to remove pro-oxidative metal catalysts and free radicals from oxidizable lipids in labile food systems or biomembrane lipid bilayer systems in vivo, especially at the site of the intestine.
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CHAPTER I: LITERATURE REVIEW

1.1 Introduction

Bovine milk and milk formula provide necessary nutritive elements, immunological agents and biologically active substances for the development of calves and human neonates (Clare and Swaisgood, 2000). Milk proteins, consisting of 30-35g per liter of milk, are also versatile food ingredients that have functional and nutritional properties (Shah, 2000). In addition to being a good resource of high-quality proteins, milk proteins are also physiologically important by providing a range of bioactive peptides upon digestive hydrolysis (Fiat et al., 1993). These peptides have diversified functions ranging from antihypertension, immunomodulation, metal binding, opiate, antithrombotic and antibacterial activity (Schanbacher et al., 1997; Schanbacher et al., 1998; Meisel, 1998; Meisel and Schlimme, 1990; Schlimme et al., 1989).

Among these peptides, caseinophosphopeptides (CPP), which are derived from the tryptic hydrolysis of casein and possessing an unique metal binding activity (Scholz-Ahrens and Schrezenmeir, 2000), warrant further attention. CPP has a high concentration of phosphorylated serine residues in close proximity on the peptide chain. A dense population of phosphate groups creates a polar, acidic domain that is electrostatically ideal for binding to divalent metals such as calcium, zinc, copper, manganese and iron (Fiat et al., 1993).

In milk, the principal cation bound to the phosphate groups is calcium. Extensive research on CPP has focused mainly on the role of enhancing solubility and hence bioavailability of calcium during milk or casein meal digestion (Brommage et al., 1991; Gerber and Jost, 1986; Lee et al., 1980; Li et al., 1989; Mykkänen and Wasserman, 1980;
Phosphorylated casein hydrolysis products effectively increased bone calcification in rachitic infants (Mellander, 1950) and inhibited precipitation of calcium phosphate in vitro (Reeves and Latour, 1958). Since the distal intestine is the location where the passive transport of calcium is dominant, an enhanced solubility would indirectly lead to greater calcium absorption from the intestinal lumen.

Being a natural digestion product of milk (Chabance et al., 1998; Naito et al., 1974; Hirayama et al., 1992a), and resistant to further proteolytic breakdown (Kasai et al., 1992; Kasai et al., 1995), CPP carries a great potential as an in vivo bioactive peptide, especially in the distal sections of the small intestine. This affinity to form organophosphate salts with calcium and other trace elements has founded new interesting applications for CPP as dietary supplements (Steijins, 1998), antihypertension agents (FitzGerald, 1998), immunostimulants (Hata et al., 1999; Otani et al., 2000) and also anticariogenic toothpaste and mouthwash (Reynolds, 1998). CPP may also potentially aid in protecting food- or bio-systems from oxidative damage by removing transition metals responsible for catalyzing lipid peroxidation.

1.2 Milk Caseins

Milk protein fractions in bovine milk include α-lactalbumin, β-lactoglobulin, caseinates, immunoglobulins, lactoferrin, proteose-peptone fractions and minor whey proteins such as transferrin and serum albumin (Clare and Swaisgood, 2000). Thus, milk proteins are mainly classified into two classes, namely caseins and whey proteins (Fiat et al., 1993). Bovine caseins are highly specific proteins synthesized in the bovine mammary gland, constitute around 78-80% of total milk proteins and are the main source
of amino acids, calcium and inorganic phosphate to the neonate (Schlimme and Meisel, 1995).

Milk caseinates associate as highly aggregated micelles that are characterized by high content of proline and ester-bound phosphate, but a low content of sulphur amino acids especially of cystine and low solubility at pH 4-5 (Hambraeus, 1985). Bovine casein consists of four gene products with substantially different properties: $\alpha_{s1}$, $\alpha_{s2}$, $\beta$ and $\kappa$, which represent approximately 38%, 10%, 36% and 12% of whole casein respectively (Fox, 2001). Caseins are mostly phosphorylated and are highly diversified in the number of phosphate groups attached to the protein, depending on the gene product. Phosphate contents range from containing only 1 mole of phosphate per mole protein for $\kappa$-casein, to 5, 8 and 13 phosphate groups respectively for $\beta$-casein, $\alpha_{s1}$-casein and $\alpha_{s2}$-casein. Casein phosphate groups are esterified as serine monoesters (Figure 1), usually highly anionic clusters that bind bivalent cations such as calcium effectively (Fox, 2001). With a complex network of calcium phosphate bridges, bovine caseins are found to contain a content of calcium and inorganic phosphate in milk, which is far higher than would be expected from the physico-chemical solubility of calcium phosphate in milk (Hambraeus, 1985).

Casein micelles are consisted of over a million submicelles enriched with $\kappa$-casein on the surface and $\alpha_{s1}$-, $\alpha_{s2}$- and $\beta$-caseins in the core, where the submicelles are linked through microcrystals of calcium phosphate and perhaps hydrophobic and hydrogen bonds (Fox, 2001). At a concentration over 6 mM at 30°C, excess calcium
binding leads to a charged neutralization of the micelle and precipitation of $\alpha_{s1}$-, $\alpha_{s2}$- and $\beta$-caseins, while $\kappa$-caseins are not affected (Fox, 2001). Precipitation and crosslinking of micelles leads to curd formation in the neonate stomach, allowing a slower release of nutrients over long time periods (Swaisgood, 1989). All caseins especially $\beta$-casein have a high content of proline, preventing the formation of secondary structures ($\alpha$ helices and $\beta$ sheets/turns), and thus allowing caseins to exist as flexible structures (Fox, 2002). Due to a flexible structure, individual caseins are especially stable to denaturing agents such as heat or urea, but are more susceptible to proteolysis than typical globular proteins through enhanced penetration by exo- and eno-peptidases (Swaisgood, 1989). A greater digestibility of casein can be associated with enhanced bioavailability of other nutrients such as trace elements, minerals and several vitamins that are bound to the protein. Thus, binding, or lack thereof, and the strength of association become the main determinants of bioavailability of the nutrient at the intestinal mucosa site (Lönnerdal, 1989).

Following curding of protein in the stomach, caseins undergo extensive enzymatic hydrolysis upon entry into the small intestine and can be recovered in the jejunum mainly in the form of degraded peptides (Mahé et al., 1996). Peptides released from gastric digestion of dietary casein stimulate release of cholecystokinin (CCK) (Beucher et al., 1994) or opioid peptides (Daniel et al., 1990), slowing gastric emptying and stimulating exocrine pancreatic secretions (Mahé et al., 1996). Gastric emptying rate and gastrointestinal transit time were shown to be significantly longer with feeding of casein protein as compared to whey protein in rats, calves and humans (Daniel et al., 1990; Yvon and Pelissier, 1987; Mahé et al., 1995). Approximately half of the milk nitrogen is absorbed only between the stomach and the proximal jejunum (42%) and half (53%)
between the proximal jejunum and the terminal ileum (Mahé et al., 1992). This indicates that the lower part of the intestine is also necessary for the completion of the casein digestion. Through an extended absorption period, small- (i.e. di- and tri-peptides) and medium-sized (10-51 amino acids) peptides are better absorbed intact through the intestine and will potentially produce biological effects at the tissue levels (Roberts et al., 1999).

1.3 Casein-derived bioactive peptides

Even though neither casein nor individual casein fractions have an established physiological role, peptides derived from casein have been shown to possess various bioactive properties (Shah, 2000). Bioactive peptides are peptides that are usually inactive within the protein sequence but become active only upon release during digestive processes (Meisel, 1998). Bioactive peptides obtained from enzymatic hydrolysis in vitro, or from digestion products in vivo, have a natural resistance to further enzymatic hydrolysis because of a high content of organically bound phosphate groups and proline residues within the peptides (Meisel and Schlimme, 1990).

Sites of action for bioactive peptides are dependent on the specific sites to which they are exposed after release by proteolysis of the native protein (Schanbacher et al. 1998). Even nutritionally insignificant amounts of liberated peptides can be adequate to exert physiological effects (Meisel and Schlimme, 1990). Effects may be localized in the gastrointestinal tract after mucosal absorption. Alternatively, effects may be systemic, taking place in peripheral organs subsequent to translocation of peptides across the gut epithelium and entry as intact peptides into the blood stream (Meisel and Schlimme, 1990).
Bioactive peptides derived from milk caseins directly influence numerous biological processes evoking behavioral, gastrointestinal, hormonal, immunological, neurological, vasoregulatory and nutritional responses (Clare and Swaisgood, 2000). Specific functional roles include opioid agonistic, opioid antagonistic, angiotensin-converting enzyme (ACE) inhibitory, immunomodulatory, antimicrobial, antithrombotic and mineral binding effects. Casein-derived peptides include casomorphins (opioid agonists), casoxins (opioid antagonists), casokinins (antihypertensive peptides), casoplatelins (antithrombotic peptides), and caseinophosphopeptides (mineral carriers) (Meisel and Schlimme, 1990). Reactions associated with each bioactivity have been well characterized and reviewed (Fiat et al., 1993; Schanbacher et al., 1998; Meisel, 1997a; Meisel, 1997b; Meisel, 1998; Shah, 2000; Meisel and Schlimme, 1990; Kitts and Kwong, 2002). Two of these peptides, β casomorphin and caseinophosphopeptide, have been identified as digestion products found in vivo (Meisel and Frister, 1989; Chabance et al., 1998; Naito et al., 1972; Hirayama et al., 1992a).

1.4 Caseinophosphopeptides (CPP)

Mineral binding bioactive peptides, caseinophosphopeptides (CPP), are classified according to the fact that they possess a dense population of phosphoseryl groups in close vicinity. The function of these peptides in bovine milk is to stabilize the emulsion by creating thermodynamically stable casein micelles that have an open sponge-like assembly for easy access of digestion enzymes (West, 1986) and can be supersaturated with protein, calcium and phosphate (Steijns, 2002). Tryptic hydrolysates of bovine casein micelles were shown to contain mineral rich peptide fractions that contain 72% of the colloidal calcium, 49% of the inorganic phosphate, 27% of the nitrogen and 82% of
the micellar phosphoseryl residues found originally in the native micelle (Gagnaire et al., 1996). Distribution of phosphoserine moieties varies in caseins (αs, β and κ) and the extent of phosphorylation directly affects CPP mineral chelating affinity with the order of αs2 > αs1 > β > κ (Kitts, 1994). β-casein phosphopeptides, with molecular weight of about 3.0 kDa, are sufficient in micromolar concentration to bind and solubilize up to 40 fold molar excess of phosphate and calcium (Naito and Suzuki, 1974).

Caseins are phosphorylated post-translationally as monoesters and clusters by specific protein kinases (Kinsella, 1989), following formation in the mammary gland. A particular anionic triplet of amino acid residues may act as a signal to kinase to indicate which residues to phosphorylate. Serine or threonine residues are converted to phosphorus monoesters that are located 2 amino acids on the N-terminal of negatively charged residues such as glutamic or aspartic acid (Ribadeau-Dumas, 1989; West, 1986).

This anionic triplet (SerP-SerP-SerP-Glu-Glu) becomes a distinctive feature for all of the major fractions of phosphopeptides characterized, whether the CPP are of in vitro or in vivo origin. The most common CPP derived from in vitro tryptic digests of whole bovine casein include β-casein-4P (1-25), αs1-casein-5P (59-79), αs2-casein-4P (1-21), and αs2-casein-4P (46-70). A wide variety of phosphopeptide sequences have been characterized using different analysis techniques or choice of hydrolytic enzymes (see Table 1, 2 and 3). Besides trypsin, protease preparations of bacterial, fungal, plant and animal origin can also be used to yield CPP (McDonagh and FitzGerald, 1998). The functional sequences of CPP required for mineral affinity have been identified to be αs1-casein (45-55), αs1-casein (56-74) αs2-casein (55-75) and β-casein (13-35) in which the anionic triplet is embedded (Kelly and McDonagh, 2000).
Table 1. Reported sequences of CPP derived from β-casein.

<table>
<thead>
<tr>
<th>Caseinate</th>
<th>Mol Wt</th>
<th>Peptide</th>
<th>Enzyme</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>3.1kDa</td>
<td>(f1-25)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Manson and Annan, 1971; Ono et al., 1994; Gagnaire et al., 1996; Cross et al., 2001; Kitts et al., 1991; Berrocal et al., 1989; Schlimme and Meisel, 1995</td>
</tr>
<tr>
<td>β</td>
<td>(f1-25)4P</td>
<td>Pepsin &amp; Trypsin</td>
<td>In vitro</td>
<td>Ono et al., 1998</td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>3.5 kDa</td>
<td>(f1-28)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Ono et al., 1994; Hirayama et al., 1992b; Gerber and Jost, 1986; Gagnaire et al., 1996</td>
</tr>
<tr>
<td>β</td>
<td>(f2-28)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Juillerat et al., 1989</td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>3.6 kDa</td>
<td>(f1-29)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Gagnaire et al., 1996</td>
</tr>
<tr>
<td>β</td>
<td>3.0kDa</td>
<td>(f2-25)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Ono et al., 1994; Reynolds et al., 1994; Kopra et al., 1992</td>
</tr>
<tr>
<td>β</td>
<td>(f7-24)4P</td>
<td>Gastric and Intestinal enzymes</td>
<td>In vivo (rats' ileum)</td>
<td>Hirayama et al., 1992a</td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>(f7-24)4P</td>
<td>Pancreatin</td>
<td>In vitro</td>
<td>Adamson and Reynolds, 1995; Feng et al., 1997</td>
<td></td>
</tr>
</tbody>
</table>

1Reynolds et al., 1994
2Ono et al., 1998
3Gagnaire et al., 1996
4Gerber and Jost, 1986
<table>
<thead>
<tr>
<th>Caseinate</th>
<th>Mol Wt</th>
<th>Peptide</th>
<th>Enzyme</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{s1}$</td>
<td>5.6 kDa$^3$</td>
<td>(f35-79)8P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Gagnaire et al., 1996</td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>5.5 kDa$^3$</td>
<td>(f37-79)7P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Gagnaire et al., 1996</td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>4.6 kDa$^{1,3}$</td>
<td>(f43-79)7P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Reynolds et al., 1994; Gagnaire et al., 1996</td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f46-70)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Ono et al., 1994</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f59-78)5P</td>
<td>Pancreatin</td>
<td>In vitro</td>
<td>Adamson and Reynolds, 1995</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>2.7 kDa$^{1,2,4}$</td>
<td>(f59-79)5P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Ono et al., 1994; Berrocal et al., 1989; Juillerat et al., 1989; Huq et al., 1995; Reynolds et al., 1994; Gerber and Jost, 1986</td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f59-79)5P</td>
<td>Gastric and Intestinal enzymes</td>
<td>In vivo (rats' ileum)</td>
<td>Brommage et al., 1991</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f59-79)5P</td>
<td>Pepsin &amp; Trypsin</td>
<td>In vitro</td>
<td>Ono et al., 1998</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f61-74)</td>
<td>Gastric and Intestinal enzymes</td>
<td>In vivo (rats' ileum)</td>
<td>Hirayama et al., 1992a</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f61-78)5P</td>
<td>Pancreatin</td>
<td>In vitro</td>
<td>Adamson and Reynolds, 1995; Feng et al., 1997</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f62-69)</td>
<td>Gastric and Intestinal enzymes</td>
<td>In vivo (rats' feces)</td>
<td>Kasai et al., 1992</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f64-73)</td>
<td>Gastric and Intestinal enzymes</td>
<td>In vivo (rats' feces)</td>
<td>Kasai et al., 1992</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f59-64)5P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Gerber and Jost, 1986</td>
<td></td>
</tr>
<tr>
<td>$\alpha_{s1}$</td>
<td>(f66-74)3P</td>
<td>Gastric and Intestinal enzymes</td>
<td>In vivo (minipigs' intestine)</td>
<td>Meisel and Frister, 1988; Meisel and Frister, 1989a; Meisel and Frister, 1989b</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Reported sequences of CPP derived from αs2-casein.

<table>
<thead>
<tr>
<th>Caseinate</th>
<th>Mol Wt</th>
<th>Peptide</th>
<th>Enzyme</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>αs2</td>
<td>2.7 kDa1,3</td>
<td>(f1-21)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Kitts et al., 1991; Reynolds et al., 1994; Gagnaire et al., 1996</td>
</tr>
<tr>
<td>αs2</td>
<td>3.1 kDa3</td>
<td>(f1-24)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Gagnaire et al., 1996</td>
</tr>
<tr>
<td>αs2</td>
<td>(f1-32)</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Hirayama et al., 1992b</td>
<td></td>
</tr>
<tr>
<td>αs2</td>
<td>2.6 kDa1</td>
<td>(f2-21)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Ellegård et al., 1999; Reynolds et al., 1994; Kopra et al., 1992</td>
</tr>
<tr>
<td>αs2</td>
<td>3.0 kDa1,4</td>
<td>(f46-70)4P</td>
<td>Trypsin</td>
<td>In vitro</td>
<td>Gerber and Jost, 1986; Ellegård et al., 1999; Juillerat et al., 1989; Reynolds et al., 1994; Kopra et al., 1992</td>
</tr>
</tbody>
</table>

1Reynolds et al., 1994
2Ono et al., 1998
3Gagnaire et al., 1996
4Gerber and Jost, 1986

Phosphopeptides prepared through different in vitro or in vivo methods were shown to share general amino acid profiles, analogous chemical natures and physiological functions (Naito and Suzuki, 1974). Several CPP derived from β-casein were shown to exist in the stomach and duodenum of adult humans after milk digestion (Chabance et al., 1998) and in intestinal digests of mini-pigs (Meisel and Frister, 1988; Hirayama et al., 1992a) and rats (Naito et al., 1972) hours following ingestion of casein diets. Because of the high concentration of negative charges on phosphopeptides, CPP becomes relatively resistant to further proteolysis (Meisel and Schlimme, 1990) and remnants of it can be found in feces of casein-fed rats, suggesting at least a part of the CPP formed in the small intestine was not hydrolyzed in the digestive tract but excreted (Kasai et al., 1992). Therefore, CPP in small intestine not only resists hydrolysis by digestive enzymes, but may even escape hydrolysis by enteric bacteria in the distal
intestine (Kasai et al., 1992). CPP found in the cecum and colon contents of rats fed diets containing purified CPP was not distinguishable from that of rats fed casein that contained a comparable amount of phosphoserines (Kasai et al., 1995).

1.5 Properties of CPP

Fractions of phosphopeptides isolated from casein hydrolysates were found to sequester calcium phosphate at neutral to alkaline pH of 7 - 10.5 (Reeves and Latour, 1958; Swaisgood, 1982) and inhibit amorphous calcium phosphate precipitation (Sato et al., 1991; Berrocal et al., 1989; Ellegård et al., 1999). By linking the seryl phosphate groups to calcium phosphate in spherical, nanometer-sized particles, CPP, specifically the β-CPP (1-25), is capable of stabilizing amorphous dicalcium phosphate. This activity will arrest the formation of hydroxyapatite crystals at an early stage (Holt et al., 1998). Phosphoseryl groups undergo different phases in binding kinetics upon increasing concentrations of inorganic calcium. At low concentrations below 1 mM calcium, phosphoseryl residues exhibit primarily exothermic binding to calcium ions. As concentration increases to 1-3 mM, a second exothermic binding phase is followed by a progressively more endothermic reaction involving the phosphoseryl residues binding to carboxylate residues, resulting in increased self-association. Above a 3 mM calcium threshold concentration, the reaction becomes very endothermic and phosphoserine residues interact predominantly with carboxylate residues. Gradually, persistent binding to carboxylate residues minimizes inter-molecular electrostatic repulsion and hydrophobic interaction of the hydrophobic domains leading to the formation of large aggregates and eventual precipitation between 5-6 mM of calcium. (Swaisgood, 1993).
Inhibition of calcium phosphate precipitation can be achieved at a concentration of 10mg/L of CPP or higher at a pH 6.5 with complete emulsion stability maintained with 100mg/L (Berrocal et al., 1989). Strength of calcium sequestering is in turn highly correlated to the extent of phosphorylation in different fractions of CPP and conditions (pH, ionic strength, temperature and enzyme selection) under which peptide-metal interactions occur. The apparent calcium binding constant, $K_{\text{app}}$ (l/mol), reported by Meisel (1997) was 328 and the lowest for $\alpha_{s1}$-casein (f43-48) that consisted of 2 phosphate groups as compared to 629 for $\beta$-casein (f1-25) and 841 for $\alpha_{s1}$-casein (f59-79) with 4 and 5 phosphate groups respectively. Optimal binding of CPP to $^{45}$Ca occurred at pH 8.0 with a binding capacity to $^{45}$Ca that was determined to be 50% at 8 µg of CPP and 100% at 25 µg CPP (Nagasawa et al., 1991).

In order to perform CPP bio-sequestering activity, phosphorylated serine clusters have a critical role to not only create an anionic environment for effective mineral binding, but also to maintain the stability of peptides towards further proteolytic hydrolysis. Peptides, in which the O-phosphorylserine groups are dephosphorylated, have been shown to be more susceptible to enzymatic hydrolysis than in cases where organic phosphate groups were still bound as monoesters, diesters or triesters (Mellander, 1963). Phosphopeptide $\alpha_{s1}$-casein (59-79) obtained most abundantly in the ileum of rats following casein diets was shown to be more slowly dephosphorylated than other peptide fragments in the intestinal lumen, thereby implicating a high in vivo stability (Brommage et al., 1991). When completely dephosphorylated, CPP loses the affinity for divalent metals (Nagasawa et al., 1991) and calcium solubilization properties (Gerber and Jost, 1986). Dephosphorylated CPP was ineffective (Berrocal et al., 1989) even at
concentrations higher than 100mg/L at preventing calcium phosphate precipitation, indicating that the binding sites for calcium and functional units of CPP bioactivity are essentially the phosphoserine residues.

Cation binding affinity (pK) of the phosphoserines can be greatly affected by an alteration in pH, temperature, ionic strength and the presence of calcium. Baumy and colleagues (1989) identified a maximum calcium binding (pK 6.57-7.10) taking place on residue 17,18 and 19 on β-casein (1-25). In accordance to the calcium binding phases of phosphoserine residues, protonation of the phosphate groups at a low pH environment hinders calcium binding and calcium phosphate solubilization, whereas competitive binding of calcium by carboxylate groups from residual dissolved carbon dioxide under alkaline conditions also inhibits binding affinity (Meisel and Olieman, 1998). Increasing temperature from 20°C to 40°C will catalyze endothermic binding of calcium, thereby greatly enhancing CPP-metal association (Swaisgood, 1993). At greater ionic strength, the presence of other cations such as sodium, with very similar binding kinetics as calcium, competes with calcium to bind phosphoseryl residues, thus lowering the calcium-binding ability of CPP. The presence of calcium is also inhibitory to calcium chelation, shown by a linear decrease in binding between 1 and 4 moles of calcium per mole of phosphopeptide. However, once the four sites on β-casein (1-25) are saturated, any excess calcium has a minor effect on binding affinity (Baumy et al., 1989).

Binding of one calcium atom to ionized phosphoserine can exert an inductive effect by attracting electrons to decrease the strength between the oxygen and hydrogen bond of the second acidic group. This observation indicates that not only is the functional binding sequence (SerP-SerP-SerP-Glu-Glu) of CPP important to mineral-
peptide interaction, but the neighboring residues are also equally essential for effective binding (West, 1986). Amino acid residues upstream and downstream from this region may also participate in binding. Evidence showed that when the sequence that spanned this domain (SerP-SerP-SerP-Glu-Glu) was mimicked synthetically, calcium binding activity was lost (Meisel and Frister, 1989a; Shah, 1990). Synthetic peptides corresponding to the phosphoserine rich region of αs1-casein bind less calcium than the entire tryptic peptide (59-79), whereas the synthetic N-terminal (59-63) and the corresponding C-terminal (71-79) do not bind calcium at all (FitzGerald, 1998). Similarly when the sequence is cleaved at the glutamic acid residue, charge redistribution in the anionic cluster significantly lowers calcium-binding activity (Park and Allen, 1998).

Besides physical parameters that are characteristics to CPP bioactivity, the enzyme selection for CPP preparation is also a main contributing factor to its bioactivity. Caseinophosphopeptides prepared from a selection of bacterial, fungal, plant and animal proteolytic enzymes could bind 0.40-0.61 and solubilize 7.4-24.0 mg Ca\(^{2+}\)/mg CPP, as compared to traditional CPP prepared with trypsin that binds 0.60 and solubilizes 14.7 mg Ca\(^{2+}\)/mg CPP (McDonagh and FitzGerald, 1998). Peptides prepared by successive digestion treatment with pepsin (pH 4.0 stomach) and trypsin (pH 7.0 intestine) were shown to have a higher capacity to bind calcium. For example, these peptides were reported to chelate 248 mg Ca\(^{2+}\)/g peptide, as compared to tryptic peptides that bind 168 mg Ca\(^{2+}\)/g peptide (Ono et al., 1998). Using an innovative *in vitro* simulation of human digestion by stepwise addition of digestive enzymes and alteration of pH, CPP that was generated by porcine pancreatic trypsin, bioprotease N100L and porcine pancreatin did
have significantly different effects on the dialysability of $^{45}$Ca (Kennefick and Cashman, 2000).

The strength of calcium binding to peptide seems to be related primarily to the nature of peptide and preparation techniques. The stability of the metal-peptide complexes, on the other hand, is dependent on the conformational structure of the peptides and metals. Information on the actual conformation of the peptides is limited but past research based on circular dichroism and optical rotatory dispersion has shown that, when compared to other globular proteins, bovine $\alpha$- and $\beta$-caseins have unusually random, open and flexible structures with little secondary structures, particularly $\alpha$-helix (Swaisgood, 1993).

Divergence from the behavior of typical globular proteins may result from the acidic, highly solvated domain, which allows the caseins to approach near random coil properties. Bovine $\alpha$- and $\beta$-caseins are characterized by a high frequency of phosphoseryl residues and proline residues, having a distinct amphipathic nature with both polar and hydrophobic domains organized in a tertiary structure (Swaisgood, 1982). Predicted secondary structures around sites of phosphorylation in the Ca$^{2+}$-sensitive caseins often comprise of an $\alpha$-helix-loop-$\alpha$-helix motif with the sites of phosphorylation in the loop region (Holt et al., 1989). The motif consisting principally of phosphoserines is essential for binding calcium, but possible conformation changes in the backbone of CPP allows for residues remote from the phosphorylated motif to also interact with calcium (Cross et al., 2001).

Recent NMR studies showed that the majority of highly phosphorylated sites preferentially exist in regions likely to be $\beta$-turns or loops in the peptide structure. This
feature corresponds to different fractions of CPP. Characterization by NOE (nuclear Overhauser effect) connectivities and \(^1\)H-NMR showed that although different fractions of CPP share analogous sequence and functions, they have distinctly different conformations. The phosphoseryl motif in the peptide \(\alpha_{\text{S1}}\)-casein (59-79), segment Glu\(^61\)-SerP\(^67\), was implicated in a loop-type structure. This motif was followed by a Pro\(^73\)-Val\(^76\) in \(\beta\)-turn conformations (Huq \textit{et al.}, 1995), while the same motif in \(\beta\)-casein (1-25) SerP\(^17\)-Glu\(^20\) was embedded in \(\beta\) turns along with sequences Val\(^8\)-Glu\(^11\), Glu\(^21\)-Thr\(^24\) and Arg\(^1\)-Glu\(^4\); all of which are located in a loop structure (Cross \textit{et al.}, 2001).

1.6 CPP and Calcium

More than 50 years ago, peptides isolated directly from enzymatic hydrolysates of casein with characteristically high phosphorus content were found to form very soluble calcium complexes. These peptides, named casein phosphopeptides (CPP), were associated with enhancing calcium absorption and hence skeletal mineralization in rachitic infants (Mellander, 1950). Since the discovery of CPP, studies have consistently shown CPP to inhibit calcium phosphate precipitation \textit{in vitro} (Reeves and Latour, 1958) and thus enhance solubility and uptake of calcium \textit{in vivo} from the distal intestine during luminal digestion of casein-containing meals (Naito \textit{et al.}, 1972; Sato, 1986; Lee \textit{et al.}, 1992). CPP formed from digestive processes, yield approximately 5mg for 200g casein/kg diet (Lee \textit{et al.}, 1980) and can improve paracellular calcium absorption \textit{in situ} in the distal intestine of rats fed casein diets (Nagasawa \textit{et al.}, 1991) and soy diets supplemented with CPP (Yuan and Kitts, 1992). In addition to alkaline conditions of the intestine, CPP was also shown to function at a low pH, gastric environment, accelerating
calcium emptying and lowering insoluble calcium level in rats following consumption of calcium fortified milk (Tsuchita et al., 2001).

Once in the systemic circulation, CPP may have an intricate role in modulating calcium metabolism. CPP has been implicated in stimulating bone and cartilage calcification, increasing bone mass and calcium content (Lee et al., 1992) and reducing bone resorption (Ashida et al., 1996; Tsuchita et al., 1996). It was suggested that CPP has the ability to enhance bone mineralization by activating alkaline phosphatase (Matsui et al., 1994), enhancing renal tubular reabsorption of calcium and increasing serum osteocalcin (Tsuchita et al., 1995) and to lower bone resorption by lowering tartrate-resistant acid phosphatase activity, osteoclast differentiation and cell number (Matsui et al., 1994) and lowering urinary cAMP concentrations (Tsuchita et al., 1995). CPP significantly enhanced the accumulation of calcium in the tibia of chicks (Mykkänen and Wasserman, 1980) and significantly improved hypertrophic cartilage calcification in explanted embryonic rat bone rudiments at a concentration of 10 mg/100ml of culture medium (Gerber and Jost, 1986). These observations suggest that the inhibitory effect of CPP on amorphous calcium phosphate precipitation does not exclude local precipitation and hence mineralization in the cartilaginous matrix.

Approximately one third of the calcium in bovine milk is present in the serum phase as free Ca\(^{2+}\) or complexed by phosphate, while two thirds is partly incorporated in micellar calcium phosphate and partly bound to casein (Tsuchita et al., 2001). Intestinal calcium absorption is the sum of two processes; a saturable transfer mediated by hormone- and vitamin D-dependent active transport via calcium binding proteins in the proximal intestinal (i.e. duodenum and upper jejunum) and a non-saturable transfer that is
concentration-dependent, diffusional and predominantly paracellular in the lower intestine (Saito et al., 1998). In the case of intestinal absorption after a meal, the concentration of calcium in the lumen reaches between 3-4 mmol/L in rats and 7-8 mmol/L in humans (Bronner et al., 1987). Calcium flows through the apical membrane of enterocytes by active and passive mechanisms, are driven by high extracellular or intracellular concentration gradient and sustained by the calcium pump located at the basal membrane that pushes calcium into the capillary compartment (Ferraretto et al., 2001). At high intraluminal calcium levels, the proportion of calcium absorbed by diffusion becomes proportionally greater than by active transport (Saito et al., 1998).

When the relationship between CPP concentration and the corresponding level of serosal calcium in chicks is mapped on a graph, the occurrence of a non-linear plot suggests that perhaps 2 different types of transport processes are involved: a saturable absorption of calcium at low peptide concentrations and a non-saturable translocation of calcium stimulated by a high concentration of peptides. The authors postulated that the peptides are capable of acting at a minimum of two sites in the distal intestine, the site of uptake and the site of release of calcium from the epithelium membrane (Mykkänen and Wasserman, 1980). Evidence of the non-saturable transfer is shown by the independence of CPP enhanced calcium transport from vitamin D status in chicks and rats (Mykkänen and Wasserman, 1980; Kopra et al., 1992).

While some researchers have theorized that the physiological role of CPP on intestinal calcium absorption is only an indirect luminal inhibition of the precipitation of phosphate calcium salts (Li et al., 1989), and not a direct effect on the intestinal mucosal membrane (Erba et al., 2001), some are convinced that CPP has a much greater potency
as a bioactive peptide. In the presence of extracellular calcium, CPP was shown to induce a transient rise of free intracellular calcium ions in intestinal tumor cells HT-29 and Caco-2 (Ferraretto et al., 1999; Ferraretto et al., 2000). Since calcium flux generated by CPP lacks desensitization, CPP appears to aggregate in oligomers suitable for insertion into plasma membrane. It is possible that CPP does not influence membrane-bound receptors or ion channels, but acts rather as a calcium carrier for calcium-selective channels on the plasma membrane or becomes internalized as a calcium-peptide complex via endocytosis (Ferraretto et al., 2001).

While many of the studies have shown that CPP has a positive effect on calcium solubility and subsequent metabolism and utilization of calcium, there exist many discrepancies of findings among different studies. For example, it is understandable that CPP functions can be influenced by variation in experimental designs, preparation methods (i.e. *in vivo* or extrinsic), extent of interaction between CPP and calcium and also end measures used to determine calcium absorption.

Dietary calcium levels used in experimental design, particularly the calcium to CPP ratios, are important for determining the effect of CPP on calcium absorption in animal studies. Saito and colleagues (1997) have identified the minimum effective dose of CPP to enhance calcium absorption, under marginal dietary calcium levels (0-0.35%), to be 0.7g/kg or a weight ratio of CPP/Ca of 0.2 in rats fed CPP supplemented soy protein diets. Similarly, CPP boosted calcium balance and plasma calcium in rats fed isolated soy protein diets in a dose-dependent manner when the CPP/Ca ratios range between 0 to 0.35, reaching constant levels when CPP/Ca ratios are above 0.35 (Lee et al., 1992).
In contrast, CPP seems to be quite ineffective in enhancing calcium absorption and utilization at higher levels of calcium intake. Soy diets and casein diets supplemented with CPP (3%) had little effect on apparent calcium absorption and utilization in rats with an adequate (0.5%) calcium intake (Yuan and Kitts, 1992). At a level of 1.8 g/100g, CPP fed as a component of whey protein diet (Ca 0.7%) did not affect plasma calcium, apparent calcium absorption or retention and femur mineralization in vitamin D-replete and -deficient rats (Kopra et al., 1992). CPP at levels of 3.8 or 7.6 g/100g in whey protein diets (Ca 0.53%) also had no effect on fractional calcium absorption in normal female rats (Brommage et al., 1991). However, calcium absorption in the same female rats was elevated when 9-27% casein was supplemented in whey protein diets when total calcium intake was dropped to 0.2%. Inclusion of CPP in low (1.5%) calcium diets successfully improved utilization of calcium and recovered specific gravity of egg shells in laying hens, but at normal (3.4%) calcium diets, calcium requirements for eggshell formation were satisfied and CPP addition did not have any effects on egg shell parameters (Ashida et al., 1996). At an adequate dietary calcium intake level, luminal calcium concentration may already be quite high so that the contribution of CPP to enhancement of calcium may be very small or negligible (Saito et al., 1998). It appears important that when casein is added as supplementation to animal diets, the basal diet must also include other dietary factors such as carbohydrate and fatty acids in order for effective in vivo release of CPP and its subsequent mineral-binding bioactivity (Lee et al., 1983).

Besides variable dietary calcium levels in experimental designs, inconsistent CPP preparation methods and associated purity standards also contribute to the diversity of
conclusions from different studies. Crude preparation and demineralization of CPP have been shown to greatly lower the affinity to sequester calcium and hence exert an in vivo effect. Scholz-Ahrens and colleagues (1990) have attributed a lack of CPP effect on calcium content and bending moment of femurs from weanling piglets and on apparent absorption or retention of calcium in vitamin-D depleted rats to the low quality of phosphopeptides in casein meals. Decalcification of CPP prior to supplementation has proven to have no effect and even negative effect on calcium absorption in rats. At a concentration of 100g “decalcified” CPP/kg in a single meal, the absorption of calcium was not affected but was significantly reduced by inclusion of 200, 350 and 500g CPP/kg (Bennett et al., 2000).

The affinity of CPP, either ingested or formed from digestive processes, to exert a function on calcium at any dietary calcium levels is dependent on the extent of interaction between CPP and the mineral. The efficiency of CPP in calcium solubilization under in vitro conditions may be compromised in the presence of phosphatases, inhibitory agents and other dietary factors, found in human and animal models. It was shown that a CPP-Ca complex could not be absorbed and remained on the mucosal side of the ileum when extracted rat intestinal tissues are mounted in an Ussing chamber (Li et al., 1989). This observation is however not valid for extrapolation to in vivo studies, since CPP and CPP-Ca complexes are capable of interacting with intestinal phosphatases, thus influencing subsequent utilization of the calcium that is released. Intestinal phosphatases were found to have the highest activity in the ileum of rats (Brommage et al., 1991), breaking calcium ions free from the CPP-Ca complex and releasing them for passive absorption.
CPP was also shown to interact with alkaline phosphatase to enhance bone mineralization and with tartrate-resistant acid phosphate to reduce bone resorption (Matusi et al., 1994).

The presence of inorganic phosphate or phytates in the diet will lead to the formation of insoluble calcium salts, significantly affecting the extent of CPP-calcium interaction and lowering the potency of CPP as mineral chelator. Any interference with the interaction between CPP and calcium from the digestion of other food ingredients will not only render CPP ineffective in enhancing calcium absorption, but also severely hinder calcium bioavailability. In the presence of inorganic phosphate, calcium transport measured using a perfused ileum was decreased by over 90% and adding CPP to the solution fraction minimized the inhibitory effect of phosphate on calcium absorption to around 46-60% in rats (Erba et al., 2001).

Using a perfused intestine technique, Hansen et al. (1996) showed a negative impact on calcium and zinc absorption in the presence of aqueous phytate solution. Although adding CPP did not completely overcome the effect of phytate, CPP was significantly better than bovine serum albumin, casein or whey proteins to inhibit the phytate effect on zinc and calcium absorption. CPP was also shown to improve calcium absorption in rat pups from oat-based and soy-based high phytate infant diets by 45 and 10%, respectively (Hansen et al., 1996). In humans, CPP was associated with an improvement of calcium and zinc absorption from phytate containing rice-based infant cereal by 26-27% and 24-32%, respectively, when the ratio of phytate to calcium or zinc was relatively low (phytate:Ca:Zn ratio (μmol) of 30:12:19.7). However, CPP did not affect calcium absorption from whole grain-based infant meals and bread meals (Hansen
et al., 1997a; Hansen et al., 1997b), when phytate to calcium or zinc ratios were much higher (phyate:Ca:Zn ratios (µmol) of 300:13.5:27.1 and 528:7.8:21.8).

Interestingly, not only is the extent of interaction between CPP and calcium influenced by other dietary factors, CPP may also be compatible with other bioactive compounds in the diet, thus enhancing calcium utilization in this way. While the isoflavone genistein was found to increase dry weight, calcium content, alkaline phosphatase activity and DNA content in femoral diaphyseal and metaphyseal tissue of young and elderly rats, supplementation of diets with CPP augmented the effect of genistein on calcium content and alkaline phosphatase activity of metaphyseal tissues in young and elderly rats in a synergistic manner (Ma and Yamaguchi, 2000).

In addition to dietary factors tested, variation in the measured effect of CPP on calcium absorption may also be associated with a wide diversity in end measure parameters used in calcium absorption studies. Many authors have persistently employed similar methods such as serum/urinary/fecal calcium (Kopra et al., 1992; Lee et al., 1992; Saito et al., 1997), ligated intestinal loop (Mykkänen and Wasserman, 1980; Lee et al., 1980; Kitts et al., 1992; Yuan and Kitts, 1991; Yuan and Kitts, 1992) and bone calcification parameters, such as femur calcium content and bending moment (Pointillart and Guégen, 1989; Scholz-Ahrens et al., 1990; Yuan and Kitts, 1994), to calculate apparent absorption of calcium. Apparent absorption, based on calcium ingested and excreted via urine or feces, may not be a sensitive method to assess bioavailability of calcium, because it is affected not only by exogenous calcium sources but also by endogenous calcium excretion in feces. It is possible that the stimulative action of CPP on calcium absorption is so slight that the changes in apparent absorption of calcium and
bone metabolism that is controlled primarily by hormones that maintain calcium homeostasis were not detected in some cases (Matsui et al., 1994). Therefore, other authors become more innovative and make use of fecal excretion radioisotope ratios $^{47}$Sc:$^{47}$Ca (Brommage et al., 1991; Bennett et al., 2000), other calcium controlled parameters such as systolic/mean blood pressure (Nagasawa et al., 1991; Kitts et al., 1992) and alternative bone mineralization indices like alkaline phosphatase (Matusi et al., 1994), osteocalcin (Tsuchita et al., 1995) and bone DNA (Ma and Yamaguchi, 2000). However, the lack of uniformity in end-point measures makes it especially difficult to make comparisons, substantiate and determine validity of results among studies.

1.7 CPP and Iron, Zinc and other metals

Phosphoserine clusters on casein-derived phosphopeptides bind calcium as well as other cations through coordinate or ionic bonding. Caseins usually bind about 95% of manganese and zinc and 50-75% of copper and iron in bovine milk (Flynn and Cashman, 1997). The specific conformations adopted by CPP are often influenced by the specific cation involved. Caseinophosphopeptides tend to configure in structures consisting of turns and loops in the presence of calcium ions but they behave markedly different in the presence of other counter-ions such as sodium or ammonia counter-ions (Cross et al., 2001). One molecule of $\alpha_{s1}$-casein can bind 14 atoms of iron and 16 atoms of copper while $\beta$-casein can bind 9 atoms of iron, 13 atoms of copper and 7 atoms of calcium at pH 7.0-8.0 (Brulé and Fauquant, 1982; Baumy and Brulé, 1988). Under physiological pH conditions, stability of manganese and zinc complexes has been shown to be greater than that for calcium and magnesium and still greater than strontium (Mellander, 1963). Calcium, magnesium, zinc and manganese are bound to phosphoserines through ionic
linkages, whereas copper and iron are bound through ionic and coordination links with NH$_2$, CO$_2$H, CONH and H$_2$O (Baumy and Brulé, 1988). Therefore, while CPP-calcium, magnesium, zinc or manganese complexes were vulnerable to changes in pH and ionic strength, CPP-iron and copper complexes were not affected. It was found that coordination linkages developed between iron and phosphoserine were approximately 100 fold stronger than the ionic bonding between phosphoserine and zinc or calcium (Bouhallab et al., 1991; Brulé and Fauquant, 1982).

### 1.7.1 Zinc absorption

Presence of inorganic iron and phytates can hinder intestinal zinc absorption in both animals and humans. CPP acts as a low-molecular chelator similar to ligands such as histidine, EDTA and organic acids to form a complex with zinc, thereby enhancing zinc absorption. CPP was also responsible for overcoming the inhibitory effect of dietary phytates in aqueous phytate solutions (Hansen et al., 1996) or phytate-containing rice meals (Hansen et al., 1997a) and that of inorganic iron on zinc absorption (Sandström et al., 1985; Lönnerdal et al., 2000; Péres et al., 1998).

### 1.7.2 Non-heme iron absorption

Casein binds about 85% of the added non-heme iron in skim bovine milk; 72% and 21% were associated with the $\alpha_s$- and $\beta$-caseins (Demott and Dincer, 1976), respectively. Most of the iron added to bovine milk binds to phosphoserine residues in the casein micelle, while some are sequestered by inorganic phosphate in both the milk serum and casein micelle (Hunt, 2001). Iron bound to phosphoserine is stable to changes in pH between 2 and 6, suggesting that iron is probably not only bound to casein by ionic bonds but also by coordination bonds via the oxygen of the phosphate group in a
tetrahedral coordination structure (Galdi and Valencia, 1988). However, at concentrations above 4mM, iron-casein complex precipitates through negative charge neutralization of caseins by iron and formation of intermolecular iron bridging (Gaucheron et al., 1996).

Non-heme iron bioavailability is usually low, ranging between 2 to 20%, and inversely related to iron stores or influenced by enhancing and inhibiting factors present in the diet (South et al., 2000). Iron from food is released under acidic conditions in the stomach and can form large, insoluble polymers of Fe$^{+3}$OH when pH rises in the duodenum. Major pathways of non-heme iron uptake involve brush border vesicles or an energy-requiring, carrier-mediated process via a transmembrane protein transporter. However, the pathway by which dietary factors have the strongest influence on divalent mineral absorption is the paracellular route. Although this route is a minor pathway, there are no adaptive regulation mechanisms involved, allowing the accommodation of significant amounts of iron when dietary iron levels are high (Benito and Miller, 1998). It is believed that the ferrous form of iron, being easily diffusible, is the form primarily absorbed in humans (Peters et al., 1971).

The presence of dietary factors may serve to enhance or inhibit non-heme iron absorption. Through multiple regression analyses on various dietary factors in humans, the main biochemical predictors of non-heme iron absorption from complex meals were identified to be sources of animal tissues, phytic acid and ascorbic acid (Reddy et al., 2000). While animal tissues (South et al., 2000) and reducing agents, such as ascorbic acid (Hallberg et al., 1986; Cook and Monsen, 1977), were found to have a positive effect on iron absorption, inorganic calcium (Hallberg et al., 1991), phytate (Hallberg et
Small-sized ligands, such as ascorbic acid, form unstable iron chelates by binding only a few of the 6 coordinating positions of iron, which serve as iron donors to mucin in the duodenal lumen at neutral pH to enhance iron absorption (Benito and Miller, 1998). However, dietary constituents such as phytate can form precipitates or stable chelates with iron that interfered with binding between iron and mucin and diminished iron absorption (Gillooly et al., 1983). Removing phytate from bran by dephytinization was shown to yield a bran fraction that had only a small remaining inhibitory effect on iron absorption in human adults (Hallberg et al., 1987).

High levels of inorganic calcium and phosphate in bovine milk are also implicated in lowering iron bioavailability (Barton et al., 1983). Due to similarity in physicochemical properties, calcium and iron may compete for common mucosal acceptors preventing movement of iron from the mucosa cell into circulation (Jackson and Lee, 1992). Inorganic phosphate, on the other hand, can oxidize ferrous iron to the ferric state and sequester it as ferric phosphate that is not readily dialyzable (Peters et al., 1971).

Dietary proteins can also either enhance or impair iron absorption depending on the food source. While meat proteins enhance iron absorption (Kim et al., 1995), egg white, bovine milk and soy proteins display an inhibiting effect (Peters et al., 1971; Kim et al., 1995; Cook et al., 1981; Derman et al., 1987). Whole proteins are assumed to be too large to diffuse across the intestinal mucosa and release iron too far along the intestine for efficient absorption. The effect of bovine milk proteins on non-heme iron
absorption can be lessened through enzymatic hydrolysis during digestive processes, thereby improving digestive solubility and availability of iron (Hurrell, 1989; Lönnertdal and Glazier, 1989). While protein digestion progresses, more soluble, low molecular weight iron peptides are likely to reach the brush border and enhance iron availability (Slatkavitz and Clydesdale, 1988).

Under physiological conditions, it is thought that inorganic forms of iron need to be reduced to the ferrous state for effective absorption. Luminal pH at the site of iron absorption is predicted to be between pH 6.0 and 7.4, whereas calcium phosphate was effective in binding ferrous iron only above pH 5.8 (Peters et al., 1971). The binding of ferrous iron to isolated rat intestinal brush borders was not affected by removal of oxygen, which indicates that the attachment of iron to brush borders does not depend on an energy requiring process (Greenberger et al., 1969).

Although protein hydrolysis yields digestion products throughout the upper part of the digestive tract, ferrous iron absorption occurs mainly at the duodenum and proximal jejunum sites of the overall intestine. Therefore, it is useful that iron remains soluble and bound to low molecular weight ligands when it reaches the upper part of the bowel, in order to allow for favorable absorption further down the digestive tract (Ait-oukhatar et al., 2000). The addition of ferrous salts to αs1- and β-casein causes a rapid uptake of oxygen (Emery, 1992), thus leading to the oxidation of iron and the formation of stable iron-phosphoprotein complexes. In the case of phosphoserine groups in a cluster similar to that of CPP, formation of these complexes is accompanied by the uptake of oxygen in excess to the theoretical requirement for the oxidation of iron to the ferric state (Manson and Cannon, 1978): $\beta$CN 1-25 + Fe$^{2+}$ + O$_2$ + 4H$^+$ $\rightarrow$ $\beta$CN 1-25/Fe$^{3+}$
+ βCN 1-25/Fe$^{2+}$ + 2H$_2$O. This oxygen uptake is absent if the caseins are first dephosphorylated, thereby indicating the importance of O-phosphoserine groups in the binding of α$_s$- and β-caseins to iron. Acting as a ligand, phosphopeptide β-casein (1-25) was shown to form a complex with ferrous chloride with a binding capacity of 4 mol Fe$^{2+}$/mole peptide and is responsible for significantly boosting the level of soluble iron at pH 7, from 23 to 81% (Bouhallab et al., 1991). This binding is extraordinarily firm suggesting that CPP-iron interactions involve coordinate bonding in addition to electrostatic bonding.

It was proposed that since the affinity of βCN (1-25) to iron is high, compared to calcium iron should be less bio-dispensable and available for absorption (Hurrell et al., 1989). However, binding iron to purified β-CN (1-25), CPP seems to improve iron bioavailability, enhancing erythropoiesis and iron storage in the liver and hastening the cure of iron deficiency in young rats more effectively than ferrous sulfate salt or whole β casein (Ait-oukhatar et al., 1997). β-CN (1-25) can also enhance iron uptake, reduce mucosal storage and improve the net absorption in control and anemic rats using a perfused vascularized duodenal loop model (Pérès et al., 1999a).

Iron bound to phosphorylated casein is relatively resistant to dissociation; therefore, in this way, iron can be kept in a protected, stable state until it reaches the enterocyte receptor and is released by phosphatases (Pointillart and Guéguen, 1989). Because of the presence of a high concentration of negatively charged phosphate groups, β-CN(1-25)-Fe complex is protected from the action of pepsin and of pancreatic endopeptidases and is hydrolysed to a lesser extent than free β-CN(1-25) during duodenal digestion (Ait-oukhatar et al., 2000). Enzymatic dephosphorylation by phosphatase of
the βCN-iron complex releases free, dialyzable iron, in the ferrous form, at the apical membrane of brush border enterocytes and not in the digestive lumen. The constant association of iron with phosphoserine in contact with the mucous membrane seems to facilitate its transfer at the membrane receptor (Galdi and Valencia, 1988). It was found that fragments of the iron-CPP complex, such as soluble Fe/βCN (15-25) with lower molecular weight, reach the brush border more readily than the whole Fe/βCN (1-25) complex and can better enhance iron absorption (Bouhallab et al., 1999).

Binding iron to phosphopeptides keeps it soluble during upper digestive tract transit, preventing against inhibitory interactions with nutrients, such as calcium and zinc, which share similar electrochemical properties and compete for a common transport mechanism at the enterocyte membrane. Although calcium does not seem to be in competition with iron at the rat duodenal brush border membrane vesicle level at a 1:1 ratio, calcium does inhibit iron absorption when present as a 10-fold or 100-fold greater concentration, relative to iron (Wien et al., 1994). The addition of calcium, at an iron:calcium ratio of 1:2, to the perfused intestinal loop of iron-deficient rats significantly lowered iron uptake, but the decrease was lowered by nearly 20% in the β-CN(1-25) group as compared to the gluconate group (Pérès et al., 1997). The presence of zinc also significantly reduced net iron absorption and disappearance from the lumen, lowering uptake by 29-50% at an iron:zinc ratio of 1 (Wien et al., 1994). However, when either element was first bound to β-CN(1-25), no inhibition was observed even when an iron:zinc ratio was as high as 1:5 (Pérès et al., 1999b). The bound form of trace elements can enhance the digestive absorption by preventing interactions with free ions during membrane transfer, since CPP-Fe and CPP-Zn complexes do not share the same transport
mechanisms as the ions. The bound ion complexes, if absorbed, are likely taken up into the enterocytes by specific pathways such as endocytosis (Pérès et al., 1999b).

1.8 Additional functions of CPP

1.8.1 Anticariogenic properties

Multi-phosphorylated peptides such as CPP have been proposed to possess anticariogenicity due to an affinity to localize amorphous calcium phosphate in dental plaque, thus maintaining a state of supersaturation with respect to tooth enamel and restricting mineral loss. Although supersaturated with amorphous and crystalline calcium phosphate, stabilization provided by CPP prevents spontaneous precipitation (Reynolds, 1998). CPP bound amorphous calcium phosphate at 0.1% reduces calcium diffusion by 65% at pH 7.0 (Rose, 2000). The amorphous calcium phosphate acts as a buffer for free calcium and phosphate ion species in plaque, thereby preventing tooth enamel demineralization by acid from bacterial plaque in rats (Reynolds et al., 1995) and humans in situ (Reynolds and Riley, 1994) and enhancing re-mineralization in subsurface lesions in human third-molar enamel (Reynolds, 1997).

1.8.2 Immunomodulating properties

Milk is usually a source of host defense for neonates (Cross and Gill, 2000; Gill et al., 2000). Since calcium flux into lymphocytes plays an important part in triggering lymphocyte stimulation, bovine milk derived CPP's association with improvement of calcium solubility and utilization has brought many researchers to investigate a possible immunological role.

Phosphopeptides, αs1-casein (59-79) and β-casein (1-25), originated from raw and pasteurized bovine milk, were shown to be responsible for inhibiting concanavalin A-
(Con A) induced proliferation of mouse spleen cells and rabbit Peyer’s patch cells, while enhancing lipopolysaccharide- (LPS) and phytohemagglutinin- (PHA) induced proliferation in these cells (Hata et al., 1998; Kihara and Otani, 2000). In addition to mouse spleen cells and rabbit cells, CPP III, mainly consisting of α_{s2}-casein (1-32) and β-casein (1-28), was also shown to have a similar effect on Con A-, LPS- and PHA-induced proliferation of nude mouse spleen cells (Hata et al., 1999). Since LPS, PHA and ConA stimulate different subsets of lymphocytes, namely the B-lymphocytes, helper T-lymphocytes and suppressor/killer T-lymphocytes respectively, it was shown that CPP suppresses killer T-cells, while also playing a role in boosting B-lymphocytes and helper T-lymphocytes (Hata et al., 1999).

Mitogenic activity of CPP-III on mouse spleen cells was unaffected by prior digestion with pepsin, trypsin/chymotrypsin or pancreatin, but was greatly severed when CPP was previously treated with potato acid phosphatase (Hata et al., 1999). Therefore, it can be concluded that mitogenic activity in cell culture is attributable to the phosphoserine residues.

When cultured with mouse spleen cells, CPP α_{s1}-casein (59-79) also significantly enhanced the immunoglobulin levels of IgG, IgM and IgA (Hata et al., 1999). Oral ingestion of CPP-III has been shown to be beneficial for the enhancement of mucosal immunity in mice. Mice fed CPP-III supplemented diets at 0.1 to 1.0% had a significantly greater intestinal specific IgA response towards antigens such as ovalbumin and β-lactoglobulin (Otani et al., 2000).

Although CPP seems to be quite a promising and beneficial immunostimulant as a dietary supplement, it must be utilized with caution since the multi-phosphorylated region
of phosphopeptides is also identified to be the epitope for anti-casein antibodies binding (Perich et al., 1999). The antigenicity of CPP was established by Park and Allen (2000) in a rat model. Tryptic casein phosphopeptides from α- and β-caseins were shown to induce a significantly lower IgG specific response (to whole α- or β-casein) than intact proteins or whole tryptic hydrolysates. Enzymatic hydrolysis of caseins served only to lower but could not remove antigenicity entirely.

1.9 Milk Protein Antioxidants

Mineral supplementation to milk as a boost to the nutritional quality may also put the milk system at risk towards oxidative changes, if bound to the milk fat fraction. For example, iron (II) can undergo cyclic autoxidation and form ferric hydroxide-protein complexes that may contribute to further instability to supplemented milk during prolonged storage, thereby severely diminishing the nutritional quality for neonates (Gutteridge and Halliwell, 1994). In order to counteract this oxidative vulnerability, the role of antioxidants is fundamental in the milk system (Lindmark-Månsson, 2000). Halliwell (1995) defined antioxidant simply as “any substance that, when present at low concentrations compared with those of an oxidizable substrate, will significantly delay or prevent oxidation of that substance”. Oxidizable substrate refers to any molecule found in foods and in living tissues, such as lipids, proteins, carbohydrates or DNA, that are vulnerable to attack by Reactive Oxygen Species (ROS). These include the superoxide anion (O$_2^-$), hydroxyl radical (·OH), and oxygen-centered radicals of organic compounds (peroxyl ROO' and alkoxyl RO') together with non-radical reactive compounds such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (¹O$_2$) (Morrissey and O’Brien, 1998).
In bovine milk, major protein fractions including whey and casein have been implicated in displaying various antioxidative characteristics. A non-heme iron-binding glycoprotein in the whey fraction of bovine milk, lactoferrin, has an affinity for iron and inhibited iron-catalyzed oxidation in iron-supplemented infant formulas (Satué-Gracia et al., 2000) and liposomal phospholipid systems (Wakabayashi et al., 1999). A high molecular weight fraction of whey was also shown to act as a scavenger for peroxyl radicals in salmon oil-in-water emulsion systems (Tong et al., 2000). Similarly, phosphate-bound phosphoserine residues in casein have the ability to scavenge superoxide, hydroxyl and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (Suetsunna et al., 2000) and to lower oxidation in iron-induced peroxidation of arachidonic acid in liposomes (Cervato et al., 1999).

Antioxidants are classified into three different categories: Primary antioxidants donate electrons and terminate free radical chain reactions through converting lipid radicals to more stable products; secondary antioxidants lower the rate of chain initiation by a variety of mechanisms: acting as an reducing agents, chelating agents for metallic catalysts such as copper and iron or as singlet oxygen quenchers; enzymatic antioxidants remove highly oxidative species such as hydrogen peroxide or superoxide (Gordon, 1990). The most common synthetic chain-breaking antioxidants used in foods are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and nordihydroguaiaretic acid (NDGA). The relatively low cost, superb efficiency and stability allow these synthetic antioxidants to lower oxidation. However, repeated concerns about the safety (i.e. possible carcinogenic effects) of these antioxidants have led to a growing interest in ‘natural’ antioxidants. Ideally, antioxidants should be found
“naturally” in living organisms, plants and animals inclusive, for example, tocopherol in vegetable oils, ascorbic acid in fruits (Gutteridge and Halliwell, 1994) or even bioactive peptides derived from milk (Meisel, 1998).

1.10 Lipid Auto-oxidation and Fenton Reaction

The most important stressors in aerobic organisms and food systems are the reduced derivatives of oxygen, free radicals such as the reactive oxygen species, which exist naturally either as a part of normal physiological and metabolic processes or as a product of food processing and manufacturing. It has been established that oxidative deterioration of lipids are caused by auto-oxidation reactions, which are essentially free radical chain reactions proceeding via defined initiation, propagation and termination steps. In the initiation step, hydrogen atoms are abstracted from the methylene (-CH2-) group between double bonds in polyunsaturated fatty acids generating free radicals that are stabilized by resonance (Richardson and Korycha-Dahl, 1983). The following reactions summarized the steps auto-oxidation:

Initiation: \[ \text{LH} \rightarrow \text{L}^\cdot \]  
\[ \text{L}^\cdot + \text{O}_2 \rightarrow \text{LOO}^\cdot \]  
\[ \text{Propagation: } \text{LOO}^\cdot + \text{L}'\text{H} \rightarrow \text{LOOH} + \text{L}'^\cdot \]  
\[ \text{Termination: } \text{LOO}^\cdot + \text{L}'' \rightarrow \text{LOOL}' \]  
\[ \text{LOO}^\cdot + \text{L'O}_2 \rightarrow \text{LOOL}' + \text{O}_2 \]  
\[ \text{L}^\cdot + \text{L}'' \rightarrow \text{LL}' \]
In initiation (e.g. Equations 1 and 2), the fatty acid (LH) forms a free radical (L'), which reacts with oxygen to form the peroxy radical (LOO'). The oxygenation of L' is very rapid and is therefore the primary product of oxidation. Oxidation can also be initiated by one electron species such as transition metals with valence states one unit apart (e.g. Fe$^{3+}$ and Fe$^{2+}$) (Larson, 1997). These radicals react with other unsaturated fatty acids by abstracting more hydrogen atoms in the propagation step (Equation 3). Reactions during the propagation step become self-catalytic and accelerate until the free radicals become stabilized during termination (Equations 4, 5 and 6).

Peroxides, formed as a result of lipid auto-oxidation, are then subsequently transformed into a variety of oxygenated substances, such as alcohols, hydroperoxides, ketones, epoxides and others reactive products, which undergo further reactions that yield a complex and potentially toxic mixture of polymers (Shahidi, 1997). In the presence of metals such as iron (II), lipid autoxidation can be induced through the Fenton reaction, which involves the oxidation of iron [Equation 7], generation of hydroxyl [Equation 7] and superoxide radicals [Equations 8 and 9].

\[
\begin{align*}
  \text{Fe}^{2+} + \text{H}_2\text{O}_2 & \longrightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^- \quad [7] \\
  \text{Fe}^{3+} + \text{H}_2\text{O}_2 & \longrightarrow \text{Fe}^{2+} + \text{O}_2^- + 2\text{H}^+ \quad [8] \\
  \cdot\text{OH} + \text{H}_2\text{O}_2 & \longrightarrow \text{H}_2\text{O} + \text{H}^+ + \text{O}_2^- \quad [9]
\end{align*}
\]

Reduced iron catalyzes the formation of hydroxyl radicals and of other reactive oxygen species via the Fenton reaction, thereby causing free radical-mediated lipid peroxidation. H$_2$O$_2$ is non-toxic and relatively stable as a molecule by itself, but can become a precursor of the highly reactive $\cdot$HO, which oxidizes all substances close to it.
Since it is nearly impossible to completely abolish the presence of initiating species such as preformed free radicals and other pro-oxidative factors such as metal ions (Richardson and Korycka-Dahl, 1983), antioxidant agents should target at stabilizing reactive species generated in the propagation phase to effectively inhibit or slow lipid oxidation in real food systems and in vivo.

In general, iron supplementation in food sources such as bovine milk can improve the bioavailability of iron for humans, but may also increase the exposure of reactive oxygen species generated by the iron-induced Fenton reaction to both the food system and the consumer. Since iron excretion from the body is limited and largely unregulated, iron is potentially more toxic than other trace elements due to the lack of effective means to protect human cells against iron overload and the role of iron in generation of free radicals. It was found that intestinal epithelial cells play a key role in regulating body iron absorption and intracellular iron levels (Arredondo et al., 1997). An excess of free dietary iron is associated with oxidative damage in DNA and lipid peroxidation at biomembranes (Wessling-Resnick, 2000), which lead to erosion of microvilli at the gastrointestinal (GI) tract absorptive surface and eventual mucosal cell apoptosis and necrosis (Srigiridhar et al., 2001). In addition to damage of the GI tract, prolonged iron overload, and the associated accumulation of oxidation products in tissues, may lead to conditions such as liver failure, diabetes, heart malfunction and joint inflammation (Halliwell and Gutteridge, 1984).

1.11 Protein Glycosylation

Since proteins and bioactive peptides derived from bovine milk have been shown to display a variety of functionalities, it is of interest so determine if these functionalities
can be improved by different modification techniques. Various functional properties of proteins have been shown to be improved by conjugation with polysaccharides or oligosaccharides. The conjugation reaction involves covalently binding ε-amino groups of the protein to the reducing-end carbonyl groups of the sugar, under controlled heating and humidity conditions, similar to that in a Maillard reaction (Figure 2). These protein-saccharide conjugates are also known as neoglycoproteins.

![Figure 2. Protein-polysaccharide conjugate product using Maillard Reaction. (Nakamura et al., 1998)](image)

To date, research on the modification of bovine milk proteins by glycosylation is scarce, but many egg proteins have been shown to display enhanced functionalities, following glycosylation with saccharides. Covalently binding egg white protein, ovalbumin, to dextran, a large neutral polysaccharide, was shown to aid in emulsification unto lipid surfaces, hence more effectively scavenging free radicals there and improving antioxidant activities (Kato et al., 1988; Kato et al., 1990; Nakamura et al., 1992). Enhanced emulsifying properties of lysozyme via glycosylation with dextran are suggested to contribute to greater antimicrobial activity targeted towards the lipopolysaccharide outer membrane in Gram-negative bacteria (Nakamura et al., 1991). In addition to dextran, antioxidant activity of ovalbumin, antimicrobial activity of lysozyme (Nakamura et al., 1994; Nakamura and Kato, 2000) and emulsifying properties
of phosvitin (Sattar Khan et al., 1999; Nakamura et al., 1998) were also significantly enhanced through conjugation of the proteins with galactomannan, a polysaccharide derived from guar gum.

The functionalities of these protein-saccharide conjugates may be affected by the length and number of saccharide chains attached. Emulsifying properties and heat stability of lysozyme-galactomannan conjugates improved with the length and number of polysaccharide chains, whereas these functionalities were not affected by conjugation with an oligosaccharide, xyloglucan (Shu et al., 1996). These researchers then concluded that conjugation with long, branched chain polysaccharides such as dextran and galactomannan efficiently improved functionality of proteins but this improvement was not as apparent when proteins are conjugated with shorter chain or straight chain oligosaccharides. However, these findings were refuted by a five fold higher emulsifying activity in lysozyme-oligosaccharide (i.e. xyloglucan) conjugates when compared to the native proteins (Nakamura et al., 2000).

Since bovine milk β-casein was found to have higher surface activity and superior adhesion to oil-in-water emulsions than milk whey protein β-lactoglobulin and egg yolk phosvitin (Dickinson et al., 1991), addition of saccharides to peptides derived from casein may serve to amplify the hydrophilicity of the complex, enhancing surface activity and possibly improving metal chelating and free radical scavenging activities in aqueous and emulsion models previously observed in these peptides. Since these peptide-saccharide conjugates are not likely to be toxic for ingestion (Nakamura and Kato, 2000), they have a great potential to be bioactive in vitro and in vivo.
CHAPTER II: OUTLINE OF RESEARCH ACTIVITIES

2.1 General Introduction

As a potent bioactive peptide with metal binding characteristics, bovine milk casein-derived caseinophosphopeptides (CPP) may serve to remove pro-oxidative metal catalysts such as iron from an environment of oxidizable lipids (Hegenauder et al., 1979), from dietary sources or at biomembrane lipid bilayer systems of potential target organs. Since the absorption of iron is mainly regulated at the gastrointestinal tract, it is important to search for potential iron chelators that have a selective affinity for iron, exhibit a low toxicity in cells upon oral intake (Faa and Crisponi, 1989) and, most importantly, remain bioactive under gastrointestinal alkaline conditions (Benito and Miller, 1998).

Since bovine milk β-casein was found to have higher surface activity and superior adhesion to oil-in-water emulsions than milk whey protein β-lactoglobulin and egg yolk phosvitin (Dickinson et al., 1991), conjugation of saccharides to peptides derived from casein may serve to amplify the hydrophilicity of the complex, enhancing surface activity and possibly improving antioxidant activities in aqueous and emulsion models. Since these peptide-saccharide conjugates are not likely to be toxic for ingestion (Nakamura and Kato, 2000), they have a great potential to be bioactive in vitro and in vivo.

The following in vitro studies were designed to examine the potential of bovine milk caseinophosphopeptides (CPP) to serve as an antioxidant, through metal chelating and/or free radical sequestering, in aqueous, emulsion and cell culture models. In addition, the effect of glycosylation on the CPP antioxidant activity was investigated.
2.2 Null Hypothesis (H₀)

1. CPP has no affinity to sequester divalent metals such as iron and calcium.
2. CPP does not behave as an antioxidant by sequestering metal catalysts and inhibiting the Fenton Reaction.
3. CPP does not exhibit both primary and secondary antioxidant activity.
4. Conjugation between CPP and saccharides will not alter antioxidant activity.
5. CPP is non-toxic to enterocytes.
6. CPP has no role in inhibiting oxidation reactions at the site of the human intestine by sequestering free metals in the intestinal lumen.

2.3 Objectives

1. To determine the affinity of CPP for sequestering iron (II), (III) and calcium.
2. To determine if CPP has the affinity to inhibit Fenton reaction-induced oxidation in various *in vitro* model systems.
3. To determine if CPP protects against oxidation reactions induced by stable free radicals or a free radical initiator.
4. To determine if the affinity of CPP in the above models is altered by conjugation with galactomannan and xyloglucan.
5. To determine if CPP can protect human intestinal carcinoma cells (Caco-2) and embryonic cells (Int 407) from bivalent metal-induced cytotoxicity.

2.4 Experimental Approach

1. To compare absorbance scans (200-600nm) for CPP, divalent metals and CPP-metal complex for the purpose of estimating CPP-mineral interaction and possible sequestering activity.
2. To perform site-specific binding and non site-specific binding assays with deoxyribose and liposome assays, using iron as an initiator for oxidation.

3. To use free radicals as an initiator for oxidation and perform stable free radical DPPH and ABTS assays and liposome assay (with AAPH) to assess primary antioxidant activity.

4. To assess the viability of human intestinal carcinoma cells (Caco-2) and embryonic cells (Int-407) following exposure to ferrous iron-induced toxicity by the Mitochondrial Tetrazolium (MTT) Test.

2.5 Comments on the methodologies for antioxidant analysis

In order to detect an interaction between CPP and metal ions, CPP will be incubated with the following metal ions (i.e. iron II, iron III and calcium) in a 37°C water bath for one hour prior to measuring for absorbance. Absorbance scanning between 200-600 nm will provide a quick and convenient method for identifying the interaction and possible sequestering between ligand and ion (Hu and Kitts, 2000). If the scan for the CPP-metal complex shows peaks not present for CPP and metal ions individually, then initial evidence for binding is obtained.

The most common methods to monitor the affinity of a potential antioxidant usually involves inducing lipid auto-oxidation in bulk oil or emulsion systems. Of all the reactive oxygen species, the hydroxyl radical is the most potent oxidant in biomolecules, making it extremely effective in starting lipid peroxidations. In the presence of divalent metal ions (Fe$^{2+}$), fatty acids can be oxidized to produce peroxyl radicals (ROO$^\cdot$), which then undergo a complex series of reactions to yield alkoxy radicals (RO$^\cdot$) and other
degradation products such as malondialdehyde-(MDA) and conjugated diene-containing substances (Gutteridge and Halliwell, 1990).

The rate constants for hydroxyl radical-induced reactions can be determined in a simple test tube assay, the deoxyribose assay. Rate constants for radical-induced oxidation reactions had been measured previously by highly expensive methods, such as pulse radiolysis. However, this inexpensive deoxyribose assay gives accurate results despite the simplicity (Halliwell et al., 1987). The sugar 2-deoxy-D-ribose is degraded (Equation 13) on exposure to hydroxyl radicals generated by the Fenton reaction (Equations 10, 11 and 12). If the resulting complex mixture of products is heated under acid conditions, a secondary product of oxidation, malondialdehyde (MDA), is formed and this may be detected by the reaction with thiobarbituric acid (TBA) to form a pink chromogen (Equation 14). The deoxyribose assay can therefore be a useful method for measuring the formation of OH radical in biochemical systems.

\[
\begin{align*}
\text{Fe}^{2+}-\text{EDTA} + O_2 & \leftrightarrow \text{Fe}^{3+}-\text{EDTA} + O_2^- \quad [10] \\
2O_2^- + 2H^+ & \rightarrow H_2O_2 + O_2 \quad [11] \\
\text{Fe}^{2+}-\text{EDTA} + H_2O_2 & \rightarrow OH^- + \cdot OH + \text{Fe}^{3+}-\text{EDTA} \quad [12] \\
\cdot OH + \text{deoxyribose} & \rightarrow \text{fragments} \rightarrow \text{heat & acid} \rightarrow \text{MDA} \quad [13] \\
2\text{TBA} + \text{MDA} & \rightarrow \text{chromogen} \quad [14]
\end{align*}
\]

There are two different ways of determining antioxidant activity using this assay. If deoxyribose is incubated with iron in the presence of EDTA, the formation of hydroxyl radicals (OH) is favored, accelerating the oxidation of the sugar. This is regarded as the non-site specific binding assay, since CPP, when added, will interact with hydroxyl
radicals generated rather than directly with the deoxyribose. On the other hand, when EDTA is replaced with an equal volume of phosphate buffer, the assay can detect site specific binding when CPP and deoxyribose compete for the metal iron. Reduction in deoxyribose degradation is indicated by a lowering in absorbance values at 532 nm, for both types of assay.

The potential antioxidant effect of CPP will also be determined in emulsion models, namely phospholipid liposomes. Liposomes were first described by Bangham (1965) while studying the nature of cell membranes. It was found that liposomes formed spontaneously when phospholipids were dispersed into water. Liposomes are simply spherical vesicles consisting of one or more phospholipid bilayers surrounding an aqueous cavity. Initial studies with liposomes focused on the cell membrane; however, the majority of studies now use liposomes for drug delivery research (Rongen et al., 1997). The liposomes used in the following studies were formed by dissolving soybean lecithin in a saline phosphate buffer and used as an emulsion system to test the extent of oxidation induced by metals or free radicals. The extent of oxidation was monitored by the formation of conjugated diene products arising from lipid hydroperoxides, lipid alcohols and fatty acids, which contain no oxygen other than the carboxyl group (Smith and Anderson, 1987). Measurement of conjugated diene at an absorbance of 234 nm is a fast, accurate and convenient method for determining the relative amount of lipid peroxidation. Oxidation can be initiated by the addition of ferrous iron (FeII) or peroxyl radicals generated by the thermolysis of 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH). The reaction system should be maintained for 100 minutes at 37°C, with
measurements taken every 4 minutes. The antioxidant potency is determined from the reduction in peroxidation propagation rate, as compared to the control.

A second method to detect the free radical scavenging potential of CPP is through the reaction with a stable free radical \( \alpha,\alpha\)-diphenyl-\( \beta \)-picrylhydrazyl (DPPH). Because of the odd electron in DPPH, it will show a strong absorption band at 517 nm in ethanol. The solution thus appears deep violet (Blois, 1958). If the electron is paired off, the absorption diminishes and the resultant decolorization correlates well with the number of electrons taken up. The test agent donating the electron or hydrogen atom would therefore be an active antioxidant.

Since the solvent of the DPPH is ethanol, a medium where CPP is not very soluble, an alternate stable radical 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS\(^+\), which is more hydrophilic can also be used. The pre-formed radical monocation ABTS\(^+\) is generated by oxidation with ammonium persulfate, resulting in the production of a blue/green chromophore with an absorption maxima of 734 nm (Pellegrini et al., 1999). The addition of antioxidants to the pre-formed radical cation would reduces ABTS\(^+\) to ABTS on a time-scale, which is dependent on the antioxidant activity and concentration of the test sample. The extent of decoloration, when expressed as % inhibition of the ABTS\(^+\), can be monitored by the change in absorbance at 734 nm.

The human colon adenocarcinoma cell (Caco 2) culture model has been exploited recently as a model for measuring bioavailabilities of iron from foods (Garcia et al., 1996), calcium (Ekmekcioglu, 1999) and zinc (Hansen et al., 1996), due to an affinity to differentiate spontaneously into biopolar enterocytes that exhibit many of the characteristics of normal epithelial cells such as microvilli junctions and the excretion of
brush border associated enzymes (Halleux and Schneider, 1994). However, since Caco-2 cells are carcinoma cells, metal-induced cytotoxicity in the Caco-2 cells in addition to non-carcinomic human embryonic intestinal cells (Int-407) will be evaluated for a comparison. Cytotoxicity can be measured using Mitochondrial Tetrazolium (MTT) Test (Mosmann, 1983). Viable cells with a functional mitochondrial enzyme cleave the mitochondrial tetrazolium salt into a blue colored formazan. The amount of formazan produced, which correlates to the number of viable cells, is monitored spectrophotometrically (A570).
CHAPTER III:
ANTIOXIDANT EFFECTS OF COMMERCIALLY AVAILABLE CPP

3.1 Materials and Methods

Ferrous chloride, calcium chloride, L-ascorbic acid, 2 deoxy-D-ribose, 2 thiobarbituric acid (TBA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), L-α-lecithin (from soybean), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (diammonium salt, ABTS), chelex-100 ion-exchange resin, and casein were obtained from Sigma Chemical Co. (St. Louis, MO). Ferrous sulfate, EDTA, hydrogen peroxide (30%) and trichoroacetic acid (TCA) were from Fisher Scientific (Fairlawn, NJ). Methanol, acetic acid and hydrochloric acid were from Fisher Scientific (Napean, ON). Ferric chloride was purchased from Mallinckrodt (Paris, Kentucky). Sodium hydroxide, potassium dihydrogen orthophosphate and sodium chloride were obtained from BDH Inc. (Toronto, ON). 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was from Wako Chemicals USA Inc. (Richmond, VA). Sodium dodecyl sulfate (SDS), ammonium persulfate, glycine, coomassie blue G-250, broad range molecular weight standards, tris base, β-mercaptoethanol, glycerol and bromophenol blue were from Bio-Rad Laboratories (Richmond, CA). Phosphate buffer was made with distilled deionized water and was eluted through a Chelex-100 column to eliminate the occurrence of transition metals.

3.1.1 CPP Formulations

Caseinophosphopeptides (CPPms) were obtained from Meiji Seika Kaisha Ltd. (Kawasaki, Japan). CPPms-I and CPPms-III were commercially produced by treating a whole casein solution [15% (w/v), pH 7.0] with trypsin (Novo Industri Japan) at 60°C for 2 hours to give the hydrolysates (Naito et al., 1990). Enzyme hydrolysis for production
of CPPms-I and CPPms-III required 0.002\% (w/w) and 0.01\% of the substrate respectively. CPPms-I was obtained from spray-drying the whole hydrolysate. CPPms-III enriched with phosphopeptides was produced by filtering the insoluble mass from the hydrolysate at pH 4.5 before precipitating the phosphopeptides by adding calcium chloride [1.1\% (w/v)] and ethanol [50\% (v/v)].

3.1.2 CPP Characterization Studies

3.1.2.1 Molecular Weight Characterization

Casein, CPPms-I and CPPms-III were analyzed by SDS-PAGE according to the method of Laemmli (1970) using 15\%T (i.e. weight percentage of total monomer including crosslinker) polyacrylamide separating gel and 4\% stacking gel containing 0.1\% SDS on the Mini-Protean I Mini-Cell slab gel electrophoresis unit (BIO-RAD Laboratories, Richmond, CA). Protein samples (40\mu g) were heated at 100\(^\circ\)C for 5 minutes in 0.5M Tris-glycine buffer, pH 6.8 containing 10\% SDS. Electrophoresis was carried out at a constant voltage of 100 volts for 1.5 hours with a Tris-glycine running buffer. Gels were placed in 0.1\% Coomassie Blue G-250 staining solution (40\% methanol and 10\% acetic acid) for 30 minutes and destained in 40\% methanol and 10\% acetic acid for 3 x 15 minute destain washes.

3.1.2.2 Iron and Calcium Chelating Capacity

A spectrophotometric method was adopted from Hu and Kitts (2000) to estimate the metal ion chelating capacities of CPPms. CPPms-I and CPPms-III (1mg/ml) were incubated with 100 \mu M FeCl\(_2\), FeCl\(_3\) or CaCl\(_2\) at 37\(^\circ\)C for 2 hours. The differential spectra of CPPms, metal chlorides, and CPPms-metal chloride complexes were recorded against 10 mM phosphate buffer (pH 7.4) over a wavelength range of 200-600 nm.
3.1.3 Determination of Antioxidant Activity

3.1.3.1 Hydroxyl radical scavenging assay in a Deoxyribose model

A Fenton reaction model containing 0.1 mM of Fe$^{3+}$ as the catalytic metal was used. The substrate 2-deoxyribose (3.6 mM) was mixed together with CPPms (0.05, 0.10, 0.50, 1.00 mg/ml) and 0.1 mM EDTA, 0.1 mM ferric chloride, 0.1 mM ascorbic acid and 1mM H$_2$O$_2$. The reaction mixture was incubated at 37°C for 1 hour. Following incubation, 1 ml of 10% (v/v) trichloroacetic acid (TCA) and 1% 2-thiobarbituric acid (TBA) were added and the mixture was boiled for 15 minutes at 95°C. Absorbance at 532 nm was recorded after cooling. The extent of deoxyribose degradation by hydroxyl radicals generated by the Fenton reaction was calculated using the equation:

\[
\text{% Inhibition} = \frac{\text{Abs}_{532\text{nm}}\text{control} - \text{Abs}_{532\text{nm}}\text{sample}}{\text{Abs}_{532\text{nm}}\text{control}} \times 100
\]

3.1.3.2 Peroxyl Radical and Metal scavenging assays in a Liposome model

Liposomes were made by sonicating soybean lecithin (α-phosphatidylcholine) in an ice-water bath for 20 minutes in 10 mM phosphate buffer (pH 7.4) according to Hu and Kitts (2000). Peroxyl radical induced liposomal peroxidation was conducted at a constant temperature of 37°C using an ATI Unicam (UV2) UV/Vis spectrophotometer. The reaction was initiated by the addition of 0.2 mM AAPH to a mixture of 0.1 mg/ml of liposome in 10 mM phosphate buffer (pH 7.4) and CPP. Generation of conjugated diene hydroperoxide was monitored by taking absorbance readings at 234 nm every 4 minutes for 100 minutes.

3.1.3.3 Free Radical Scavenging Activity

The affinity of CPPms to scavenge a stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured according to Blois (1958). CPPms (0.05, 0.10, 0.50, 1.00 mg/ml)
was mixed with 0.1 mM DPPH radical in ethanol solution prior to an incubation period of 20 minutes at room temperature. Discoloration was monitored by measuring the absorbance at 519 nm following incubation.

The (2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical cation model was also used to evaluate the free radical scavenging effect of CPPs (Pellegrini et al, 1999). Ethanol was replaced by distilled deionized water in the radical preparation. Discoloration is determined by comparing the absorbance at 734 nm of the treatment groups with the control after an 8-minute incubation at room temperature.

The percentage inhibition in the DPPH and ABTS assays was calculated according to the following equation:

\[
\% \text{ Inhibition} = \frac{\text{Abs}_\text{control} - \text{Abs}_\text{sample}}{\text{Abs}_\text{control}} \times 100
\]

3.1.4 Statistical Analysis

All data (with the exception of the liposome assays) were collected in triplicates and analysed by one way ANOVA (\(\alpha \leq 0.05\)), followed by a multiple range Tukey post test analysis using the GraphPad Prism Analysis software (GraphPad Software Inc., San Diego CA) to identify significant difference among treatment means (\(P \leq 0.01\)). Data were obtained from 3 different individual experiments for the liposome assays and submitted to linear regression using GraphPad software. Rates of propagation (i.e. slope of regression equation) were then analyzed by one way ANOVA (\(\alpha \leq 0.05\)) and Tukey test (\(P \leq 0.01\)).
3.2 Results

3.2.1 Molecular Characterization of CPP

Caseinophosphopeptides (CPPms-I and -III), purchased from Meiji Seika Kaisha Ltd., were analyzed using SDS PAGE electrophoresis to estimate the apparent molecular weight of peptides present (Figure 3). Individual protein bands for the whole casein sample had apparent molecular weights of approximately 22-23 kDa, 25 kDa, 23-34 kDa and 19 kDa, respectively. These sizes are consistent with the reported molecular weights of $\alpha_{s1}$, $\alpha_{s2}$, $\beta$ and $\kappa$ respectively (Swaisgood, 1982). As for the CPPms-I and CPPms-III samples, a number of peptides having a molecular weight of lower than 6 kDa was observed. This electrophoresis method used did not however provide a precise description of the exact molecular weights of small peptides. Other workers have reported CPP preparations consisting of 40% $\alpha_{s1}$-CN(43-79) and 36% $\beta$-CN(1-25) with 4.6 kDa and 3.1 kDa respectively (Naito et al., 1990; Reynolds et al., 1994; Ono et al., 1998; Gaignaire et al., 1996).
Figure 3. SDS PAGE Gel (15% T) using the Laemmli buffer system (0.375 M Tris, pH 8.8). Lane 1: 40 µg Casein, Lane 2: 40 µg CPPms-III, Lane 3: 40 µg CPPms-I, Lane 4: Broad Range Molecular Weight Marker.
3.2.2 Qualitative Metal Ion Sequestering Activity of CPPms

Absorbance scanning between 200 and 600 nm was performed to detect interactions between CPPms and metal ions of interest. Absorbance scans were performed (Figures 4 to 9) for Fe (II), Fe (III), Ca (II), CPPms-I, CPPms-III and the different CPPms-metal mixtures following one hour incubation at 37°C. The differential spectra for each metal and CPPms revealed characteristic absorbance peaks, whereas peaks for CPPms-metal mixtures resembled neither the metal ion nor the peptide, indicating that an interaction between these two substances had occurred. Although the strength of binding cannot be quantified from the results, the spectra provided qualitative evidence for an interaction between CPPms and Fe$^{2+}$ or Fe$^{3+}$, while also distinguishing the binding characteristics between CPPms-I and III.
Figure 4. Differential spectra of CPPms-I with ferrous iron: Spectrum 1 represents the spectrum of CPPms-I against 10mM phosphate buffer (pH 7.4); spectrum 2 represents the spectrum of a mixture of CPPms-I and Fe$^{2+}$ against phosphate buffer; spectrum 3 represents the spectrum of Fe$^{2+}$ against phosphate buffer.
Figure 5. Differential spectra of CPPms-I with ferric iron: Spectrum 1 represents the spectrum of CPPms-I against 10mM phosphate buffer (pH 7.4); spectrum 2 represents the spectrum of a mixture of CPPms-I and Fe$^{3+}$ against phosphate buffer; spectrum 3 represents the spectrum of Fe$^{3+}$ against phosphate buffer.
Figure 6. Differential spectra of CPPms-I with calcium: Spectrum 1 represents the spectrum of CPPms-I against 10mM phosphate buffer (pH 7.4); spectrum 2 represents the spectrum of a mixture of CPPms-I and Ca\textsuperscript{2+} against phosphate buffer; spectrum 3 represents the spectrum of Ca\textsuperscript{2+} against phosphate buffer.
Figure 7. Differential spectra of CPPms-III with ferrous iron: Spectrum 1 represents the spectrum of CPPms-III against 10mM phosphate buffer (pH 7.4); spectrum 2 represents the spectrum of a mixture of CPPms-III and Fe$^{2+}$ against phosphate buffer; spectrum 3 represents the spectrum of Fe$^{2+}$ against phosphate buffer.
Figure 8. Differential spectra of CPPms-III with ferric iron: Spectrum 1 represents the spectrum of CPPms-III against 10mM phosphate buffer (pH 7.4); spectrum 2 represents the spectrum of a mixture of CPPms-III and Fe$^{3+}$ against phosphate buffer; spectrum 3 represents the spectrum of Fe$^{3+}$ against phosphate buffer.
Figure 9. Differential spectra of CPPms-III with calcium: Spectrum 1 represents the spectrum of CPPms-III against 10mM phosphate buffer (pH 7.4); spectrum 2 represents the spectrum of a mixture of CPPms-III and Ca$^{2+}$ against phosphate buffer; spectrum 3 represents the spectrum of Ca$^{2+}$ against phosphate buffer.
3.2.3 Site-Specific and non site-specific scavenging activity of CPPms

The affinities of CPPms to sequester iron and quench hydroxyl radicals were evaluated using two different assays in a deoxyribose model. In the site-specific assay (e.g. $\text{Fe}^{2+} + \text{H}_2\text{O}_2$), the affinity of CPPms to chelate iron and minimize the direct interaction between the pro-oxidative iron and deoxyribose is tested. With the addition of EDTA in the non site-specific assay (e.g. $\text{Fe}^{2+}$-EDTA + $\text{H}_2\text{O}_2$), direct $\text{Fe}^{2+}$ binding to deoxyribose is restricted which favors the formation of OH in the aqueous medium. In the latter assay, the affinity of CPPms to quench hydroxyl radicals was tested.

CPPms was found to be highly effective in suppressing radical induced damage of deoxyribose in both assays (Table 4; Figures 10a and 10b). This result suggests that not only does CPP possess secondary antioxidant characteristics, as a metal chelator, but CPPms can also act as a primary antioxidant via direct interference with free radicals. Both samples of CPPms exhibited a concentration dependent increase in activity. CPPms-I inhibited up to 50% of the deoxyribose degradation, at 1.0 mg/ml, in both assays, whereas CPPms-III, at 1.0mg/ml, lowered deoxyribose degradation by 50% in site-specific assay, but only 25% in the non site-specific assay. In the non site-specific assay, CPPms-I was significantly more effective in inhibiting the deoxyribose oxidation than CPPms-III ($p<0.01$) at the 2 highest concentrations (i.e. 0.5 and 1.0 mg/ml).
Table 4. Effect of CPPms-I and -III on Percent Inhibition of Deoxyribose Degradation.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Concentration of CPP (mg/ml)</th>
<th>CPPms</th>
<th>0.05</th>
<th>0.10</th>
<th>0.50</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site-Specific Binding</td>
<td>I</td>
<td>33.6 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.2 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.2 ± 1.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>46.2 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>27.1 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.8 ± 0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>40.5 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.5 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Non Site-Specific Binding</td>
<td>I</td>
<td>-0.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.4 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.7 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>-0.5 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-0.5 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.5 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

1. % Inhibition = \( \frac{\text{Abs}_{532\text{nm}_{\text{control}}} - \text{Abs}_{532\text{nm}_{\text{sample}}}}{\text{Abs}_{532\text{nm}_{\text{control}}}} \times 100; \)

   Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (e.g. a, b, c and d) are significantly differently (p<0.01);
   Means within the same column for each assay that do not share a common superscript letter (e.g. x and y) are significantly differently (p<0.01)

2. Ascorbic acid mediated Fenton Reaction in the absence of EDTA
3. Ascorbic acid mediated Fenton Reaction in the presence of EDTA
Figure 10a. Effect of CPPms-I (■) and CPPms-III (□) on Percent Inhibition of Deoxyribose Degradation (Site-Specific Assay)\(^1\).

1. \( \% \) Inhibition = \( \frac{\text{Abs } 532\text{nm}_{\text{control}} - \text{Abs } 532\text{nm}_{\text{sample}}}{\text{Abs } 532\text{nm}_{\text{control}}} \times 100; \)

Results are expressed as Mean \( \pm \) SD, \( n = 3; \)
Reaction initiated by ascorbic acid mediated Fenton Reaction in the absence of EDTA.
Figure 10b. Effect of CPPms-I (■) and CPPms-III (□) on Percent Inhibition of Deoxyribose Degradation (Non Site-Specific Assay)\(^1\).

1. \[
\text{% Inhibition} = \frac{\text{Abs}_{532\text{nm}}^{\text{control}} - \text{Abs}_{532\text{nm}}^{\text{sample}}}{\text{Abs}_{532\text{nm}}^{\text{control}}} \times 100;
\]

Results are expressed as Mean ± SD, n = 3;

Reaction initiated by ascorbic acid mediated Fenton Reaction in the presence of EDTA.
3.2.4 Effect of CPPms on the formation of Conjugated Diene

The effect of CPPms in protecting lecithin liposomes (i.e. an emulsion system) from oxidation, induced by pro-oxidative free radicals or bivalent metals, was determined by monitoring the formation of conjugated diene at an absorbance of 234 nm. The rates of propagation, expressed as percent of control, were calculated by applying linear regression to the kinetic plots of absorbance (A234nm), against time of incubation (minutes) and comparing the slopes obtained for each treatment (Appendix; Figures A1 and A2). Inhibition in the rate of propagation was most significant (p<0.01) at the highest concentration of CPPms used (i.e. 0.50 mg/ml). CPPms-I found to be two fold more effective than CPPms-III (p<0.01) in quenching the free radical AAPH at the liposomal interface, lowering oxidation to 23.0 % control as compared to 43.3 % control for CPPms-III.

The addition of ferrous ions initiated liposome oxidation possibly through an intricate series of free radical chain reactions. Since plots of absorbance against incubation time showed a more rapid progression of oxidation, an alternate method was used to analyze the kinetics of propagation. Percent inhibition was calculated at each time point of the plot to achieve a plateau (Appendix; Figures A3 and A4) and the y-intercept was used for comparison (Table 5).

In this assay, CPPms-I and CPPms-III were not significantly different in activity (p>0.05) at the highest concentration (i.e. 0.50 mg/ml) and lowest concentration (i.e. 0.05 mg/ml). However, CPPms-III was more effective (p<0.01) in lowering iron-induced oxidation in liposomes at a concentration of 0.10 mg/ml. Both CPPms-I and -III were shown to possess the most antioxidative activity at the highest concentration, 0.50 mg/ml,
inhibiting 66.4 and 53.4% of the oxidation respectively. Other known metal chelators such as phytic acid and EDTA (0.5mM) were also used in the Fe(II)-induced liposome oxidation as positive controls (Appendix; Figure A5).

Table 5. Rate of Propagation\(^2\) (% Control) of Liposome Peroxidation induced by Peroxyl Radical (AAPH) and % Inhibition\(^3\) of Liposome Peroxidation induced by Transition Metal Ion (Fe\(^{2+}\))\(^1\).

<table>
<thead>
<tr>
<th>Assay</th>
<th>CPPms</th>
<th>Concentration of CPPms (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>AAPH(^2)</td>
<td>I</td>
<td>73.1 ± 1.4 (^{ax})</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>76.8 ± 1.8 (^{ax})</td>
</tr>
<tr>
<td>(Fe(^{2+}))(^3)</td>
<td>I</td>
<td>11.4 ± 2.4 (^{ax})</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>25.8 ± 1.3 (^{ax})</td>
</tr>
</tbody>
</table>

1. Results are expressed as Mean ± SD, n = 3 individual experiments; Means within the same row that do not share a common superscript letter (e.g. a, b and c) are significantly differently (p<0.01); Means within the same column that do not share a common superscript letter (e.g. x and y) are significantly differently (p<0.01).
2. Rate of Propagation was determined by application of linear regression on kinetic graphs and expressed as % control.
3. % Inhibition \([\frac{(1-A_{234\text{nm(sample)}}/A_{234\text{nm(control)}})}{1}]*100\) is calculated for individual time points and application of linear regression on kinetic graphs was used to achieve a constant (y-intercept).
3.2.5 Effect of CPPms on scavenging stable DPPH and ABTS radicals

The free radical scavenging activity of CPPms was determined by measuring the direct effect of CPPms on pre-formed stable radicals. The odd electron in the DPPH radical displays a strong absorption band at an absorbance of 517 nm, which is absent once the odd electron is paired off by a hydrogen- or electron-donating antioxidant. No significant hydrogen/electron donating activity was observed for either preparation of CPPms (Table 6), possibly due to the low solubility of CPPms in the solvent, ethanol, used in this assay.

A more hydrophilic free radical, ABTS, was also used to evaluate the free radical quenching activity of CPPms. The reduction of monocation, ABTS\(^+\), accompanied by a reduction in absorbance at 734 nm, thus the extent of discoloration of ABTS\(^+\), when expressed as a percentage inhibition, is a function of the radical quenching affinity of CPPms.

CPPms-I was shown to be a potent electron/proton donor with concentration dependent activity that stabilized up to 92.8% of the radicals at 1.0 mg/ml (Table 7). This activity was equivalent to the activity of 23.7 \(\mu\)M Trolox. Hydrogen/electron donating activity was not as apparent in the CPP-III samples, which stabilized only 8.4% of the radicals at the highest concentration (1.0 mg/ml).
Table 6. Effect of CPPms-I and -III on Scavenging Stable DPPH Radicals.\(^1\)

<table>
<thead>
<tr>
<th>Concentration of CPPms (mg/ml)</th>
<th>CPPms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>I</td>
<td>-5.94 ± 4.8(^{ax})</td>
</tr>
<tr>
<td>III</td>
<td>-7.10 ± 2.12(^{ax})</td>
</tr>
</tbody>
</table>

1. Values represent \% scavenging = \(\frac{\text{Abs} \: 519\text{nm}_{\text{control}} - \text{Abs} \: 519\text{nm}_{\text{sample}}}{\text{Abs} \: 519\text{nm}_{\text{control}} \times 100}\); Results are expressed as Mean ± SD, \(n = 3\); Means within the same row that do not share a common superscript letter (e.g. a, b, c and d) are significantly differently (\(p<0.01\)); Means within the same column that do not share a common superscript letter (e.g. x and y) are significantly differently (\(p<0.01\)).
Table 7. Effect of CPPms-I and -III on Scavenging Stable ABTS Radicals.\(^1\)

<table>
<thead>
<tr>
<th>Concentration of CPPms (mg/ml)</th>
<th>CPPms</th>
<th>0.05</th>
<th>0.10</th>
<th>0.50</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (TE)(^2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.5 ± 0.7 \textsuperscript{ax}</td>
<td>7.9 ± 1.0 \textsuperscript{by}</td>
<td>75.1 ± 1.6 \textsuperscript{cy}</td>
<td>91.8 ± 0.5 \textsuperscript{dy}</td>
<td></td>
</tr>
<tr>
<td>(TE)(^2)</td>
<td>(0.5)</td>
<td>(2.2)</td>
<td>(19.4)</td>
<td>(23.7)</td>
<td></td>
</tr>
<tr>
<td>III (TE)(^2)</td>
<td>0.2 ± 1.0 \textsuperscript{ax}</td>
<td>1.1 ± 0.8 \textsuperscript{ax}</td>
<td>3.2 ± 0.7 \textsuperscript{ax}</td>
<td>8.4 ± 0.8 \textsuperscript{ex}</td>
<td></td>
</tr>
<tr>
<td>(TE)(^2)</td>
<td>(0.2)</td>
<td>(0.4)</td>
<td>(1.0)</td>
<td>(2.3)</td>
<td></td>
</tr>
</tbody>
</table>

1. Values represent \(\%\) scavenging = \(\frac{A_{734\text{nm}}^{\text{control}} - A_{734\text{nm}}^{\text{sample}}}{A_{734\text{nm}}^{\text{control}}} \times 100\); 
Results are expressed as Mean ± SD, \(n = 3\); 
Means within the same row that do not share a common superscript letter (e.g. a, b, c and d) are significantly different (\(p<0.01\)); 
Means within the same column that do not share a common superscript letter (e.g. x and y) are significantly different (\(p<0.01\));

2. TE = the calculated Trolox equivalence (\(\mu\text{M}\)) using the standard curve equation 
\(y = 3.896x - 0.5844\); where \(x\) stands for micromolars of Trolox and \(y\) for \(\%\) scavenging.
3.3 Discussion

As an in vitro antioxidant assessment, caseinophosphopeptides, CPPms-I and -III from Meiji Seika Kaisha Ltd. (Japan), were analyzed for mineral sequestering and free radical quenching activities in both aqueous and emulsion models. Both samples of CPPms contained peptides with a molecular weight of less than 6kDa, which were consistent with the peptides that are derived from α_{s1}-, α_{s2}- and β-caseins reported by other researchers (Naito et al., 1990; Reynolds et al., 1994; Ono et al., 1998; Gagnaire et al., 1996). Other higher molecular weight peptides, derived from trypsin hydrolysis of casein, were found only in CPPms-I but not in CPPms-III.

A qualitative analysis of metal sequestering activity, using a simple absorbance scan over the range of 200 to 600 nm, showed the interaction between CPPms and metal ions such as ferrous iron and ferric iron. Spectra for the CPPms-metal complexes exhibited distinctly different apparent absorbance peaks than the corresponding CPPms and metal ions. The metal sequestering affinity of CPP has been attributed to the presence of a high concentration of phosphoserine residues in close proximity, thereby creating a polar and anionic domain that is highly electrostatically favorable for cationic metal ions (Reeve and Latour, 1958). The observed affinity of phosphoseryl residues, present in α_{s1}-casein (64-68) and β-casein (15-19), towards iron was reported by Manson and Cannon (1978). Phosphoseryl residues catalyzed oxidation of iron from ferrous to the ferric state, thereby resulting in the formation of highly stable iron-phosphoprotein complexes. CPP can sequester up to 4 mM of ferrous iron, creating iron adducts through delicate conformational changes in the peptide and attachment of iron to the oxygen on
phosphoserine in a tetrahedral coordination structure (Gaucheron et al., 1996). Once the free ferrous iron is bound by CPP, iron can be oxidized but partial oxidation may yield 2 different species of CPP-iron complexes. This confirms the affinity of phosphoserines to form stable chelates with ferrous as well as ferric iron (Galdi and Valencia, 1988), as shown by the reported detection of both βCN(1-25)/Fe$^{3+}$ and βCN (1-25)/Fe$^{2+}$ complexes when the β-casein derived CPP, βCN(1-25), was incubated with ferrous iron (Bouhallab et al., 1991). Affinity of phosphoserine residues towards Ca$^{2+}$ can be affected by changes in pH and ionic strength with a lower binding ability occurring at high ionic strength and pH lower than 7, while the affinity of phosphoserine residues towards Fe$^{2+}$ was unaffected (Baumy and Brulé, 1988). A pH of 7.0 and ionic concentration of 0.10 mM of phosphate was used in this study and provided an adequate sequestering environment for the peptides to interact effectively with the metal ions of interest.

An assay involving hydroxyl radical-induced degradation of deoxyribose, the backbone sugar found in deoxyribonucleic acid (DNA) in an ascorbate mediated Fenton reaction was used to investigate the potential antioxidant activity of CPP. Since deoxyribose has an affinity towards iron, the free ferrous iron, in the site-specific assay (e.g. Fe$^{2+}$ + H$_2$O$_2$), interacts directly with deoxyribose before generation of hydroxyl radicals (·OH) from Fenton reaction. With the addition of EDTA in the non site-specific assay (e.g. Fe$^{2+}$-EDTA + H$_2$O$_2$), direct Fe$^{2+}$ binding to deoxyribose is restricted which favors the formation of ·OH in the aqueous medium. The antioxidant affinity of CPP is then directly related to its affinity for Fe$^{2+}$ in the former assay, and to its scavenging capacity for ·OH radicals in the latter.
The lower hydroxyl radical scavenging activity in the presence of EDTA (non-site-specific assay) for highly purified CPP-III samples indicates that antioxidant activity was comparatively more pronounced as a ferrous iron chelator, than as a hydroxyl radical quencher. In contrast, CPP-I was equally effective with either role. This observation is interesting in view of reports showing that \( \alpha_{s1} \) and \( \beta \)-caseins can scavenge hydroxyl radicals in a deoxyribose model (Cervato et al., 1999).

In addition to aqueous models, the antioxidant activity of CPP was also investigated in a phospholipid liposome (i.e. emulsion) model. Spherical liposomes were formed spontaneously when phospholipids (e.g. soybean lecithin) dispersed into an aqueous buffer. Oxidation was initiated by the addition of ferrous ions or free radicals, such as AAPH (2,2'-azobis-2-amidinopropane-dihydrochloride), and the extent of oxidation was monitored by the formation of conjugated diene products at an absorbance of 234 nm. AAPH is a hydrophilic radical initiator that produces carboxy radicals and peroxyl radicals in the presence of molecular oxygen. Trace amounts of metal ions were eliminated by chelax ion-exchange chromatography prior to the exposure to AAPH to eliminate the possibility that decomposition of lipid peroxides was attributed to metal ion-catalyzed oxidation.

Both CPP-I and -III were found to effectively lower the iron- and AAPH-induced oxidative damage of liposomes. The results provided strong evidence that that phosphopeptides are potent antioxidants not only in an aqueous model but also in a lipophilic model. Phosphate groups in both free and serine-bound forms (orthophosphate and phosphoserine) were shown previously to have a lower effect on oxidation, compared to the corresponding phosphorylated caseins (Cervato et al., 1999). Thus, it is logical to
conclude that phosphoserine groups in CPP may be capable of partitioning into emulsion droplets and available to lower oxidative damage triggered by free radicals and ferrous iron.

CPP at the liposomal surface can donate hydrogen atoms to stabilize peroxyl radicals and therefore diminishing the peroxidative effect of radicals on the emulsion droplet. Thermolysis of azo-substances, such as AAPH, generates a continuous flux of peroxyl radicals (ROO') that react with nearby substrates, extracting hydrogen atoms from substrates such as lipids (L-H) to generate a new radical L' [Equation 15]. Formation of this radical invokes a free radical chain reaction resulting in the deterioration of lipids. However, in the presence of a chain-breaking antioxidant (AH), such as CPP, the chain reaction is terminated [Equation 16] and oxidation inhibited.

\[
\text{ROO}^+ + \text{L-H} \rightarrow \text{ROOH} + \text{L}' \quad [15]
\]

\[
\text{ROO}^+ + \text{A-H} \rightarrow \text{ROOH} + \text{A}' \quad [16]
\]

Higher phosphopeptide content, as in CPP-III, were likely to be responsible for the two-fold greater AAPH quenching affinity at the liposome surface, when compared to CPP-I that has a lower phosphopeptide content.

In addition to the free radical initiator, ferrous iron was also used as a pro-oxidant in this study because it has a high reactivity in emulsion models. Positively charged metal ions are attracted to the negatively charged droplet surface resulting in generation of more free radicals, at or near the droplet surface (Mei et al., 1999). It has been shown that Fe^{2+} associated much better with anionic rather than with cationic or non-ionic hexadecane emulsions (Mei et al., 1998). Oxidation thereby increases as iron-emulsion
droplet interactions increase. Iron-induced oxidation levels in salmon oil-in-water emulsions were shown to be higher at pH 7.0 than lower pH values, due to a lower solubility and a resultant precipitation of the metal onto the lipid droplet surface. The net effect of this is the closer association of iron with the lipid, which enhances oxidation rates (Mancuso et al., 1999). Phosphoserines were shown to partition into the lipid phase and successfully chelate iron donated by ligands, thereby significantly impeding oxidative deterioration of iron-fortified milk (Hegenauer et al., 1979).

In the case of the positively charged phospholipid liposomes used in this study, the affinity of Fe\(^{2+}\) towards the emulsion droplet was apparent since the oxidation proceeded in an exponential manner. Negatively charged CPP may play a role in the repulsive electrostatic interactions occurring between charged emulsion droplets and charged oxidant by promoting CPP-metal association and lowering attachment of the metal onto the emulsion droplet.

Metal chelators, such as phytic acid and EDTA, were chosen as positive controls to validate the efficacy of the ferrous iron-induced liposome oxidation. Both of these species were capable of lowering the extent of oxidation, as observed for different concentrations of CPP, confirming the previous findings that demonstrated the efficacy of EDTA and phytate (50-2000 \(\mu\)M) in protecting SDS-salmon oil emulsions from ferrous chloride (500 \(\mu\)M) induced oxidation (Mei et al., 1998).

Caseins have been shown to be most active in lowering TBARS formation in Fe/ascorbate-induced peroxidation of arachidonic acid supplemented liposomes when compared to lactoferrin and lactoglobulin. Among the caseins, \(\alpha\)-casein, carrying 10 phosphate groups, was shown to be most potent in extending the latency of peroxidation
when compared to β-casein with 5 phosphate groups and κ-casein with only one phosphate group (Cervato et al., 1999). In addition to iron chelating activity, caseins also prevented hydroxyl radical formation, thereby suggesting that the mechanisms of antioxidant action were complex involving mechanisms other than simple iron-chelating activity. In a similar copper (Cu²⁺) metal-induced oxidation model, caseins were more effective at protecting trilinolein emulsions than whole whey protein and α-lactalbumin (Allen and Wrieden, 1982).

Other proteins derived from bovine milk, such as whey, are also active in lowering oxidation but through different mechanisms. Bovine whey proteins including iron-binding lactoferrin and serum albumin were shown to block transition metals and scavenge free radicals via aminio acid residues tyrosine and cysteine. With sulfhydryl residues, whey proteins scavenged peroxyl radicals and chelate iron to significantly lower formation of lipid peroxides in salmon-in-oil emulsions (Tong et al., 2000), corn oil emulsions (Huang et al., 1999), phosphatidylcholine liposomes (Colbert and Decker, 1991; Shinmoto et al., 1992; Heinonen et al., 1998; Wakabayashi et al., 1999) and infant formulas (Satué-Gracia et al., 2000).

In order to verify the free radical quenching affinity of CPP, two additional free radical assays were used. The first assay involves a stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical that can accept an electron or hydrogen to become a stable, diamagnetic molecule invulnerable to oxidation. Because of its odd electron, DPPH shows a strong absorption band at 517 nm in ethanol (Blois, 1958). When this odd electron is paired-off by an antioxidant (AH), the absorption vanishes and the resulting discoloration becomes a measure of the radical scavenging activity (Equation 17).
However, CPP did not show any significant hydrogen/electron donating activity in this assay. This lack of activity could possibly be due to the low solubility of CPP in the solvent, ethanol, used. In a polar solvent, CPP structures can undergo conformation changes, possibly reducing the extent of contact between the peptide and the free radicals and rendering the acidic, hydrophilic functional domain ineffective.

A second assay involves the radical monocations 2,2'-azinobis-(3-ethylenothiazoline-6-sulfonic acid (ABTS') that are generated by oxidation of ABTS with ammonium persulfate and reduced in the presence of hydrogen-donating antioxidants (Re et al, 1999). A similar hydrogen donation occurred in the ABTS assay:

\[(\text{ABTS})^+ + \text{A-H} \rightarrow (\text{ABTS}):\text{H} + \text{A}^-\]  

The addition of hydrogen/electron donating antioxidants (AH) to the monocation reduces it back to ABTS, thereby diminishing the strong absorption reading at 734 nm. Thus the extent of discoloration of ABTS⁺ as percentage inhibition is a function of the radical quenching ability. Because of the higher hydrophilicity in the medium (i.e. deionized water), CPP was shown to effectively scavenge and hence stabilize the ABTS⁺ cations. In contrast to the findings from the emulsion model, the crude CPP-I sample had a nine-fold more efficient hydrogen/electron donating than the CPP-III sample. The saturation of calcium ions, in the final steps of CPP-III preparation, may have interfered with the anionic functional domain created by the phosphoserines and contributed to a lowered activity to interact with ABTS⁺ cations, despite the higher concentration of phosphoserine residues in the CPP-III sample.
In previous studies, this ABTS assay had been used extensively to detect antioxidant activity of various phenolic compounds in beverages and food extracts, but has yet to be applied to the milk system. However, free radical scavenging activity was identified in an isolated peptic hydrolysate of casein with a primary structure of Tyr-Phe-Tyr-Pro-Glu-Leu (1kDa) (Suetsuna et al., 2000). This peptide also had a potent superoxide anion scavenging activity specifically at the Glu-Leu residues and also played an important role in quenching hydroxyl and DPPH radicals (Suetsuna et al., 2000). Although CPP does not contain this particular peptide, it is highly likely that the functional domain in CPP with the sequence SerP-SerP-SerP-Glu-Glu has comparable activity not only in metal chelation but also in hydrogen/electron donation and free radical scavenging.

3.4 Conclusion

Caseinophosphopeptides (CPP) derived from tryptic digests of bovine casein, molecular weight 19 – 25kDa, were characterized to consist of molecular weights less than 6kDa. Two different CPP fractions, CPP-I and CPP-III, were shown to interact with ferrous iron, ferric iron and calcium ions and were screened for in vitro antioxidant activity. CPP-I and CPP-III exhibited significant (p<0.01) inhibition of both site-specific and non site-specific degradation of deoxyribose in a Fenton reaction oxidation test and protected liposomes from oxidation induced by ferrous iron and free radical AAPH. CPP-I was more effective (p<0.01) at quenching ABTS radicals than CPP-III. These results demonstrate a potential affinity for bioactive casein derived peptides to prevent peroxidation reactions in labile food systems and reduce oxidative stress in the digestive tract.
Figure A1. Effect of CPP I at 0 mg/ml (●), 0.05 mg/ml (□), 0.10 mg/ml (■) and 0.50 mg/ml (X) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by peroxyl radicals generated by addition of 2mM AAPH at 37°C.
Figure A2. Effect of CPP III at 0 mg/ml (●), 0.05 mg/ml (□), 0.10 mg/ml (■) and 0.50 mg/ml (×) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by peroxyl radicals generated addition of 2mM AAPH at 37°C.
Figure A3. Effect of CPP I at 0 mg/ml (◆), 0.05 mg/ml (□), 0.10 mg/ml (■) and 0.50 mg/ml (X) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by Fenton reaction generated by addition of 0.50 mM FeSO₄ at 37°C.
Figure A4. Effect of CPP III at 0 mg/ml (◆), 0.05 mg/ml (□), 0.10 mg/ml (■) and 0.50 mg/ml (×) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by Fenton reaction generated by addition of 0.50 mM FeSO₄ at 37°C.
Figure A5. Effect of Control (●), 0.5 mM Phytic Acid (□) and 0.5 mM EDTA (×) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by Fenton reaction generated by addition of 0.50 mM FeSO₄ at 37°C.
CHAPTER IV: Antioxidant effects of laboratory prepared CPP and conjugates

4.1 Materials and Methods

4.1.1 CPP and conjugates formulations

CPP-I, II and III were prepared by dissolving bovine milk casein in water making a 10% solution, with a pH adjusted to 7.5 to 8.5 (Kawano et al., 1991). Crystalline trypsin from porcine pancrease was added to a 0.01% (w/v) final concentration against the substrate and incubated at 50°C for 6 hours. CPP-I was obtained by spray-drying the whole hydrolysate. The hydrolysate was further purified by ion-exchange chromatography using Dowex 50wx8 cation exchange column (Dow Chemical Co.), followed by activated charcoal column (American Norit Co.) and was labeled CPP-II. CPP-III enriched with phosphopeptides was obtained by filtrating the insoluble mass from the hydrolysate at pH 4.5 before precipitating the phosphopeptides by adding calcium chloride (1.1% w/v) and ethanol (50% v/v) to the filtrate (Figure A12).

CPP and galactomannan, a mannase hydrolysate of guar gum, (Taiyo Chemicals Co. Japan), or xyloglucan, a β-glucanase hydrolysate of tamarind seed (Dainihon Pharmaceuticals Osaka), were mixed in water at a weight ratio of 1:1 and lyophilized. The resulting powder mixture was incubated for 3 days at 60°C under 65% relative humidity. The incubated sample was applied on an open column (1.6 x 5.5cm) with DEAE-Toyopearl (Tosoh, Tokyo) equilibrated with 20 mM phosphate buffer (pH 7.4) and the column was washed with the same buffer to separate free carbohydrate. The CPP conjugates were eluted with 500 mM NaCl buffer (pH 7.4). Peak fractions were collected together and applied to an open column with Sephacryl S-300HR (Amersham/Pharmacia Biotech). The conjugates were eluted with 0.1M phosphate
buffer (pH 7.4). Concentration of protein was directly determined by absorbance at 280 nm, while that of carbohydrate was determined by the absorbance at 470 nm after color development using phenol-sulfuric acid reaction. The molar binding ratio between CPP-III and galactomannan was 1:1.9 and for CPP-III and xyloglucan was 1:2.2.

Calcium free samples of CPP conjugates were prepared by dissolving 100mg CPP conjugates in 10 mM EDTA and stirring the mixture for 2 hours at room temperature before dialyzing against deionized water for 2 days at 4°C using Spectra/Por MWCO 1000 (SPECTRUM LAB Inc.).

4.1.2 Molecular Weight and Mineral Characterization

Casein, CPP-I, CPP-II and CPP-III were analyzed by electrophoresis using 16.5% acrylamide Tris-Tricine (N-tris [hydroxymethyl] methyl glycine) SDS ready gel (10 well), according to the method of Laemmli (1970), and a Mini-Protean I Mini-Cell slab gel electrophoresis unit (BIO-RAD Laboratories, Richmond, CA). A 4% acrylamide stacking gel, pH 6.8, was used. Protein samples (40 μg) and polypeptide molecular standards were heated at 100°C for 5 minutes in 1.0 M Tris-tricine buffer, pH 6.8 containing 10% SDS. Electrophoresis was carried out at a constant voltage of 100 volts for 100 minutes with a Tris-tricine running buffer. Gels were placed in 40% methanol and 10% acetic acid fixative solution for 30 minutes, stained in 0.025% Coomassie Blue G-250 solution (10% acetic acid) for 1 hour and destained in 10% acetic acid for 3 x 15 minute destain washes.

Mineral characterization of CPP was performed on 10mg CPP-I, II and III by ICP-MS analysis for calcium, iron, magnesium, zinc and copper at Elemental Research Inc. (Appendix; Table A1).
4.1.3 Hydroxyl Radical Scavenging Assay in a Deoxyribose model

A Fenton reaction model containing 0.1 mM of Fe$^{3+}$ as the catalytic metal was used. The substrate 2-deoxyribose (3.6 mM) was mixed together with CPP (0.05, 0.10, 0.50, 1.0 mg/ml), 0.1 mM EDTA, 0.1 mM Ferric chloride, 0.1 mM ascorbic acid and 1 mM H$_2$O$_2$. The reaction mixture was incubated at 37°C for 1 hour. Following incubation, 1 ml of 10% (v/v) trichloroacetic acid (TCA) and 1% 2-thiobarbituric acid (TBA) were added and the mixture was boiled for 15 minutes at 95°C. Absorbance at 532 nm was recorded after cooling. The extent of deoxyribose degradation by hydroxyl radicals generated by the Fenton reaction was calculated using the equation:

\[
\% \text{ Inhibition} = \frac{\text{Abs}_{532\text{nm}_{\text{control}}} - \text{Abs}_{532\text{nm}_{\text{sample}}}}{\text{Abs}_{532\text{nm}_{\text{control}}}} \times 100
\]

4.1.4 Peroxyl Radical Scavenging Assays in a Liposome model

Liposomes were made by sonicating soybean lecithin (α-phosphatidylcholine) in 10 mM phosphate buffer (pH 7.4) in an ice-water bath for 20 minutes, according to Hu and Kitts (2000). Peroxyl radical induced-liposomal peroxidation was conducted at a constant temperature of 37°C using an ATI Unicam (UV2) UV/Vis spectrophotometer. The reaction was initiated by the addition of 0.2 mM AAPH to a mixture of 0.1 mg/ml of liposome in 10 mM phosphate buffer (pH 7.4) and CPP. Generation of conjugated diene hydroperoxide was monitored by taking absorbance readings at 234 nm every 4 minutes for 100 minutes.

4.1.5 Free radical scavenging assay

The affinity of CPP to scavenge stable 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cations was investigated (Pellegrini et al., 1999). Distilled deionized water was used in place of ethanol for radical preparation. Discoloration was
determined by comparing the absorbance at 734 nm of the treatment groups with the control after an 8-minute incubation at room temperature. The inhibitory percentage of ABTS was calculated according to the following equation:

\[
\% \text{ Inhibition} = \frac{\text{Abs}_{734\text{nm}}^{\text{control}} - \text{Abs}_{734\text{nm}}^{\text{sample}}}{\text{Abs}_{734\text{nm}}^{\text{control}}} \times 100
\]

4.1.6 Statistical Analysis

All data (with the exception of the liposome assays) were collected in triplicates and analysed by one way ANOVA (\(\alpha \leq 0.01\)), followed by a multiple range Tukey post test analysis using the GraphPad Prism Analysis software (GraphPad Software Inc., San Diego CA) to identify significant differences among treatment means (\(P \leq 0.01\)). Data were obtained from 3 different individual experiments for the liposome assays and submitted to linear regression using GraphPad software. Rates of propagation (i.e. slope of regression equation) were then analyzed by one way ANOVA (\(\alpha \leq 0.01\)) and Tukey test (\(P \leq 0.01\)).
4.2 Results

4.2.1 Molecular weight and mineral content of CPP

Using a tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis, TSDS-PAGE (Lesse et al., 1990), a higher resolution molecular weight characterization for CPP was achieved. It was found that CPP-I, II and III consisted of peptides of sizes that range from 3.5 to 6.5 kDa (Figure 11). Other researchers have reported that CPP contained peptides corresponding to the 2.6 kDa $\alpha_s$2-casein (2-21)-4P (Reynolds et al., 1994) or the 2.7 kDa $\alpha_s$1-casein (59-79)-5P (Reynolds et al., 1994; Ono et al., 1998; Gerber and Jost, 1986). The higher molecular weight peptides present in CPP-I and II seemed to be larger hydrolysis fragments of casein proteins. Casein on the other hand had a range of peptides with molecular weights of 24 to 35 kDa, which seemed to correspond to the reported molecular weights of $\alpha_s$, $\alpha_s$2 and $\beta$, around 22-23 kDa, 25 kDa and 23-34 kDa (Swaisgood, 1982).

Each gram of CPP-I and II was shown to contain 400 to 500 µg of calcium, whereas CPP-III had exceedingly high calcium content of 58.6 mg (Appendix; Table A1). All CPP preparations had a similar iron content of 200 to 400 µg iron per gram peptide. The mineral profiles of CPP reflect the preparation methods employed. CPP-II had a relatively low calcium content, since the casein hydrolysates underwent cation-exchange chromatography as part of the purification procedures. In contrast, CPP-III was saturated with calcium ion because of the addition of calcium chloride in the final preparation steps.
Figure 11. 16.5% Tris-Tricine SDS-PAGE Gel stained with Coomassie G-250 using the Laemmli buffer system (1.0 M Tris, pH 8.45). Lane 1: 40 μg CPP-I, Lane 2: 40 μg CPP-II, Lane 3: 40 μg CPP-III, Lane 4: 40 μg Casein, Std: Polypeptide Standards.
Phosphopeptide content in CPP-I, II and III were 12.3%, 12.3% and 86.1%, respectively (Saito et al., 1993). Phosphoserine concentration in CPP-I and CPP-III measured by HPLC was reported to be 0.16 and 1.22 mmol/g, respectively (Kasai et al., 1995). HPLC analysis of CPP-III also confirmed the peptide content to be 83-93% (w/w), mainly consisting of residues (1-32) of bovine $\alpha_{s2}$-casein and residues (1-28) $\beta$-casein (Hirayama et al., 1992b) and a nitrogen:phosphorus ratio of 8.0 (Saito et al., 1998).

4.2.2 Site-Specific and Non Site-Specific Scavenging Activity of CPP and Conjugates

CPP inhibited the radical-induced degradation of deoxyribose by 2 processes, through metal chelating and hydroxyl radical scavenging. Oxidative degradation of deoxyribose was inhibited to a greater extent through free radical scavenging up to 60% at 1.00mg/ml CPP (Table 8; Figure 12a). In comparison, 32% inhibition occurred at the same concentration through ferrous iron chelation (Table 8; Figure 12b). CPP concentration was a greater factor in the metal chelating activity of CPP, than either the purity or calcium content. Although free radical scavenging activity also followed a concentration dependent increase, CPP-III was consistently more potent at inhibiting hydroxyl radical generation (Figure 12a), thus demonstrating the importance of the higher purity peptides in order to fully exploit CPP bioactivity.

CPP-I, II and III were conjugated to polysaccharide galactomannan (GAL) and oligosaccharide xyloglucan (XYL), and the effect of glycosylation on the antioxidant activity of CPP was explored. Both saccharides are highly hydrophilic and upon attachment on the reducing ends of CPP result in an increase in surface activity and possibly altered structural conformation.
Hydroxyl radical scavenging activity and ferrous iron binding activity were both dramatically lower for the peptide-saccharide conjugates than for the peptides alone (Tables 9 and 10). Peptide-saccharide conjugates inhibited significantly (p<0.01) less deoxyribose oxidation than the corresponding peptide, at all concentrations tested in both assays. GAL-CPP conjugates inhibited, on average, 10-20% less deoxyribose oxidation than the corresponding CPP. In comparison, XYL-CPP conjugates had at least 30% less inhibition of deoxyribose oxidation, or no inhibition at all. GAL alone resulted in 28.8% inhibition of deoxyribose oxidation, while XYL exhibited 8.2% (Table 11; Table 13). It was found that GAL-CPP conjugates were not superior in antioxidant activity compared to simple, control mixtures of peptides and sugars. GAL and CPP mixtures were significantly more effective (p<0.01) in scavenging hydroxyl radicals at all concentrations tested, but had better (p<0.01) or equivalent (p>0.05) metal chelating activities than the GAL-CPP conjugates. XYL-CPP conjugates had greater (p<0.01) radical scavenging activity, but lower ferrous iron binding activity (p<0.01) than corresponding mixtures. Removal of calcium via EDTA treatment following conjugation significantly lowered (p<0.01) the activity of the GAL-CPP conjugates regardless of concentration (Table 12), but improved (p<0.01) that of XYL-CPP conjugates at concentration of 0.50 mg/ml and higher (Table 14).
Table 8. Effect of CPP I, II and III on Percent Inhibition of Degradation of Deoxyribose.\textsuperscript{1}

<table>
<thead>
<tr>
<th>Assay</th>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>0.10</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Non Site-Specific Binding\textsuperscript{2}</td>
<td>CPPI</td>
<td>25.3 ± 0.3 \textsuperscript{ax}</td>
<td>34.2 ± 1.1 \textsuperscript{bx}</td>
<td>53.6 ± 0.6 \textsuperscript{cx}</td>
<td>61.3 ± 0.7 \textsuperscript{dxy}</td>
</tr>
<tr>
<td></td>
<td>CPPII</td>
<td>29.9 ± 0.6 \textsuperscript{ay}</td>
<td>34.9 ± 0.7 \textsuperscript{bx}</td>
<td>52.9 ± 0.5 \textsuperscript{cx}</td>
<td>60.4 ± 0.7 \textsuperscript{dx}</td>
</tr>
<tr>
<td></td>
<td>CPPIII</td>
<td>34.7 ± 0.8 \textsuperscript{az}</td>
<td>50.7 ± 0.9 \textsuperscript{by}</td>
<td>60.0 ± 0.7 \textsuperscript{cy}</td>
<td>63.3 ± 0.7 \textsuperscript{dy}</td>
</tr>
<tr>
<td>Site-Specific Binding\textsuperscript{3}</td>
<td>CPPI</td>
<td>17.8 ± 0.5 \textsuperscript{ax}</td>
<td>16.2 ± 0.9 \textsuperscript{ax}</td>
<td>24.5 ± 0.5 \textsuperscript{bx}</td>
<td>32.1 ± 1.1 \textsuperscript{cx}</td>
</tr>
<tr>
<td></td>
<td>CPPII</td>
<td>18.2 ± 0.1 \textsuperscript{ay}</td>
<td>18.4 ± 0.1 \textsuperscript{axy}</td>
<td>26.5 ± 0.4 \textsuperscript{bx}</td>
<td>32.9 ± 0.9 \textsuperscript{cx}</td>
</tr>
<tr>
<td></td>
<td>CPPIII</td>
<td>16.8 ± 1.4 \textsuperscript{ax}</td>
<td>19.5 ± 0.9 \textsuperscript{ay}</td>
<td>26.8 ± 0.3 \textsuperscript{bx}</td>
<td>32.1 ± 1.0 \textsuperscript{cx}</td>
</tr>
</tbody>
</table>

1. \% Inhibition = $100 \times \frac{(A_{532\text{nm control}} - A_{532\text{nm sample}})}{A_{532\text{nm control}}}$; Results are expressed as Mean ± SD, n = 3; Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01); Means within the same column of CPP and concentration that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01)

2. Ascorbic acid mediated Fenton Reaction in the presence of EDTA

3. Ascorbic acid mediated Fenton Reaction in the absence of EDTA
% Inhibition = $100 \times \frac{A_{532\text{nm}}\text{control} - A_{532\text{nm}}\text{sample}}{A_{532\text{nm}}\text{control}}$

Results are expressed as Mean ± SD, n = 3;
Reaction initiated by ascorbic acid mediated Fenton Reaction in the presence of EDTA (Non Site-Specific Binding Assay).

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Figure 12a. Effect of CPP I (■), CPP II (□) and CPP III (☒) on Percent Inhibition of Degradation of Deoxynribose.¹
Figure 12b. Effect of CPP I (■), CPP II (□) and CPP III (■■) on Percent Inhibition of Degradation of Deoxyribose.¹

1. % Inhibition = 100x (A532nm\textsubscript{control} – A532nm\textsubscript{sample})/ A532nm\textsubscript{control};
Results are expressed as Mean ± SD, n = 3;
Reaction initiated by ascorbic acid mediated Fenton Reaction in the absence of EDTA (Site-Specific Binding Assay).
Table 9. Effect of CPP I, II and III and Conjugates on Percent Inhibition\(^1\) of Degradation of Deoxyribose\(^2\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>CPPI</td>
<td>25.3 ± 0.3 (^{ ay} )</td>
</tr>
<tr>
<td>Gal-CPPI</td>
<td>8.5 ± 0.9 (^ { ax} )</td>
</tr>
<tr>
<td>Xyl-CPPI</td>
<td>11.8 ± 1.3 (^ { ax} )</td>
</tr>
<tr>
<td>CPPII</td>
<td>29.9 ± 0.6 (^ { ay} )</td>
</tr>
<tr>
<td>Gal-CPPII</td>
<td>13.0 ± 0.7 (^ { ax} )</td>
</tr>
<tr>
<td>Xyl-CPPII</td>
<td>12.4 ± 0.9 (^ { ax} )</td>
</tr>
<tr>
<td>CPPIII</td>
<td>34.7 ± 0.8 (^ { ay} )</td>
</tr>
<tr>
<td>Gal-CPPIII</td>
<td>9.8 ± 0.8 (^ { ax} )</td>
</tr>
<tr>
<td>Xyl-CPPIII</td>
<td>10.6 ± 1.0 (^ { ax} )</td>
</tr>
</tbody>
</table>

1. % Inhibition = \( 100 \times \frac{A_{532\text{nm}_{\text{control}} - A_{532\text{nm}_{\text{sample}}}}}{A_{532\text{nm}_{\text{control}}}} \);
   Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
   Means within the same column of CPP and concentration that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
   Gal-CPP denotes conjugates of galactomannan and CPP, while Xyl-CPP denotes conjugates of xyloglucan and CPP.

2. Ascorbic acid mediated Fenton Reaction in the presence of EDTA (Non Site-Specific Binding Assay)
Table 10. Effect of CPP I, II and III and Conjugates on Percent Inhibition\(^1\) of Degradation of Deoxyribose\(^2\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>CPPI</td>
<td>17.8 ± 0.5 (^{\text{ay}})</td>
</tr>
<tr>
<td>Gal-CPPI</td>
<td>0.3 ± 0.7 (^{\text{ax}})</td>
</tr>
<tr>
<td>Xyl-CPPI</td>
<td>2.8 ± 1.5 (^{\text{bx}})</td>
</tr>
<tr>
<td>CPPII</td>
<td>18.2 ± 0.1 (^{\text{ay}})</td>
</tr>
<tr>
<td>Gal-CPPII</td>
<td>7.1 ± 1.5 (^{\text{ax}})</td>
</tr>
<tr>
<td>Xyl-CPPII</td>
<td>2.1 ± 1.0 (^{\text{bx}})</td>
</tr>
<tr>
<td>CPPIII</td>
<td>16.8 ± 1.4 (^{\text{ay}})</td>
</tr>
<tr>
<td>Gal-CPPIII</td>
<td>8.2 ± 1.7 (^{\text{ax}})</td>
</tr>
<tr>
<td>Xyl-CPPIII</td>
<td>5.3 ± 1.7 (^{\text{bx}})</td>
</tr>
</tbody>
</table>

1. % Inhibition = 100x\((A_{532\text{nm}}^{\text{control}} - A_{532\text{nm}}^{\text{sample}})/A_{532\text{nm}}^{\text{control}}\);
   Results are expressed as Mean ± SD, \(n = 3\);
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (\(p<0.01\));
   Means within the same column of CPP and concentration that do not share a common superscript letter (x, y and z) are significantly differently (\(p<0.01\));
   Gal-CPP denotes conjugates of galactomannan and CPP, while Xyl-CPP denotes conjugates of xyloglucan and CPP.

2. Ascorbic acid mediated Fenton Reaction in the absence of EDTA (Site-Specific Binding Assay)
Table 11. Effect of Galactomannan and CPP I, II and III Conjugates and Mixtures on Percent Inhibition of Degradation of Deoxyribose. 1

<table>
<thead>
<tr>
<th>Assay</th>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
<th>Concentration of CPP (mg/ml)</th>
<th>Concentration of CPP (mg/ml)</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>0.10</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Non Site-Specific Binding 2</td>
<td>Gal</td>
<td>13.9 ± 0.7 a</td>
<td>21.6 ± 0.5 b</td>
<td>24.4 ± 1.3 b</td>
<td>28.8 ± 1.2 c</td>
</tr>
<tr>
<td></td>
<td>Gal&amp;CPPI</td>
<td>14.6 ± 0.3 ay</td>
<td>23.1 ± 0.8 by</td>
<td>42.3 ± 0.4 by</td>
<td>50.0 ± 0.6 dy</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPI</td>
<td>8.5 ± 0.9 ax</td>
<td>13.4 ± 0.4 bx</td>
<td>28.3 ± 0.5 cx</td>
<td>34.9 ± 0.8 dx</td>
</tr>
<tr>
<td></td>
<td>Gal&amp;CPPII</td>
<td>20.6 ± 0.8 ay</td>
<td>35.1 ± 0.2 by</td>
<td>53.3 ± 1.0 cy</td>
<td>58.0 ± 1.1 dy</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPII</td>
<td>13.0 ± 0.7 ax</td>
<td>21.3 ± 0.5 bx</td>
<td>32.9 ± 0.4 cx</td>
<td>36.6 ± 1.0 dx</td>
</tr>
<tr>
<td></td>
<td>Gal&amp;CPPIII</td>
<td>15.4 ± 0.5 ay</td>
<td>33.8 ± 0.7 by</td>
<td>63.5 ± 0.4 cy</td>
<td>65.3 ± 0.6 cy</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPII</td>
<td>9.8 ± 0.8 ax</td>
<td>19.9 ± 0.5 bx</td>
<td>47.0 ± 1.1 cx</td>
<td>47.8 ± 0.8 cx</td>
</tr>
<tr>
<td>Site-Specific Binding 3</td>
<td>Gal</td>
<td>5.6 ± 0.8 a</td>
<td>4.0 ± 1.0 a</td>
<td>8.1 ± 1.4 ab</td>
<td>10.2 ± 1.5 b</td>
</tr>
<tr>
<td></td>
<td>Gal&amp;CPPI</td>
<td>-3.8 ± 0.5 ax</td>
<td>5.8 ± 1.8 bx</td>
<td>7.6 ± 1.0 bx</td>
<td>13.8 ± 2.4 cx</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPI</td>
<td>0.3 ± 0.7 ax</td>
<td>3.1 ± 1.4 abx</td>
<td>7.9 ± 1.9 bcx</td>
<td>12.2 ± 0.5 cx</td>
</tr>
<tr>
<td></td>
<td>Gal&amp;CPPII</td>
<td>14.7 ± 1.3 ay</td>
<td>12.0 ± 1.4 aby</td>
<td>14.3 ± 1.5 aby</td>
<td>17.6 ± 1.3 bx</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPII</td>
<td>7.1 ± 1.5 ax</td>
<td>6.5 ± 1.4 ax</td>
<td>9.2 ± 1.2 ax</td>
<td>14.4 ± 1.7 bx</td>
</tr>
<tr>
<td></td>
<td>Gal&amp;CPPIII</td>
<td>12.2 ± 0.8 ay</td>
<td>15.7 ± 0.5 aby</td>
<td>19.1 ± 0.7 bx</td>
<td>24.3 ± 0.6 cy</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPIII</td>
<td>8.2 ± 1.7 ax</td>
<td>10.0 ± 0.5 ax</td>
<td>16.0 ± 2.21 bx</td>
<td>19.6 ± 0.5 bx</td>
</tr>
</tbody>
</table>

1. % Inhibition = 100x(A532nm control − A532nm sample)/ A532nm control;
   Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
   Means within the same column of CPP and concentration that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
   Gal-CPP denotes conjugates of galactomannan and CPP, while Gal&CPP denotes mixtures of galactomannan and CPP.
2. Ascorbic acid mediated Fenton Reaction in the presence of EDTA
3. Ascorbic acid mediated Fenton Reaction in the absence of EDTA
Table 12. Effect of Galactomannan and CPP I, II and III Conjugates and Calcium Free (CF) Conjugates on Percent Inhibition of Degradation of Deoxyribose.¹

<table>
<thead>
<tr>
<th>Assay</th>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
<th>0.05</th>
<th>0.10</th>
<th>0.50</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Site-Specific Binding²</td>
<td>Gal-CPPI</td>
<td></td>
<td>8.5 ± 0.9  ax</td>
<td>13.4 ± 0.4  by</td>
<td>28.3 ± 0.5  cy</td>
<td>34.9 ± 0.8  dy</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPI-CF</td>
<td></td>
<td>5.0 ± 1.4  ax</td>
<td>8.0 ± 1.5  ax</td>
<td>16.2 ± 0.6  bx</td>
<td>28.0 ± 1.8  cx</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPII</td>
<td></td>
<td>13.0 ± 0.7  ay</td>
<td>21.3 ± 0.5  by</td>
<td>32.9 ± 0.4  cy</td>
<td>36.6 ± 1.0  dy</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPII-CF</td>
<td></td>
<td>3.9 ± 1.2  ax</td>
<td>7.4 ± 1.3  ax</td>
<td>17.8 ± 1.1  bx</td>
<td>28.8 ± 1.9  cx</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPIII</td>
<td></td>
<td>9.8 ± 0.8  ay</td>
<td>19.9 ± 0.5  by</td>
<td>47.0 ± 1.1  cy</td>
<td>47.8 ± 0.8  by</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPIII-CF</td>
<td></td>
<td>2.3 ± 0.6  ax</td>
<td>3.9 ± 0.4  ax</td>
<td>12.4 ± 1.1  bx</td>
<td>26.0 ± 1.4  cx</td>
</tr>
<tr>
<td>Site-Specific Binding³</td>
<td>Gal-CPPI</td>
<td></td>
<td>0.3 ± 0.7  ay</td>
<td>3.1 ± 1.4  aby</td>
<td>7.9 ± 1.9  bey</td>
<td>12.2 ± 0.5  cy</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPI-CF</td>
<td></td>
<td>-34.6 ± 1.5  ax</td>
<td>-36.1 ± 1.9  ax</td>
<td>-23.2 ± 1.2  bx</td>
<td>-6.4 ± 1.1  cx</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPII</td>
<td></td>
<td>7.1 ± 1.5  ay</td>
<td>6.5 ± 1.4  ay</td>
<td>9.2 ± 1.2  ay</td>
<td>14.4 ± 1.7  by</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPII-CF</td>
<td></td>
<td>-36.1 ± 1.4  ax</td>
<td>-38.2 ± 1.2  ax</td>
<td>-24.3 ± 1.6  bx</td>
<td>-5.8 ± 1.6  cx</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPIII</td>
<td></td>
<td>8.2 ± 1.7  ay</td>
<td>10.0 ± 0.5  ay</td>
<td>16.0 ± 2.2  by</td>
<td>19.6 ± 0.5  by</td>
</tr>
<tr>
<td></td>
<td>Gal-CPPIII-CF</td>
<td></td>
<td>-44.6 ± 1.4  ax</td>
<td>-44.4 ± 1.7  ax</td>
<td>-31.1 ± 0.8  bx</td>
<td>-12.7 ± 1.8  cx</td>
</tr>
</tbody>
</table>

1. % Inhibition = 100x(A532nm control – A532nm sample)/A532nm control;
   Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
   Means within the same column of CPP and concentration that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
   Gal-CPP denotes conjugates of galactomannan and CPP, while Gal-CPP-CF denotes calcium free conjugates of galactomannan and CPP.

2. Ascorbic acid mediated Fenton Reaction in the presence of EDTA

3. Ascorbic acid mediated Fenton Reaction in the absence of EDTA
Table 13. Effect of Xyloglucan and CPP I, II and III Conjugates and Mixtures on Percent Inhibition of Degradation of Deoxyribose. 1

<table>
<thead>
<tr>
<th>Assay</th>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>0.10</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Non Site-Specific Binding 2</td>
<td>Xyl</td>
<td>12.4 ± 1.7 b</td>
<td>7.9 ± 1.1 ab</td>
<td>5.2 ± 1.2 a</td>
<td>8.2 ± 1.5 ab</td>
</tr>
<tr>
<td></td>
<td>Xyl&amp;CPPI</td>
<td>10.1 ± 0.2 ax</td>
<td>11.8 ± 1.4 ax</td>
<td>20.9 ± 0.8 bx</td>
<td>27.6 ± 1.0 cx</td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPI</td>
<td>11.8 ± 1.3 ax</td>
<td>11.8 ± 1.2 ax</td>
<td>20.5 ± 0.8 bx</td>
<td>26.3 ± 0.6 cx</td>
</tr>
<tr>
<td>Site-Specific Binding 3</td>
<td>Xyl</td>
<td>-4.0 ± 2.3 b</td>
<td>-10.3 ± 0.8 a</td>
<td>-11.3 ± 0.8 a</td>
<td>-11.5 ± 1.8 a</td>
</tr>
<tr>
<td></td>
<td>Xyl&amp;CPPI</td>
<td>12.2 ± 0.6 by</td>
<td>13.9 ± 0.2 by</td>
<td>7.6 ± 0.5 ay</td>
<td>11.4 ± 0.2 by</td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPI</td>
<td>2.8 ± 1.5 bx</td>
<td>2.6 ± 0.8 bx</td>
<td>-5.4 ± 1.7 ax</td>
<td>-6.1 ± 1.8 ax</td>
</tr>
<tr>
<td></td>
<td>Xyl</td>
<td>8.2 ± 2.1 ax</td>
<td>9.3 ± 1.8 aby</td>
<td>8.7 ± 0.6 ay</td>
<td>12.6 ± 0.5 by</td>
</tr>
<tr>
<td></td>
<td>Xyl&amp;CPPI</td>
<td>5.3 ± 1.7 bx</td>
<td>2.5 ± 1.6 abx</td>
<td>0.2 ± 2.0 abx</td>
<td>-0.4 ± 1.2 ax</td>
</tr>
<tr>
<td></td>
<td>Xyl</td>
<td>14.7 ± 1.3 cy</td>
<td>9.8 ± 1.2 aby</td>
<td>6.9 ± 0.6 ay</td>
<td>10.6 ± 0.9 by</td>
</tr>
<tr>
<td></td>
<td>Xyl&amp;CPPI</td>
<td>2.1 ± 1.0 bx</td>
<td>2.5 ± 1.5 bx</td>
<td>-6.0 ± 1.7 ax</td>
<td>-4.8 ± 1.6 ax</td>
</tr>
</tbody>
</table>

1. % Inhibition = 100x(A532nm\text{control} - A532nm\text{sample})/A532nm\text{control};
   Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
   Means within the same column of CPP and concentration that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
   Xyl-CPP denotes conjugates of xyloglucan and CPP, while Xyl&CPP denotes mixtures of xyloglucan and CPP.
2. Ascorbic acid mediated Fenton Reaction in the presence of EDTA
3. Ascorbic acid mediated Fenton Reaction in the absence of EDTA
Table 14. Effect of Xyloglucan and CPP I, II and III Conjugates and Calcium Free (CF) Conjugates on Percent Inhibition of Degradation of Deoxyribose.\(^1\)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
<th>0.05</th>
<th>0.10</th>
<th>0.50</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.10</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Non Site-Specific Binding(^2)</td>
<td>Xyl-CPP</td>
<td>11.8 ± 1.3(^{ax})</td>
<td>11.8 ± 1.2(^{ax})</td>
<td>20.5 ± 0.8(^{bx})</td>
<td>26.3 ± 0.6(^{cx})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPP-CF</td>
<td>12.5 ± 1.0(^{ax})</td>
<td>10.3 ± 1.6(^{ax})</td>
<td>28.4 ± 0.3(^{by})</td>
<td>40.3 ± 0.3(^{cy})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPII</td>
<td>12.4 ± 0.9(^{ax})</td>
<td>16.2 ± 1.5(^{bx})</td>
<td>23.4 ± 0.6(^{cx})</td>
<td>27.2 ± 0.2(^{dx})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPII-CF</td>
<td>12.3 ± 0.4(^{ax})</td>
<td>13.0 ± 1.2(^{ax})</td>
<td>29.4 ± 1.6(^{by})</td>
<td>41.4 ± 0.4(^{cy})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPIII</td>
<td>10.6 ± 1.0(^{ax})</td>
<td>16.8 ± 0.9(^{by})</td>
<td>34.6 ± 0.4(^{cy})</td>
<td>36.8 ± 1.1(^{ex})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPIII-CF</td>
<td>10.6 ± 0.9(^{ax})</td>
<td>9.2 ± 0.6(^{ax})</td>
<td>22.8 ± 0.6(^{bx})</td>
<td>35.1 ± 1.3(^{ex})</td>
<td></td>
</tr>
<tr>
<td>Site-Specific Binding(^3)</td>
<td>Xyl-CPP</td>
<td>2.8 ± 1.5(^{by})</td>
<td>2.6 ± 0.8(^{by})</td>
<td>-5.4 ± 1.7(^{ax})</td>
<td>-6.1 ± 1.8(^{ax})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPP-CF</td>
<td>-14.6 ± 2.8(^{ax})</td>
<td>-13.8 ± 2.5(^{ax})</td>
<td>1.7 ± 0.4(^{by})</td>
<td>18.7 ± 0.7(^{by})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPII</td>
<td>2.1 ± 1.0(^{by})</td>
<td>2.5 ± 1.5(^{by})</td>
<td>-6.0 ± 1.7(^{ax})</td>
<td>-4.8 ± 1.6(^{ax})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPII-CF</td>
<td>-15.9 ± 1.4(^{ax})</td>
<td>-12.23 ± 1.4(^{ax})</td>
<td>3.8 ± 0.9(^{by})</td>
<td>21.6 ± 0.5(^{by})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPIII</td>
<td>5.3 ± 1.7(^{by})</td>
<td>2.5 ± 1.6(^{aby})</td>
<td>0.2 ± 2.0(^{aby})</td>
<td>-0.4 ± 1.2(^{ax})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xyl-CPPIII-CF</td>
<td>-18.5 ± 1.3(^{ax})</td>
<td>-14.5 ± 2.0(^{ax})</td>
<td>-5.9 ± 1.5(^{bx})</td>
<td>5.6 ± 1.6(^{cy})</td>
<td></td>
</tr>
</tbody>
</table>

1. % Inhibition = 100\(|\text{A532nm}_{\text{control}} - \text{A532nm}_{\text{sample}}|\)\text{A532nm}_{\text{control}};
   Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
   Means within the same column of CPP and concentration that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
   Xyl-CPP denotes conjugates of xyloglucan and CPP, while Xyl-CPP-CF denotes calcium free conjugates of xyloglucan and CPP.

2. Ascorbic acid mediated Fenton Reaction in the presence of EDTA

3. Ascorbic acid mediated Fenton Reaction in the absence of EDTA
4.2.3 Effect of CPP and Conjugates on the formation of Conjugated Diene

Addition of ferrous ion to the liposome emulsion system resulted in unexpected instability. Thus, kinetics for propagation varied and could not be reliably reproduced. Therefore, although trends were present for a lower oxidation (Figures A6, A7 and A8) in the presence of CPP with the positive control, phytic acid (1mM), the variation in propagation kinetics of iron-induced oxidation in this assay did not allow for reliable calculation of propagation rates.

In order to better demonstrate the bioactivity of CPP in scavenging free radical induced-oxidation by AAPH in an emulsion model, Trolox was added as a positive control at a concentration of 1.0 μg/ml (Hu and Kitts, 2000). CPP was most potent at lowering peroxyl radical-induced damage on liposomes to 30% of control at the highest concentration (0.50 mg/ml) tested (Figures A9, A10 and A11). This effect was comparable to the Trolox control (Table 15). Antioxidant activity of CPP-I, II and III followed a concentration dependent effect at lowering % control lipid propagation rate and was not significantly different (p>0.05) among preparations at each concentration tested. Conjugation of CPP-I, II and III to GAL and XYL did not affect CPP affinity to sequester peroxyl radical AAPH and lower liposome oxidation (Table 16). The presence of galactomannan at 0.50mg/ml significantly (p<0.01) lowered the propagation rate to 35.8%, having comparable effects as the Trolox control (Table 17). In contrast, xyloglucan at 0.50mg/ml did not have an effect at lowering propagation rate by maintaining 82.0 % control (Table 19). When the GAL and CPP-I or II were added to the emulsion as a mixture, the polysaccharide and the CPP when present as separate entities, lowered oxidation to a greater extent than when conjugated at concentrations of
0.10 mg/ml and 0.50 mg/ml, respectively (Table 17). A similar effect was observed for XYL and CPP-I or II mixtures at higher concentrations (Table 19). However, mixtures of GAL or XYL with CPP-III were not different from the conjugates in antioxidant activity. Calcium free conjugates of GAL and XYL did not significantly improve calcium-containing conjugates activity (Table 18; Table 20).

### Table 15. Effect of CPP I, II and III on the rate of propagation (% Control) of liposome peroxidation induced by peroxyl radicals (AAPH).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of Trolox (µg/ml)</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>CPPI</td>
<td>31.5±3.3&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>61.8±2.3&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPPII</td>
<td>28.1±2.3&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>63.7±1.6&lt;sup&gt;cx&lt;/sup&gt;</td>
</tr>
<tr>
<td>CPPIII</td>
<td>32.0±1.3&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>67.7±4.3&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. Rate of Propagation (% Control) was determined by application of linear regression on kinetic graphs; Results are expressed as Mean ± SD, n = 3 individual experiments; Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01); Means within the same column that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of Trolox (µg/ml)</th>
<th>Concentration of CPP (mg/ml)</th>
<th>1.0</th>
<th>0.05</th>
<th>0.10</th>
<th>0.25</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPPI</td>
<td>1.0</td>
<td>0.05</td>
<td>31.5±3.3&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>61.8±2.3&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>57.0±3.6&lt;sup&gt;by&lt;/sup&gt;</td>
<td>45.6±3.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>30.6±1.2&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPI</td>
<td>28.3±2.5&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>65.2±7.0&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>53.5±8.7&lt;sup&gt;by&lt;/sup&gt;</td>
<td>ND</td>
<td>22.3±2.3&lt;sup&gt;ax&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-CPPI</td>
<td>29.0±1.0&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>49.8±11.1&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>33.4±7.2&lt;sup&gt;abx&lt;/sup&gt;</td>
<td>ND</td>
<td>24.2±1.1&lt;sup&gt;ax&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPPII</td>
<td>28.1±2.3&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>63.7±1.6&lt;sup&gt;ex&lt;/sup&gt;</td>
<td>56.9±4.7&lt;sup&gt;bey&lt;/sup&gt;</td>
<td>44.3±4.0&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>33.8±2.7&lt;sup&gt;ax&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-CPPII</td>
<td>28.7±5.4&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>66.6±10.3&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>41.5±7.9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>ND</td>
<td>21.4±2.7&lt;sup&gt;ax&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-CPPII</td>
<td>24.5±0.5&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>42.5±8.3&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>25.1±2.6&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>ND</td>
<td>19.0±1.1&lt;sup&gt;ax&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPPIII</td>
<td>32.0±1.3&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>67.7±4.3&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>57.6±3.6&lt;sup&gt;by&lt;/sup&gt;</td>
<td>46.3±6.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>38.3±6.8&lt;sup&gt;abx&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-CPPIII</td>
<td>27.2±7.8&lt;sup&gt;abx&lt;/sup&gt;</td>
<td>70.5±7.0&lt;sup&gt;ex&lt;/sup&gt;</td>
<td>49.3±7.0&lt;sup&gt;bexy&lt;/sup&gt;</td>
<td>ND</td>
<td>23.3±5.7&lt;sup&gt;ax&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-CPPIII</td>
<td>28.1±6.2&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>73.2±8.5&lt;sup&gt;ey&lt;/sup&gt;</td>
<td>50.1±3.2&lt;sup&gt;bex&lt;/sup&gt;</td>
<td>ND</td>
<td>26.6±7.0&lt;sup&gt;ax&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Rate of Propagation (% Control) was determined by application of linear regression on kinetic graphs; Results are expressed as Mean ± SD, n = 3 individual experiments; Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01); Means within the same column that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01); ND represents values that were not determined; Gal-CPP denotes conjugates of galactomannan and CPP, while Xyl-CPP denotes conjugates of xyloglucan and CPP.
Table 17. Effect of Galactomannan and CPP I, II and III Conjugates and Mixtures on the rate of propagation (% Control) of liposome peroxidation induced by peroxyl radicals (AAPH).  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of Trolox (µg/ml)</th>
<th>Concentration of Conjugate or Mixture (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>G</td>
<td>24.3 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.5 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G&amp;CPPI</td>
<td>40.9 ± 5.0&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>84.9 ± 3.7&lt;sup&gt;cy&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPI</td>
<td>28.3 ± 2.5&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>65.2 ± 7.0&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td>G&amp;CPPII</td>
<td>40.9 ± 5.0&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>83.5 ± 4.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPII</td>
<td>28.7 ± 5.4&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>66.6 ± 10.3&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td>G&amp;CPPIII</td>
<td>26.0 ± 1.7&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>68.9 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPIII</td>
<td>27.2 ± 7.8&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>70.5 ± 7.0&lt;sup&gt;cx&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. Rate of Propagation (% Control) was determined by application of linear regression on kinetic graphs;
Results are expressed as Mean ± SD, n = 3 individual experiments;
Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
Means within the same column that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
Gal-CPP denotes conjugates of galactomannan and CPP, while Gal&CPP denotes mixtures of galactomannan and CPP.
Table 18. Effect of Galactomannan and CPP I, II and III Conjugates and Calcium Free (CF) Conjugates on the rate of propagation (% Control) of liposome peroxidation induced by peroxyl radicals (AAPH).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of Trolox (µg/ml)</th>
<th>Concentration of Conjugate (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>G-CPPI</td>
<td>28.3 ± 2.5&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>65.2 ± 7.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPI-CF</td>
<td>28.3 ± 2.5&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>64.1 ± 10.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPII</td>
<td>28.7 ± 5.4&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>66.6 ± 10.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPII-CF</td>
<td>24.0 ± 4.3&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>57.4 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPIII</td>
<td>27.2 ± 7.8&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>70.5 ± 7.0&lt;sup&gt;ex&lt;/sup&gt;</td>
</tr>
<tr>
<td>G-CPPIII-CF</td>
<td>27.2 ± 7.8&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>67.1 ± 8.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. Rate of Propagation (% Control) was determined by application of linear regression on kinetic graphs; Results are expressed as Mean ± SD, n = 3 individual experiments; Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01); Means within the same column that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01); Gal-CPP denotes conjugates of galactomannan and CPP, while Gal-CPP-CF denotes calcium free conjugates of galactomannan and CPP.
Table 19. Effect of Xyloglucan and CPP I, II and III Conjugates and Mixtures on the rate of propagation (% Control) of liposome peroxidation induced by peroxyl radicals (AAPH).  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of Trolox (µg/ml)</th>
<th>Concentration of Conjugate or Mixture (mg/ml)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>X</td>
<td>24.3 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.7 ± 1.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98.1 ± 3.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>X &amp; CPPI</td>
<td>26.0 ± 1.7&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>67.1 ± 3.7&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>54.5 ± 3.4&lt;sup&gt;by&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-CPPI</td>
<td>29.0 ± 1.0&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>49.8 ± 11.1&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>33.4 ± 7.2&lt;sup&gt;abx&lt;/sup&gt;</td>
</tr>
<tr>
<td>X &amp; CPPII</td>
<td>27.4 ± 3.1&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>71.1 ± 5.5&lt;sup&gt;by&lt;/sup&gt;</td>
<td>65.1 ± 4.7&lt;sup&gt;by&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-CPPII</td>
<td>24.5 ± 0.5&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>42.5 ± 8.3&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>25.1 ± 2.6&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>X&amp;CPPIII</td>
<td>27.4 ± 3.1&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>77.7 ± 3.4&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>68.8 ± 4.8&lt;sup&gt;by&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-CPPIII</td>
<td>28.1 ± 6.2&lt;sup&gt;abx&lt;/sup&gt;</td>
<td>73.2 ± 8.5&lt;sup&gt;cx&lt;/sup&gt;</td>
<td>50.1 ± 3.2&lt;sup&gt;bxc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. Rate of Propagation (% Control) was determined by application of linear regression on kinetic graphs; 
   Results are expressed as Mean ± SD, n = 3 individual experiments; 
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01); 
   Means within the same column that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01); 
   Xyl-CPP denotes conjugates of xyloglucan and CPP, while Xyl&CPP denotes mixtures of xyloglucan and CPP.
Table 20. Effect of Xyloglucan and CPP I, II and III Conjugates and Calcium Free Conjugates on the rate of propagation (% Control) of liposome peroxidation induced by peroxyl radicals (AAPH). 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of Trolox (µg/ml)</th>
<th>Concentration of Conjugate (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>X-CPPI</td>
<td>29.0 ± 1.0&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>49.8 ± 11.1&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-CPPI-CF</td>
<td>29.0 ± 1.0&lt;sup&gt;abx&lt;/sup&gt;</td>
<td>60.5 ± 6.6&lt;sup&gt;bcx&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-CPPII</td>
<td>24.5 ± 0.5&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>42.5 ± 8.3&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-CPPII-CF</td>
<td>24.5 ± 0.5&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>52.3 ± 5.7&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-CPPIII</td>
<td>28.1 ± 6.2&lt;sup&gt;abx&lt;/sup&gt;</td>
<td>73.2 ± 8.5&lt;sup&gt;cx&lt;/sup&gt;</td>
</tr>
<tr>
<td>X-CPPIII-CF</td>
<td>28.1 ± 6.2&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>70.4 ± 7.5&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1. Rate of Propagation (% Control) was determined by application of linear regression on kinetic graphs; Results are expressed as Mean ± SD, n = 3 individual experiments; Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01); Means within the same column that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01); Xyl-CPP denotes conjugates of xyloglucan and CPP while Xyl-CPP-CF denotes calcium free conjugates of xyloglucan and CPP.
4.2.4 Effect of CPP and Conjugates on Scavenging ABTS radicals

Assessment of CPP affinity to scavenge aqueous, stable free radical ABTS showed that CPP-II was the most pronounced scavenger, quenching up to 93.9% of the radicals (equivalent to activity of 24.3 μM Trolox) at 1.00 mg/ml. CPP-III at 67.6% scavenging (equivalent to 17.5 μM Trolox) and CPP-I were relatively less effective at stabilizing ABTS (Table 21; Figure 13). This finding demonstrated the importance of CPP purification involving cation-exchange, for obtaining maximum performance of the functional phosphoseryl domain and hydrogen/electron donating activity.

Conjugation of polysaccharide galactomannan and oligosaccharide xyloglucan improved the radical stabilizing activity of CPP-I, but lowered the activity of CPP-II. There was no effect at improving CPP-III activity (Table 22). Radical scavenging of XYL conjugates with CPP-I and II were significantly (p<0.01) greater than counterpart GAL-CPP-I and GAL-CPP-II conjugates, respectively at 0.50 and 1.00 mg/ml.

Galactomannan was capable of scavenging 86.8% of the ABTS radicals, which is equivalent to the activity of 22.4 μM Trolox, while xyloglucan did not further stabilize radicals to a significant extent (Table 23; Table 25). At concentrations of 0.50 mg/ml and higher, galactomannan conjugates with CPP-II and III were more potent hydrogen/electron donors, than corresponding mixtures. Regardless of this finding, the antioxidant activity for GAL-CPP-I were not different from simple mixtures of GAL and CPP-I. However, XYL-CPP conjugates significantly (p<0.01) improved ABTS stability than XYL and CPP mixtures at all concentrations tested. Applying EDTA to GAL and XYL conjugates to remove free calcium was shown to improve the functionality of the conjugates especially at higher concentrations (Table 24; Table 26). The only exception
to this observation was the activity of calcium free XYL-CPP-III conjugates, which had an activity that was equivalent to that of the calcium containing conjugates.

Table 21. Effect of CPP I, II and III on scavenging stable ABTS radicals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>CPPI (^1) (TE) (^2)</td>
<td>0.4 ± 2.6 (^{ax}) (0.3)</td>
</tr>
<tr>
<td>CPPII (^1) (TE) (^2)</td>
<td>1.0 ± 0.9 (^{ax}) (0.9)</td>
</tr>
<tr>
<td>CPPIII (^1) (TE) (^2)</td>
<td>2.3 ± 0.9 (^{ax}) (0.7)</td>
</tr>
</tbody>
</table>

1. % Scavenging = 100 x (A734 nm \(_{\text{control}}\) - A734 nm \(_{\text{sample}}\)) / A734 nm \(_{\text{control}}\);
   Results are expressed as Mean ± SD, \(n = 3\)
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (\(p<0.01\))
   Means within the same column that do not share a common superscript letter (x, y and z) are significantly differently (\(p<0.01\))
2. TE denotes the calculated Trolox equivalence using the standard curve equation \(y = 3.896x - 0.5844\), where \(x\) stands for micromolar (\(\mu M\)) of Trolox and \(y\) for % scavenging.
Figure 13. Effect of CPP I (■), CPP II (□) and CPP III (■) on scavenging stable ABTS radicals. ¹

1. % Scavenging = 100 x (A734nm\(_{\text{control}}\) - A734nm\(_{\text{sample}}\)) / A734nm\(_{\text{control}}\)
Results are expressed as Mean ± SD, n = 3
Table 22. Effect of CPP I, II and III and Conjugates on scavenging stable ABTS radicals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>CPPI(^1) (TE)(^2)</td>
<td>0.4 ± 2.6 (^{ax})</td>
</tr>
<tr>
<td>Gal-CPP(^{1}) (TE)(^2)</td>
<td>4.7 ± 1.1 (^{axy})</td>
</tr>
<tr>
<td>Xyl-CPP(^{1}) (TE)(^2)</td>
<td>6.1 ± 1.4 (^{ay})</td>
</tr>
<tr>
<td>CPPII(^1) (TE) (^x)</td>
<td>1.0 ± 0.9 (^{ax})</td>
</tr>
<tr>
<td>Gal-CPPII(^{1}) (TE)(^2)</td>
<td>4.9 ± 0.5 (^{axy})</td>
</tr>
<tr>
<td>Xyl-CPPII(^{1}) (TE)(^2)</td>
<td>5.3 ± 1.1 (^{ax})</td>
</tr>
<tr>
<td>CPPIII(^1) (TE) (^x)</td>
<td>2.3 ± 0.9 (^{ax})</td>
</tr>
<tr>
<td>Gal-CPPIII(^{1}) (TE)(^2)</td>
<td>4.8 ± 0.7 (^{ax})</td>
</tr>
<tr>
<td>Xyl-CPP III(^{1}) (TE)(^2)</td>
<td>5.8 ± 0.7 (^{ax})</td>
</tr>
</tbody>
</table>

1. % Scavenging = 100 x (A\(734\)nm\(_{\text{control}}\) - A\(734\)nm\(_{\text{sample}}\) / A\(734\)nm\(_{\text{control}}\)); Results are expressed as Mean ± SD, n = 3; Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01); Means within the same column of CPP and concentration that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01); Gal-CPP denotes conjugates of galactomannan and CPP, while Xyl-CPP denotes conjugates of xyloglucan and CPP.

2. TE denotes the calculated Trolox equivalence using the standard curve equation \(y = 3.896x - 0.5844\), where x stands for micromolar (µM) of Trolox and y for % scavenging.
Table 23. Effect of Galactomannan (G) and CPP I, II and III Conjugates and Mixtures on scavenging stable ABTS radicals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td><strong>G</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-0.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(TE)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(0.0)</td>
</tr>
<tr>
<td><strong>G &amp; CPPI</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.0 ± 0.4&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>(TE)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(0.2)</td>
</tr>
<tr>
<td><strong>G-CPPI</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.7 ± 1.1&lt;sup/ay&lt;/sup&gt;</td>
</tr>
<tr>
<td>(TE)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(1.3)</td>
</tr>
<tr>
<td><strong>G &amp; CPPII</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.0 ± 2.0&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>(TE)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(0.7)</td>
</tr>
<tr>
<td><strong>G-CPPII</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.9 ± 0.5&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>(TE)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(1.4)</td>
</tr>
<tr>
<td><strong>G &amp; CPPIII</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>-1.0 ± 0.3&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>(TE)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(-0.1)</td>
</tr>
<tr>
<td><strong>G-CPPIII</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.8 ± 0.7&lt;sup&gt;ay&lt;/sup&gt;</td>
</tr>
<tr>
<td>(TE)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>(1.4)</td>
</tr>
</tbody>
</table>

1. % Scavenging = 100 x (A<sub>734nm control</sub> - A<sub>734nm sample</sub>) / A<sub>734nm control</sub>
   Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
   Means within the same column of CPP and concentration group that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
   Gal-CPP denotes conjugates of galactomannan and CPP, while Gal&CPP denotes mixtures of galactomannan and CPP.

2. TE denotes the calculated Trolox equivalence using the standard curve equation y = 3.896x - 0.5844, where x stands for micromolar (µM) of Trolox and y for % scavenging.
Table 24. Effect of Galactomannan and CPP I, II and III Conjugates and Calcium Free (CF) Conjugates on scavenging stable ABTS radicals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>G-CPPI (TE)²</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>4.7 ± 1.1&lt;sup&gt;ax&lt;/sup&gt; (1.3)</td>
</tr>
<tr>
<td>G-CPPI-CF (TE)²</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>2.7 ± 0.3&lt;sup&gt;ax&lt;/sup&gt; (0.9)</td>
</tr>
<tr>
<td>G-CPPII (TE)²</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>4.9 ± 0.5&lt;sup/ay&lt;/sup&gt; (1.4)</td>
</tr>
<tr>
<td>G-CPPII-CF (TE)²</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>2.6 ± 0.1&lt;sup&gt;ax&lt;/sup&gt; (0.8)</td>
</tr>
<tr>
<td>G-CPPIII (TE)²</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>4.8 ± 0.7&lt;sup&gt;ax&lt;/sup&gt; (1.4)</td>
</tr>
<tr>
<td>G-CPPIII-CF (TE)²</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>3.9 ± 0.8&lt;sup&gt;ax&lt;/sup&gt; (1.1)</td>
</tr>
</tbody>
</table>

1. % Scavenging = 100(A734nm_sample - A734nm_control) / A734nm_control; Results are expressed as Mean ± SD, n = 3; Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01); Means within the same column of CPP and concentration group that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01); Gal-CPP denotes conjugates of galactomannan and CPP, while Gal-CPP-CF denotes calcium free conjugates of galactomannan and CPP.

2. TE denotes the calculated Trolox equivalence using the standard curve equation y = 3.896x - 0.5844, where x stands for micromolar (μM) of Trolox and y for % scavenging.
Table 25. Effect of Xyloglucan (X) and CPP I, II and III Conjugates and Mixtures on scavenging stable ABTS radicals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>X (TE)</td>
<td>1.0 ± 1.5 (^a) (0.4)</td>
</tr>
<tr>
<td>X &amp; CPPI (TE)</td>
<td>0.9 ± 0.6 (^ax) (0.4)</td>
</tr>
<tr>
<td>X-CPPI (TE)</td>
<td>6.1 ± 1.4 (^ay) (1.7)</td>
</tr>
<tr>
<td>X &amp; CPPII (TE)</td>
<td>0.9 ± 1.4 (^ax) (0.4)</td>
</tr>
<tr>
<td>X-CPPII (TE)</td>
<td>5.3 ± 1.1 (^ay) (1.5)</td>
</tr>
<tr>
<td>X &amp; CPPIII (TE)</td>
<td>2.6 ± 0.5 (^ax) (0.8)</td>
</tr>
<tr>
<td>X-CPPIII (TE)</td>
<td>5.8 ± 0.7 (^ay) (1.7)</td>
</tr>
</tbody>
</table>

1. % Scavenging = 100 x (A734nm\text{control} - A734nm\text{sample})/A734nm\text{control};
   Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
   Means within the same column of CPP and concentration group that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
   Xyl-CPP denotes conjugates of xyloglucan and CPP, while Xyl&CPP denotes mixtures of xyloglucan and CPP.
2. TE denotes the calculated Trolox equivalence using the standard curve equation y = 3.896x - 0.5844, where x stands for micromolar (μM) of Trolox and y for % scavenging.
Table 26. Effect of Xyloglucan and CPP I, II and III Conjugates and Calcium Free (CF) Conjugates on scavenging stable ABTS radicals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration of CPP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>X-CPPI (^1) (TE)(^2)</td>
<td>6.1 ± 1.4 (^{ax}) (1.7)</td>
</tr>
<tr>
<td>X-CPPI-CF (^1) (TE)(^2)</td>
<td>5.3 ± 0.1 (^{ax}) (1.5)</td>
</tr>
<tr>
<td>X-CPPII (^1) (TE)(^2)</td>
<td>5.3 ± 1.1 (^{ax}) (1.5)</td>
</tr>
<tr>
<td>X-CPPII-CF (^1) (TE)(^2)</td>
<td>4.9 ± 0.9 (^{ax}) (1.4)</td>
</tr>
<tr>
<td>X-CPPIII (^1) (TE)(^2)</td>
<td>5.8 ± 0.7 (^{ax}) (1.7)</td>
</tr>
<tr>
<td>X-CPPIII-CF (^1) (TE)(^2)</td>
<td>3.2 ± 0.4 (^{ax}) (1.0)</td>
</tr>
</tbody>
</table>

1. % Scavenging = 100 x (A\(^{734\text{nm}}\)\(^\text{control}\) - A\(^{734\text{nm}}\)\(^\text{sample}\))/A\(^{734\text{nm}}\)\(^\text{control}\); Results are expressed as Mean ± SD, n = 3;
   Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01);
   Means within the same column that do not share a common superscript letter (x, y and z) are significantly differently (p<0.01);
   Xyl-CPP denotes conjugates of xyloglucan and CPP, while Xyl-CPP-CF denotes calcium free conjugates of xyloglucan and CPP.

2. TE denotes the calculated Trolox equivalence using the standard curve equation \( y = 3.896x - 0.5844 \), where x stands for micromolar (µM) of Trolox and y for % scavenging.
4.3 Discussion

An antioxidant effect of different protein hydrolysates formed by enzymatic hydrolysis of food protein by-products, such as malt sprouts, brewer’s grains and bovine erythrocytes has been shown to be significantly improved by heating them in the presence of glucose (Lingnert and Eriksson, 1980). Pure amino acid preparations in place of the peptides from the protein hydrolysates did not have the same effect at lowering linoleic acid oxidation, therefore implicating the importance of peptide chains over the amino acid composition in eliciting antioxidant potential. Milk β-casein was found to display higher surface activity and superior adhesion to oil-in-water emulsions than milk whey protein β-lactoglobulin and egg yolk phosvitin (Dickinson et al., 1991). Therefore it is logical that CPP, derived from casein, would also assume an amphiphilic nature, having versatile surface-active behavior attributed to peptide backbone flexibility and hydrophobic-hydrophilic balance. The effect of this physiochemical property on antioxidant activity was tested with CPP in this experiment.

The purpose of this study was to determine the antioxidant effect of laboratory synthesized CPP, and whether conjugation with polysaccharides such as galactomannan or an oligosaccharide, such as xyloglucan, would alter the activity of CPP in both aqueous (water-water interface) and emulsion (oil-water interface) model systems. Conjugation of CPP with saccharides induces conformational change to peptide structure, possibly exposing the functional domains and altering interfacial adsorptivity and hydrophilicity.

Preparation of CPP involved the hydrolysis of bovine casein by trypsin at the carboxyl ends of L-arginine or L-lysine on the protein, freeing lysine groups on CPP
which in turn serve as attachment sites for the reducing ends of galactomannan and xyloglucan during Maillard reactions. CPP derived from α_{s1}-casein have 7 phosphate groups and 3 free amino groups, ideal as attachment sites for galactomannan and xyloglucan molecules. As for peptides derived from β-casein with 4 phosphate groups, there is only 1 free amino group for sugar attachment.

The polysaccharide galactomannan is composed of a (1→4)-β-D-mannan backbone, substituted with a side chain of α-D-galactose, linked (1→6) to mannan residues (Grant Reid, 1985). The oligosaccharide xyloglucan, with a smaller molecular weight distribution range (hepta-, octa-, and nonamer), is derived from tamarind seed and composed of a (1→4)-β-D-glucan backbone substituted with a side chain of α-D-xylose and β-D-galactosyl-(1→2)-α-D-xylose linked (1→6) to glucose residues (Brinson and Dey, 1985). Steric hindrance that occurs during peptide conjugation to long chained galactomannan (MW 14kDa) during Maillard reactions keeps the reaction under control, whereas the lack of hindrance for short-chained xyloglucan (MW 1.4kDa) conjugation may cause the Maillard reaction to go much further.

Three different preparations of CPP as well as conjugation products were used in the antioxidant characterization studies. CPP-I was a crude spray-dried product of whole bovine casein hydrolysis while CPP-II was derived from hydrolysates that were subjected to cation-exchange chromatography. CPP-III was enriched with phosphopeptides through filtration and precipitation via calcium chloride and ethanol, increasing the content of phosphopeptides from 12.3% in CPP-I and II to 86.1% in CPP-III (Saito et al., 1993). Mineral analyses of these peptides showed that CPP-III was highly saturated with calcium, whereas CPP-I and II contained relatively lower calcium contents. Using a
higher resolution Tris-tricine PAGE, it was shown that peptides with approximate
molecular weights that correspond to α_{s2}-casein (2-21), α_{s1}-casein (59-79) and β-casein
(1-28) were identified in synthesized CPP fractions. A number of higher molecular
weight peptides were also identified in CPP-I and II, suggesting the presence of other
hydrolysis fragments of casein proteins.

Table 27. Primary amino sequences of common CPP (Swaisgood, 1982).

<table>
<thead>
<tr>
<th>Casein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>α_{s1}-casein</td>
<td>α_{s1}-casein (57-79) 4P 3<em>Ile</em>-Lys-Gln-Met*-Glu-Ala-Glu-SerP-Ile*-SerP-SerP-SerP-Glu-Glu-Ile*-Val*-Pro*-Asn-SerP-Val*-Glu-Gln-Lys_{79}</td>
</tr>
<tr>
<td>α_{s2}-casein</td>
<td>α_{s2}-casein (2-21) 5P 2<em>Asn-Thr-Met</em>-Glu-His-Val*-SerP-SerP-SerP-SerP-Glu-Glu-SerP-Ile*-Ile*-SerP-Gln-Glu-Thr-Tyr*-Lys_{21}</td>
</tr>
<tr>
<td>β-casein</td>
<td>β-casein (1-28) 4P 1<em>Arg-Glu-Leu</em>-Glu-Glu-Leu*-Asn-Val*-Pro*-Gly-Glu-Ile*-Val*-Glu-SerP-Leu*-SerP-SerP-SerP-SerP-Glu-Glu-SerP-Ile*-Thr-Arg-Ile*-Asn-Lys_{28}</td>
</tr>
</tbody>
</table>

*hydrophobic residues

The presence of a series of phosphoserine residues followed by 2 glutamate
residues is common among all caseinophosphopeptides, and creates a highly
electrostatically ideal domain for cationic metal binding (Table 28). The functionality of
this domain is supported by the conformational structure of the various peptides. With a
relative high content of phosphoseryl residues in the vicinity of hydrophobic residues
such as valine, isoleucine, leucine and proline resides, the CPP can develop a distinct
amphipathic nature with both polar and hydrophobic domains organized in a tertiary
structure. Secondary structures around sites of phosphorylation were predicted to assume
a α-helix-β-loop-α-helix motif with the heavily phosphorylated domain in the loop
region (Holt et al., 1989). CPP is also known to have a highly flexible backbone
allowing residues remote from phosphorylated motif to also interact with metal ions
(Cross et al., 2001).
The bioactivity of this functional domain can be challenged using different experimental conditions. For example, the potential antioxidant activity of CPP and associated conjugates in aqueous conditions was determined using an ascorbate mediated Fenton reaction that resulted in hydroxyl radical-induced degradation of deoxyribose. The presence of EDTA (Fe^{2+}-EDTA + H_2O_2) favors oxidation of ferrous iron and the generation of hydroxyl radicals (OH) in the non site-specific binding assay. In the site-specific binding assay, EDTA is absent, allowing ferrous iron (Fe^{2+} + H_2O_2) to interact directly with deoxyribose before proceeding to generate hydroxyl radicals from Fenton reaction. These two assays were used to determine the affinity of CPP to scavenge OH radicals and to chelate Fe^{2+} respectively.

It was found that not only did CPP successfully inhibit oxidative damage of deoxyribose through chelating catalytic metals to reduce initiation reactions as predicted, but CPP was also shown to stabilize hydroxyl radicals, thus terminating oxidation. CPP-III was consistently stronger than CPP-I and II in hydroxyl radical scavenging activity. On the other hand, the different CPP preparations were not significantly different in relative affinity for ferrous iron. This result suggested that prior saturation with calcium, as in CPP-III, would not affect iron binding activity, simply because CPP had a much stronger affinity towards iron relative to calcium. CPP bound calcium ions would therefore be readily exchanged for iron in the aqueous system.

The eight-fold higher phosphoserine content in CPP-III participated in greater hydroxyl radical quenching but did not make a difference in metal binding activity. Perhaps the majority of ferrous iron in the Fenton reaction was already bound by the 12% phosphoserine as in CPP-I and II, and therefore any further increase in phosphoserine
residues would not lower deoxyribose oxidation to a significant extent. A greater phosphoserine concentration, however, gave the CPP-III peptides a greater potential in electron donating activity to stabilize hydroxyl radicals, thus minimizing initiation and terminating the chain oxidation reactions.

It was proposed that saccharides, being hydrophilic substances, would upon conjugation with CPP improve solubility of the peptides and enhance antioxidant activity in an aqueous model. In spite of an enhanced hydrophilicity, conjugation did not improve the affinity for CPP to exert antioxidant activity by quenching hydroxyl radicals, or binding to metal iron. In contrast, the polysaccharide and oligosaccharides actually reduced the natural antioxidant activity of CPP to different extents. Among the conjugates tested, CPP-GAL conjugates retained CPP antioxidant activity to a greater extent than CPP-XYL conjugates, suggesting that saccharide chain length and possibly extent of the Maillard conjugation might have a role in determining antioxidant activity. It has been shown that xyloglucan, an oligosaccharide derived from tamarind seeds, is highly prone to hydroxyl radical attack (Miller and Fry, 2001), increasing the vulnerability of XYL conjugates to free radical damage and significantly deterring antioxidant capacity. Addition of saccharides to the CPP backbone may have altered the configuration of phosphoseryl residues by disrupting the β-loop groove of the phosphorylated sections of the peptide and thus exposing and rendering the reactive domain ineffective. The distortion in CPP conformation by glycosylation may contribute to the observed reduction in antioxidant activity of CPP-sugar conjugates, when compared to CPP alone.
In addition to the Fenton reaction model, an additional aqueous model was used to confirm the radical scavenging activity of CPP and conjugates. Primary antioxidant activity in CPP and conjugates was measured by the extent of reducing radical monocations (ABTS\(^+\)) by hydrogen/electron donating activity. This assay seemed to be more sensitive with regards to the different relative compositions of CPP. While the crude CPP-I samples had the poorest electron donating power, the calcium-saturated, but higher purity CPP-III, exhibited moderate activity. The calcium free CPP-II proved the strongest (p<0.01) in stabilizing effect of ABTS\(^+\) free radical cations. It was apparent that the presence of calcium cations on CPP seemed to obstruct the electron donating activity of phosphoserine residues. The stable ABTS\(^+\) radicals in this assay are positively charged and electrostatically similar to calcium (Ca\(^{2+}\)), thereby causing a possible charge repulsion at the functional domain which lowers activity to quench free radicals. Conjugation of CPP with galactomannan and xyloglucan lowered the activity of the most potent scavenger CPP-II, did not affect CPP-III scavenging activity, while slightly improved the activity of the weakest scavenger, CPP-I. Similar to the CPP, removing calcium from the conjugates seemed to significantly enhance antioxidant functionality. In comparison to the GAL-CPP-I and GAL-CPP-II conjugates, the XYL-CPP conjugates were consistently more effective at stabilizing the free radical through electron donation, possibly due to a lower steric hindrance amidst the phosphoserine-ABTS interaction. While glycosylation did not improve or affect the activity of more purified samples (i.e. CPP-II and III), it did help to improve in the electron donating activity of CPPI from 3.2% scavenging for CPP-I alone to 67.1% and 72.4% scavenging, for GAL-CPP-I and XYL-CPP-I conjugates respectively. The mechanism by which
polysaccharide and oligosaccharide attachment improved the activity of CPP-I is not known at this time, however it is interesting that the crudest CPP samples showed comparable radical scavenging activity to the more purified CPP through conjugation with saccharides that had different chain lengths.

Aside from aqueous models, the radical scavenging activity of CPP and conjugates in an emulsion model was also determined. Thermolysis of azo substances such as AAPH generates a continuous flux of peroxyl radicals (ROO\(^\cdot\)). Soybean phosphatidylcholine liposomes were shown to have peroxidizable membranes (Porter et al., 1980) that accelerated the peroxidation reactions of AAPH derived radicals generated in the membrane phase (Dean et al., 1991). The extent of antioxidant activity is limited by the chain-breaking affinity of CPP and associated conjugates to localize at the liposome membrane surface (i.e. oil-water interface), the site of radical generated oxidation. Linkage between surface-active peptides and saccharides could result in stabilization of the hydrophilic saccharide at the portion of the aqueous phase, while hydrophobic peptide residues are anchored in the oil droplets (Shu et al., 1996). The change in surface activity should place the peptide-saccharide immediately adjacent to the emulsion, where free radical oxidations occur (Frankel et al., 1994; Shepherd et al., 2000).

It was shown that peroxyl radical scavenging activity was not different among the different preparations of CPP, thereby indicating that phosphoserine and calcium content were not as important as other surface activity factors. The amphiphilic property of CPP provide an ideal conformation for localization at the oil-water interface where it could proficiently quench peroxyl radicals and significantly lower the rate of propagation
reactions to 30.6-38.3% control. Conjugation of CPP with highly hydrophilic polysaccharides and oligosaccharides did not significantly affect the affinity of CPP to localize at the liposome membrane and improve CPP activity.

It has been previously shown that conjugation of galactomannan with phosvitin (an iron-chelating egg yolk phosphoglycoprotein of 35kDa) increased the antioxidant activity of phosvitin in both iron-induced oxidation in phosphatidylcholine liposomes at pH 7.0 (Lee et al., 2002) and linoleic acid models (Uma Maheswari et al., 1997), or a powdered celite model system (Nakamura et al., 1998). No studies to date have established the radical scavenging activity of phosvitin in emulsion models, though phosvitin was capable of scavenging superoxide radicals in an aqueous model (Nakamura et al., 1998).

Enhancement in protein functionality, through glycosylation with galactomannan was shown for whole proteins such as ovalbumin and lysozyme (Nakamura and Kato, 2000). However, perhaps protein hydrolysates in the case of CPP, which have a much lower molecular weight, behaved differently as compared to whole globular proteins. It appeared from this study that glycosylation did not improve the bioactivity of CPP to an extent that can be considered worthwhile for the cumbersome modification reactions. The polar acidic domain, which is a characteristic requirement for metal chelating affinity and also free radical scavenging for CPP in both aqueous and emulsion conditions, does not require further modification to enhance antioxidant activity.
4.4 Conclusion

Results of this experiment showed that the different preparation techniques to produce CPP yield different calcium concentrations and peptide sizes. It appears that all CPP peptides carry the required polar, acidic Ser-P-Ser-P-Ser-P-Glu-Glu domain for ideal conformation for metal binding. CPP III was shown to be the most effective at binding iron and lowering deoxyribose oxidation, while CPP-II was more effective at sequestering ABTS radicals. All of the CPP preparations were equally effective at quenching peroxyl radicals in the liposome peroxidation model. These observations showed that CPP is both a primary and also a secondary antioxidant. Conjugation of CPP with galactomannan and xyloglucan did not enhance the bioactivity of CPP as predicted.
4.5 Appendix

Table A1. ICP-MS analysis of CPP for minerals\(^1\).

<table>
<thead>
<tr>
<th>CPP</th>
<th>Calcium</th>
<th>Iron</th>
<th>Magnesium</th>
<th>Zinc</th>
<th>Copper</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPP-I</td>
<td>400</td>
<td>300</td>
<td>37</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>CPP-II</td>
<td>500</td>
<td>200</td>
<td>59</td>
<td>30</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CPP-III</td>
<td>58600</td>
<td>400</td>
<td>163</td>
<td>70</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

\(^1\)Results shown in micrograms per gram (ppm)

Figure A6. Effect of CPP-I at 0 mg/ml (●), 0.05 mg/ml (□), 0.10 mg/ml (◆) and 0.25 mg/ml (X) and 1mM Phytic Acid (■) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by Fenton reaction generated by addition of 0.50mM FeSO\(_4\) at 37°C.
Figure A7. Effect of CPP-II at 0 mg/ml (●), 0.05 mg/ml (□), 0.10 mg/ml (◆) and 0.25 mg/ml (×) and 1mM Phytic Acid (■) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by Fenton reaction generated by addition of 0.50mM FeSO₄ at 37°C.
Figure A8. Effect of CPP-III at 0 mg/ml (●), 0.05 mg/ml (□), 0.10 mg/ml (◆) and 0.25 mg/ml (X) and 1mM Phytic Acid (■) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by Fenton reaction generated by addition of 0.50mM FeSO₄ at 37°C.
Figure A9. Effect of CPP-I at 0 mg/ml (●), 0.05 mg/ml (□), 0.10 mg/ml (◆), 0.25 mg/ml (×) and 0.50 mg/ml (△) and 1.0 μg/ml Trolox (■) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by addition of 2mM AAPH at 37°C.
Figure A10. Effect of CPP-II at 0 mg/ml (●), 0.05 mg/ml (□), 0.10 mg/ml (◆), 0.25 mg/ml (×) and 0.50 mg/ml (△) and 1.0 µg/ml Trolox (■) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by addition of 2mM AAPH at 37°C.
Figure A11. Effect of CPP-III at 0 mg/ml (●), 0.05 mg/ml (□), 0.10 mg/ml (◆), 0.25 mg/ml (△) and 0.50 mg/ml ( ● ) and 1.0 µg/ml Trolox ( ■ ) on the time course of formation of conjugated diene in the oxidation of structured liposome mediated by addition of 2mM AAPH at 37°C.
Figure A13. Preparation of CPP. (By: Nakamura, S., Personal Communications)
CHAPTER V: CYTOPROTECTIVE EFFECTS OF CPP ON COLON CARCINOMA CELLS (Caco-2) AND INTESTINAL EMBRYONIC CELLS (INT 407)

5.1 Materials and Methods

5.1.1 Cell Culture conditions

Caco-2 cells (HTB-37; American Type Culture Collection, Rockville, MD), derived from human colon carcinoma, and Int-407 cells (CCL-6; ATCC), derived from a human embryonic intestinal epithelial cell line, were used to study the protective effect of caseinophosphopeptides (CPP) on intestinal cell iron (Fe^{2+}) toxicity. Cells were grown as monolayers at 37°C with 5% CO₂ in Eagle’s minimum essential medium EMEM, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, (1.5 g/L sodium bicarbonate), 1% (v/v) essential amino acids and 10% (v/v) fetal calf serum (FCS). Culture media and supplements were purchased from Invitrogen Canada Inc. (Burlington, ON). Cells were split at a ratio of 1:3 when cells reached confluency and viability was assayed.

5.1.2 Cytotoxicity assay

Caco-2 cells and Int-407 cells were cultured at a density of 5.0 x 10⁴ cells/100μl on a 96-well plate until a tight monolayer was formed (1 to 2 days following seeding). The cells were washed twice with phosphate buffered saline solution (PBS) and then re-incubated with Fetal Calf Serum (FCS) reduced EMEM (Invitrogen Canada Inc., Burlington, ON) for 24 hours at 37°C prior to the addition of ferrous sulfate. Concentrations of ferrous (II) sulfate (Fisher Scientific, Fairlawn, NJ) ranging from 0.01 to 200 mM were dissolved in FCS-reduced EMEM prior to addition to cell cultures. Smaller increments of iron concentration were used in subsequent experiments to
determine an effective concentration that reduced cell viability to 50% of control (EC50) following a 24-hour incubation at 37°C.

Caco-2 cells and Int-407 cells were prepared similarly as described above, with an additional 1-hour incubation period containing CPP-III before the addition of ferrous sulfate. Viability of cells supplemented with 5, 10, 25, 50, 100, 250, 500 and 1000 μg CPP-III per ml of FCS-reduced EMEM prior to iron treatment were compared to cells treated with iron alone to monitor the effect of CPP-III on cell toxicity. Viability of cells treated with CPP-III only was also determined to rule out any possible cytotoxic effects of the peptide alone. Concentration of ferrous iron used to achieve a 50% viability in Caco-2 cells was determined to be 2.5 mM, compared to the 4.0 mM in the Int-407 cells (Appendix; Figures A10 and A11).

5.1.3 Cell Viability

Viability of cells was assayed by monitoring the inhibition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma Chemical Co., St. Louis, MO) reduction in the colorimetric Mitochondrial Tetrazolium (MTT) assay (Mosmann, 1983). Cells were first rinsed twice with PBS before the addition of 75μl MTT stock solution at a concentration of 5 mg/ml PBS. Viable cells with a functional mitochondrial enzyme cleave the MTT salt to a dark blue formazan product. Following a 4 hour incubation at 37°C, 100 μl of acid isopropanol (0.04N HCl in 2-propanol; Fisher Scientific, Fair Lawn NJ) was added to all wells and vortexed thoroughly to dissolve the dark blue crystals. Viable cell count correlated directly with the concentration of formazan determined at absorbance 570 nm and was compared against a control to indicate the effect of various treatments on cell survival.
5.1.4 Statistical Analysis

All experiments were done in duplicates with six replicates per experiment. All data were analyzed by one way ANOVA ($\alpha \leq 0.01$) followed by a multiple range Tukey post test analysis with the GraphPad Prism Analysis software (GraphPad Software Inc., San Diego CA) to identify significant different among treatment means ($P \leq 0.01$).
5.2 Results

5.2.1 Effect of Iron on cells

Reduction of cell viability to nearly 60% of control was achieved with the addition of FeSO₄ for Int-407 (4.0 mM) and Caco-2 cells (2.5 mM). These specific concentrations of ferrous iron were selected according to a near 50% mortality observed for both cell lines in preliminary studies (Figures 14 and 15).

5.2.2 Effect of CPP-III on cells

Laboratory synthesized, phosphoserine-enriched CPP-III was added to the apical surface of human intestinal embryonic Int-407 and human colon carcinoma Caco-2 cell monolaters in serum-reduced media. Viability (% control) of the 2 cell lines was measured by the MTT assay. CPP-III was shown to have no significant cytotoxic effects on the undifferentiated embryonic cells maintaining 98.5 to 106.2% viability of Int-407 cells (Table 29). CPP-III did not reduce the viability of the human colon cancer Caco-2 cells (100.4-103.6%) at a concentration range of 5-250 μg/ml. This result contrast the effect of CPP-III at 500 and 1000 μg/ml that significantly (p<0.01) lowered the % of cell survival to 98.0 and 82.8% respectively (Table 30).

5.2.3 Effect of CPP-III on iron toxicity of cells

CPP-III was found to inhibit the damage of iron-induced cytotoxicity in a dose-response manner, successfully recovering Int-407 cell viability to 94.2 and 98.4% of control at 500 and 1000 μg/ml respectively (Table 28). The cytoprotective effect of CPP-III in the Caco-2 cells increased with peptide concentration, resulting in a gradual rise in viable cell numbers to 87.8 and 88.6% at 500 and 1000 μg/ml respectively (Table 29).
Figure 14. Viability of Int-407 cells following a 24-hour incubation with FeSO₄ at 37°C.

1. Viability (% control) = \(\frac{\text{Abs } 570 \text{nm}_{\text{sample}}}{\text{Abs } 570 \text{nm}_{\text{control}}} \times 100\);

Results are expressed as Mean ± SD, n = 6;
Figure 15. Viability of Caco-2 cells following a 24-hour incubation with FeSO₄ at 37°C.¹

1. Viability (% control) = \( \frac{\text{Abs}_{570\text{nm}}_{\text{sample}}}{\text{Abs}_{570\text{nm}}_{\text{control}}} \times 100; \)

Results are expressed as Mean ± SD, n = 6;
Table 28. Effect of CPP-III on the viability of Int-407 cells following a 24-hour incubation with 4 mM FeSO$_4$ at 37°C.

<table>
<thead>
<tr>
<th>Concentration of CPP (µg/ml)</th>
<th>Treatment</th>
<th>CPP only</th>
<th>CPP &amp; Fe$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>60.3±3.9$^a$</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>103.9±5.3$^{ay}$</td>
<td>66.9±4.7$^{abx}$</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>105.5±5.3$^{ay}$</td>
<td>69.4±5.3$^{bx}$</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>100.2±4.5$^{ay}$</td>
<td>66.1±5.1$^{abx}$</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>100.4±5.2$^{ay}$</td>
<td>70.2±5.1$^{bx}$</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>103.3±4.6$^{ay}$</td>
<td>78.1±6.4$^{cx}$</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>98.5±2.9$^{ay}$</td>
<td>89.4±6.9$^{dx}$</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>101.7±3.6$^{ax}$</td>
<td>94.2±6.3$^{dex}$</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>106.2±4.0$^{ax}$</td>
<td>98.4±4.6$^{ex}$</td>
<td></td>
</tr>
</tbody>
</table>

1. Viability (% control) = \( \frac{A_{570nm_{sample}}}{A_{570nm_{control}}} \times 100 \);

Results are expressed as Mean ± SD, n = 6;

Means within the same column that do not share a common superscript letter (e.g. a, b, c, d and e) are significantly different (p<0.01);

Means within the same row that do not share a common superscript letter (e.g. x and y) are significantly different (p<0.01).
Table 29. Effect of CPP-III on the viability of Caco-2 cells following a 24-hour incubation with 2.5 mM FeSO$_4$ at 37°C$^1$.

<table>
<thead>
<tr>
<th>Concentration of CPP (µg/ml)</th>
<th>Treatment</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CPP only</td>
<td>CPP &amp; Fe$^{2+}$</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>60.9 ± 3.4$^a$</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>103.6 ± 1.7$^{cy}$</td>
<td>78.9 ± 4.5$^{bcdx}$</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>102.5 ± 1.6$^{bcy}$</td>
<td>76.5 ± 4.3$^{bex}$</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>100.4 ± 2.9$^{bcy}$</td>
<td>74.2 ± 4.4$^{bx}$</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>101.9 ± 3.6$^{bcy}$</td>
<td>75.5 ± 6.2$^{bex}$</td>
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</tr>
<tr>
<td>100</td>
<td>101.3 ± 2.4$^{bcy}$</td>
<td>85.9 ± 4.3$^{cdex}$</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>100.4 ± 2.1$^{bcy}$</td>
<td>84.6 ± 4.7$^{cdex}$</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>98.0 ± 2.5$^{by}$</td>
<td>87.8 ± 4.8$^{dex}$</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>82.8 ± 2.7$^{ax}$</td>
<td>88.6 ± 5.1$^{ex}$</td>
<td></td>
</tr>
</tbody>
</table>

1. Viability (% control) = \( \frac{\text{Abs}_{570\,\text{nm}}_{\text{sample}}}{\text{Abs}_{570\,\text{nm}}_{\text{control}}} \times 100; \)

Results are expressed as Mean ± SD, n = 6;

Means within the same column that do not share a common postscript letter (e.g. a, b, c, d and e) are significantly different (p<0.01);

Means within the same row that do not share a common postscript letter (e.g. x and y) are significantly different (p<0.01).
5.3 Discussion

The human intestine is constantly challenged by the presence of dietary oxidants, mutagens and oxygen species or related reactive oxygen metabolites that reside in the luminal contents. With the consumption of diets containing highly unsaturated fats, luminal accumulation of lipid hydroperoxides can be considerable, promoting oxygen radical generation and propagation and accelerating lipid peroxidation (Aw, 1999). Notwithstanding this, subtoxic levels of lipid peroxides (10μM) can potentially disrupt intestinal redox homeostasis and initiate an apoptotic cascade that cannot be reversed even when the redox balance is restored (Wang et al., 2000). In addition to preformed radicals, diet-derived minerals, such as iron, may also act as oxidants, catalyzing Fenton reaction-induced oxidations and altering the cellular oxidation-reduction (redox) balance at the absorptive surface of the intestines (Núñez et al., 2001).

Imposing severe oxidant stress can lead to cytotoxicity of human intestinal epithelial cells. Intestinal cells respond differently to the degree of oxidative stress. For example, at mild oxidant concentrations (<10μmol/L), intestinal proliferative activity increases, whereas at high oxidant concentrations (10-50μmol/L), intestinal cells die by apoptosis (i.e. cell shrinkage). At even higher concentrations of oxidants (>100μmol/L), there is significant necrotic cell death (i.e. cell lysis) (Aw, 1999).

Through the Fenton reaction, the prooxidant iron can initiate a series of chain reactions resulting in the generation of free radicals such as hydroxyl radicals at the site of the intestine, causing severe damage to tissues and biomolecules. These events, in turn, may lead to cell ageing and death. Human colon carcinoma cells (Caco-2) are a suitable model for the study of transport and metabolism of possible toxicological
substances, such as iron, because Caco-2 cells accumulate iron in a time- and concentration-dependent manner, specifically from the apical pole (Halleux and Schneider, 1994). Excessive intracellular accumulation would then lead to cytotoxicity (Rossi et al., 1996). However, the iron uptake from the apical surface into enterocytes and transport across the Caco-2 cells are affected both by the valency of the iron and the iron status of the cells. Previous workers have shown that the uptake of ferrous iron ($Fe^{2+}$) was 200 fold greater than that of the ferric form ($Fe^{3+}$), and the iron uptake was significantly higher for cells grown in conditions of low iron concentrations (Alvarez-Hernandez et al., 1991). The greater affinity for ferrous uptake at the brush border membrane of cells suggests that the intake of iron at the apical pole first requires a reduction of ferric iron prior to entry (Halleux and Schneider, 1994). Iron accumulation in Caco-2 cells was shown to increase protein and DNA oxidative damage, resulting in the loss of cell viability (Núñez et al., 2001). Iron induced DNA damage of intestinal epithelial cells in vitro and in vivo occurred by incubation of mucosal cells with 800 $\mu$M FeSO$_4$ and 12 $\mu$M H$_2$O$_2$ and oral supplementation of iron to normal and iron-deficient rats (Srigiridhar et al., 2001). While a single oral dose of 8 mg iron caused apoptotic damage to rat intestinal mucosal cells, the continuous administration of the same dose for a period of 15 days resulted in necrosis of the GI tract absorptive surface as evidenced by the reduction in microvilli for normal rats and a complete erosion of villi in iron deficient rats. The oxidative factors involved were identified in these studies to be hydroxyl and methoxyl radicals in the luminal and mucosal contents of rats (Srigiridhar et al., 2001).

Ferrous iron crosses the enterocyte by 2 processes, which include a paracellular passage or a membrane receptor-facilitated pathway that is followed by intracellular
shuttling to the basolateral surface. It is hypothesized that ferrous iron chelators serve to inhibit iron uptake (Han et al., 1995) and accumulation in enterocytes, while free radical quenchers target the hydroxyl radicals generated at the cell membrane. The collective effect of these processes is to reduce the probability for the initiation and propagation of cell damage by free radical chain reactions.

In this study, CPP-III was shown to be an effective iron-binding and radical quenching agent with potential cytoprotective effects on intestinal cells upon the insult of iron-induced cytotoxicity. The human colon adenocarcinoma cells, Caco-2 cells, in addition to a non-carcinomic human intestinal embryonic cell line Int-407 were used to test this effect. Caco-2 cells are adherent epithelial cells originally isolated from primary colonic tumor cells in humans. Upon reaching confluence, Caco-2 cells express characteristics of enterocytic differentiation in monolayers, with typical asymmetric morphology and the presence of glycogen deposits and microvilli. The Caco-2 cells are seeded with the basolateral membrane attached to the surface of the culture dish and the microvillus-studded apical membrane facing the media. Integrity of monolayers was reinforced by tight junctions in the apical zone and demosomes along the cells (Dharmsathaphorn et al., 1984). In spite of its cancerous origin, Caco-2 cells appear to behave similarly to the normal in vivo counterpart particularly under the differentiated state (Pageot et al., 2000). Similar to the Caco-2 cells, the Int-407 cells are also adherent epithelial cells, which are grown in monolayers with the only difference being the non-carcinomic nature. Int-407 cells, derived from human normal embryonic intestinal tissues isolated from the jejunum and ileum of embryos, were chosen in this study as a control, normal cell line for cytotoxicity assays.
Cell viability, monitored by the MTT assay, was used as an end-measure for the iron toxicity assays. The tetrazolium salt (MTT, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was metabolized to a colored formazan salt by mitochondrial enzyme activity found only in living cells and the extent of coloration correlated with viable cell numbers. This assay measures metabolic events that can more accurately quantify living cells and is more sensitive than dye exclusion assays that tend to overestimate viability as some reproductively dead cells fail to take up the dye (Wilson, 1992).

Reduced serum and serum-free culture systems were shown recently to be superior to fetal bovine serum-containing systems because of a lower incidence of protein interference (Halleux and Schneider, 1994; Shinmoto et al., 1992; Maurer, 1992). Replacement of serum-containing medium by a defined nutritive medium, could increase the reproducibility of experiments and avoid interactions between serum constituents, while sustaining viability, growth rate and normal structural/functional differentiation in Caco-2 cells (Jumarie and Malo, 1991).

It was found that CPP inhibited ferrous sulphate-induced toxicity in both the Caco-2 and Int-407 cells. Through tight binding with the ferrous iron, CPP removed the ferrous iron in the media and prevented iron uptake and intracellular accumulation. CPP alone displayed no cytotoxic effect on the Int-407 cells and only slightly reduced the viability of Caco-2 cells at the 2 highest concentrations. This slight reduction in viability of the Caco-2 cells by CPP alone may also be attributed to the unusually high calcium content of these CPP samples. This finding is consistent with the observed reduction of colonic epithelial cell proliferation, particularly that of precancerous or cancerous cells,
by dietary calcium supplements in rats (Liu et al., 2001; Li et al., 1998), thereby indicating the potential for calcium-containing ligands such as CPP to inhibit colon carcinogenesis in vivo (Viñas-Salas et al., 1998; Kampman et al., 2000). A high concentration of calcium was shown to lower the cell proliferation effect of free bile acids or fatty acids by converting them to insoluble calcium salts (McSherry et al., 1989) or binding to colon cell surface calcium receptors that signals the cells to stop proliferation (Pazianas et al., 1995).

As the Caco-2 epithelial cells differentiate, they also express alkaline phosphatase activity (Jovani et al., 2001), with the majority of phosphatase activity located at the apical side of the brush border membrane (Hidalgo et al., 1989). Yeung and colleagues have found that through partial hydrolysis of casein peptide bonds, phosphoserines residues become exposed and as a result vulnerable to attack by intestinal alkaline phosphatase (IAP). This effect leads to the release of bound iron or the inhibition of iron chelation, which facilitates iron absorption (Yeung et al., 2001). The CPP dephosphorylation by IAP and the corresponding release of iron from the CPP-Fe complex may have contributed to the relative lower recovery in viability of Caco-2 cells, as compared to the control Int-407 cells in the present study.

In vivo studies have shown that CPP exists not only in intestinal digests (Hirayama et al., 1992a) of rats fed casein or CPP-III diets, but also in the cecum and colon (Kasai et al., 1995) and subsequently in the fecal matter of rats (Kasai et al., 1992). The possibility exists therefore that the CPP-Fe complex would also bypass hydrolysis from proteolytic enzyme activity in the lumen or by enteric bacteria. At the apical pole of the brush border membrane, the CPP-Fe complex is likely not absorbed into the
intestinal cells since the complex is neither recognized by the iron receptor on the membrane surface, or alternatively is small enough to enter through tight junctions for paracellular absorption. It is possible therefore that once the iron is bound to CPP, it collectively bypasses all digestive and absorptive processes and is excreted in a manner similar to CPP alone. Though derived from bovine milk, CPP behaves quite differently from other bovine milk proteins, such as β-lactoglobulin, α-lactalbumin and lactoferrin that have all been shown to bind to brush border membranes of Caco-2 cells (Bolte et al., 1998) and transport through Caco-2 cell monolayers through absorptive endocytosis, releasing its iron following lysosomal degradation (Caillard and Tomé, 1995; Sánchez et al., 1996).

Although the levels of oxidation products were not determined in this study, the dual functionality of CPP as a metal chelator and also a free-radical stabilizer provides strong evidence that there is significant potential for CPP to prevent iron-induced oxidative damage to intestinal tissues associated with excessive and prolonged iron supplementation in vivo. CPP may also participate in quenching other pre-formed radicals, such as lipid peroxides or oxygen species, which have dietary origin. Studies to date have not been able to quantify the exact levels of CPP generated from CPP- or casein-containing diets, or to identify possible inhibitors present in the diet that counteract the bioactivity of CPP. Therefore, further research is warranted to establish the role of CPP as an active antioxidant in the context of dietary antioxidative supplements, or a therapeutic agent for correcting iron overload.
5.4 Conclusion

Using a reduced serum culture system, CPP-III was shown to be relatively non-toxic to cells and to successfully recover human intestinal embryonic Int-407 and colon carcinoma Caco-2 cells from ferrous sulphate-induced cytotoxicity. Alkaline phosphatase activity present on the apical surface of differentiated Caco-2 cells may have contributed to a comparatively lower recovery of cell viability from cytotoxicity as compared to the control Int-407 cells. It seemed likely that iron-bound CPP would not be taken up by the enterocytes, but rather exit the digestive tract while bypassing most of the hydrolysis activity of proteolytic enzymes and enteric bacteria.
GENERAL CONCLUSION

A series of experiments were conducted to evaluate the potential in vitro antioxidant activity of caseinophosphopeptides (CPP), a tryptic hydrolysis product of bovine milk casein. First of all, CPP-I and CPP-III from Meiji Seika Kaisha Ltd. (Japan) were analyzed for mineral sequestering and free radical quenching activities in both aqueous and emulsion models. CPP-III had a higher purity than CPP-I, but both were shown to contain peptides lower than 6kDa. A qualitative analysis of metal scavenging activity showed the interaction between CPP-I and III with metal ions such as ferrous iron, ferric iron and calcium. CPP suppressed the oxidation of deoxribose through site-specific binding with the pro-oxidant iron and through non site-specific binding with hydroxyl radicals. CPP-I and III were equally (p>0.05) effective in inhibiting deoxyribose oxidation through iron chelation, but CPP-I exhibited significantly (p<0.01) higher hydroxyl radical scavenging activity than CPP-III, at 0.50 and 1.00mg/ml. In an emulsion model, CPP was shown to lower oxidation through scavenging AAPH radicals and chelating ferrous iron at the surface of phospholipid liposomes. The ferrous ion sequestering activities of CPP-I and III in an emulsion were not significantly different (p>0.05) at 0.50mg/ml. However, CPP-I had a 2 fold lower (p<0.01) rate of propagation (% control) than CPP-III. In an aqueous free radical assay, CPP-I exhibited a significant (p<0.01) 91.8% scavenging for hydrophilic ABTS radicals while CPP-III had 8.4%. Hence, commercially available CPP acted as a primary and also a secondary antioxidant by displaying both metal sequestering and free radical quenching activity in aqueous and emulsion models.
Secondly, the antioxidant activities of laboratory synthesized CPP, along with CPP-saccharide conjugates, were evaluated to determine the effect of glycosylation on CPP antioxidant activity. CPP-I, II and III were shown to consist of peptides with molecular weights of 3.5 to 6.5 kDa, which correspond to peptides reported by other studies (Reynolds et al., 1994; Gerber and Jost, 1986; Gagnaire et al., 1996). CPP-III had the highest calcium concentration (58.6mg), as compared to CPP-I and II that contained 400 and 500µg of calcium. CPP inhibited degradation of deoxyribose via 2 processes, through metal chelating and hydroxyl radical scavenging. Oxidative degradation of deoxyribose was inhibited to a greater extent through free radical scavenging up to 60% at 1.00mg/ml CPP, in comparison to inhibition of 32% at the same concentration through ferrous iron chelation. CPP-III was consistently more effective at inhibiting hydroxyl radical generation. Conjugation of CPP with polysaccharide galactomannan (GAL) or oligosaccharide xyloglucan (XYL) was shown to negatively impact the hydroxyl radical scavenging activity and ferrous iron binding activity of CPP, thereby inhibiting significantly (p<0.01) less deoxyribose oxidation than the corresponding peptide. In an emulsion model, CPP lowered AAPH-induced damage on liposomes to 30% of control at the highest concentration (0.50 mg/ml) and did not differ significantly (p>0.05) among preparations at each concentration tested. This antioxidant effect was comparable to the Trolox control. Conjugation of CPP-I, II and III to GAL and XYL did not affect (p>0.05) CPP affinity to sequester peroxyl radical AAPH and lower liposome oxidation. Assessment of CPP affinity to scavenge aqueous, stable free radical ABTS showed that CPP-II was the most pronounced scavenger, quenching up to 93.9% of the radicals at 1.00mg/ml. At 1.00mg/ml, CPP-III scavenged 67.6% of the
ABTS radicals, while CPP-I only scavenged 3.2% of the radicals. Conjugation of polysaccharide galactomannan and oligosaccharide xyloglucan improved the radical stabilizing activity of CPP-I, but lowered the activity of CPP-II. There was no effect at improving CPP-III activity.

Laboratory synthesized, phosphoserine-enriched CPP-III was added to the apical surface of human intestinal embryonic Int-407 and human colon carcinoma Caco-2 cell monolaters in serum-reduced media. CPP-III was shown to have no significant cytotoxic effects on Int-407 cells, maintaining 98.5 to 106.2% viability. CPP-III did not reduce the viability of Caco-2 cells (100.4-103.6%) at a 5-250 μg/ml but significantly (p<0.01) lowered the % viability to 98.0 and 82.8% at 500 and 1000 μg/ml CPP-III, respectively.

CPP-III was found to inhibit the damage of iron-induced cytotoxicity, recovering Int-407 cell viability from 60% (control) to 94.2 and 98.4% of control at 500 and 1000μg/ml respectively. Similarly in the Caco-2 cells, the cytoprotective effect of CPP-III induced a rise in viable cell numbers from 60% (control) to 87.8 and 88.6% at 500 and 1000μg/ml, respectively.

These results demonstrate the potential affinity for bovine milk derived phosphopeptides to serve as a natural antioxidant, through metal sequestering and free radical scavenging activities, to lower the oxidative deterioration of lipids in food systems and the oxidative stress-related injury to biomolecules in vivo, especially in the intestine.
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