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UMI
Characterization of the Interaction of Human Salivary Proline-rich Proteins with Tannins

by

Ying Lu

A thesis submitted in conformity with the requirements for the degree of Master of Science in the Graduate Department of Dentistry University of Toronto

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Tannins are plant-derived polyphenolic compounds that are found widely in foods, particularly fruits and beverages. There is a large body of evidence showing that tannin can interfere with normal growth and metabolism and cause systemic toxic effects in many animals including humans. Proteins rich in proline have been shown to have high affinity for tannins.

In human parotid saliva proline-rich proteins (PRPs) constitute approximately 70% of total protein. They are usually divided into acidic (APRP), basic (BPRP) and glycosylated PRPs (GPRP). To study the formation of insoluble complexes of tannin and PRPs, the BPRPs named IB-1, IB-4, IB-6, IB-9, and GPRP as well as an APRP named PIF-s were purified from parotid saliva. These proteins were chosen as representative of different types of PRPs in studies on interaction with tannin. Tryptic digest of PIF-s as well as 3 purified tryptic peptides of PIF-s and deglycosylated GPRP core were used to evaluate the effect of proteolytic digestion of PRPs and deglycosylation of GPRP on their ability to bind tannin. PRPs were incubated with varying concentrations of tannins at neutral pH, and their capacities for tannin binding were determined. Furthermore, the stability of insoluble BPRP tannin complexes under physiological conditions was studied by incubating the insoluble complexes under conditions similar to those prevailing in the alimentary canal.

The results show that: (1) BPRPs are the PRPs which are able to precipitate most tannin at neutral pH and very little tannin binding occurs to APRP and GPRP. They have higher capacity for tannin-binding than gelatin which is considered to have high tannin binding ability. The size and sequence of BPRPs have little or no effect on tannin binding. (2) Because of the low tannin binding ability of APRPs their biological activities are not affected by the presence of tannin. Proteolytic cleavage of APRPs enhances their ability to bind tannin via the pro-rich region.
interaction of tannin and GPRPs. Since there is little tannin binding to GPRPs, these proteins in the presence of tannin will largely retain their other functions such as lubrication which depend on the carbohydrate sidechains. (4) PRPs can be expected to still be effective tannin binders after degradation and deglycosylation in the mouth. Indeed the activity of APRP and GPRP is increased. (5) The tannin-PRP complexes are very stable under conditions prevailing in the stomach. (6) More than half of the insoluble condensed tannin-PRP complexes is still stable under conditions similar to those in the small intestine. (7) This study supports the hypothesis that PRPs act as a defense against tannins in humans.
I would like to express my most sincere gratitude to my supervisor Dr. Anders Bennick for his support and professional guidance over the past two years and his advice and encouragement during the preparation of my thesis. I thank him for always having an open door for me no matter how busy he was. I have learned a tremendous amount about science and life from him. His wisdom and hard work always impressed me and will be a good model in my entire scientific career.

Many thanks to my lab colleagues, Marlene Stubbs, John Chan, Yesi Nam, Luke Drxymala and Anita Kwan, for their friendship, encouragement, helpful suggestions and discussion.

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I am grateful to Dr. David Williams for letting me use his lab computer and scanner during my thesis preparation.

Finally, I would like to say a very special thank you to my wife Catherine Lui. Without her, I would never have accomplished my work for the graduate study and thesis. Her support and dedication as a mother, wife and companion have made it possible for me to complete my research.
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<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
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<td>PRG</td>
<td>A proline-rich glycoprotein</td>
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<tr>
<td>PRPs</td>
<td>Proline-rich proteins</td>
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<td>Rotations per minute</td>
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<td><strong>TFMA</strong></td>
<td>Trifluoromethanesulfonic acid</td>
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<td><strong>Tris</strong></td>
<td>Tris(hydroxymethyl)amino-methane</td>
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1. **Tannins**

1.1 **Chemistry and Biological Function of Tannins.**

Tannins are plant-derived polyphenolic compounds which are widely found in foods, particularly fruits and beverages (Kuhnau, 1976; Mehansho *et al.*, 1987). On the basis of their chemical structures, tannins are usually divided into two groups: hydrolyzable and condensed tannins (Hagerman & Butler, 1981). The major distinction between these two groups is their reaction to hydrolysis agents, especially acids.

Hydrolyzable tannins are readily degraded by acids and gastrointestinal esterases (Mehansho *et al.*, 1987). They are composed of gallic acid (Fig. 1a) or its condensation product ellagic acid esters of glucose (Haslam, 1979) and are called gallotannins and ellagitannins. The common name "tannic acid" has been wrongly used as applying to all hydrolyzable tannins, and should be used only for gallotannins from Chinese or Turkish nutgall. This group of tannins of MW of 500-2000 is composed of glucose esterified to 4 to 10 gallic acids (Fig 1b). They are widely used in testing biological effects of tannins. The commercial tannic acid is a mixture of gallic acid, digallic acid, trigallic acid, and galloylated glucose (Joslyn & Glick, 1969). Of these the smaller molecules, i.e. gallic acid to trigallic acid, cannot precipitate proteins and must be removed from the preparation before it is used (Hagerman *et al.*, 1992).
Fig. 1. Typical structure of hydrolyzable tannins Adapted from: Mehansho et al. [1987]

(a) Gallic Acid
(b) Gallotannin
structure is more complex. They are mainly polymers of flavan-3-ol (Fig. 2a) or flavan-3, 4 diols (Fig. 2b) or a mixture of these two molecules (Mehansho et al., 1987). Gupta and Haslam (1978) determined the basic structure of a typical condensed tannin from sorghum grain (Fig 2c). Using hot, strong, mineral acids, Mehansho et al. (1987) oxidatively depolymerized condensed tannins and obtained anthocyanidin and other less well characterized compounds. This is why condensed tannins are often referred to proanthocyanidins. Compared to hydrolyzable tannins, condensed tannins differ widely in their degree of polymerization and may contain up to hundreds of flavanol units. Most condensed tannins are composed of 4 to 40 units of flavanol (Williams et al., 1983). The condensed tannins generally used for testing biological effects include quebracho (from heartwood of a South African tree), sorghum, and wattle tannins. Epigallocatechin gallate (EGCG, MW: 458), a representative component of condensed tannin, has been purified from green tea (Shirai et al., 1994) and its structure has been determined (Fig. 3a). It is very useful for studying the interaction between protein and tannin at the molecular level. Other simple representatives of the proanthocyanidins are epicatechin (Fig. 3b), a monomer of condensed tannin, and its dimer, procyanidin B-2 (Fig. 3c) (Charlton et al., 1996).
Fig. 2. Typical structure of a sorghum condensed tannin and its related compounds. Adapted from: Mehansho et al. [1987]
Fig 3. The chemical structure of (a) epigallocatechin gallate (EGCg) (b) (-)-epicatechin, a proanthocyanidin monomer (c) B-2, a procyanidin dimer. Adapted from: Shirai et al., 1994, Charlton et al., 1996
Tannins have for a long time been thought of as metabolic waste products of plants without any biological functions, but several studies now show that tannins have pertinent metabolic functions. Tipton et al. (1970) found that the tannin content of sorghum, a grain widely grown for human consumption in various African and Asiatic countries, was positively correlated to resistance to depredation by birds. The content of tannin in seeds has also been linked to the inhibition of preharvest seed germination and seed molding (Harries et al., 1969, 1970). Moreover the astringency of tannins may prevent herbivores from eating plants with high tannin content (Bate-Smith, 1954).

These observations have led to the suggestion that the biological role of tannin in plants is to act as repellent to predators (Bate-Smith, 1973). On the other hand, it would be a great advantage for the herbivores to develop mechanisms which would allow them to eat plants with high tannin content since it would allow them to broaden their food supply.

1.2 Effect of Tannins

Tannins have been reported to have a number of local and systemic detrimental effects when ingested by animals, including human beings.

1.2.1 Metabolism of Food

Various effects of tannins on food intake of experimental rats have been reported (Joslyn & Glick, 1969; Mitjavila et al., 1977; Hale & McCormick, 1981). Some of these experiments showed that tannin depressed food intake of animals, but other studies found no effect. It is still unclear if tannins decrease food intake of animals. However, feeding tannin to experimental animals results in a decreased growth rate and weight gain. When young rats were fed a diet containing tannic acid,
and it was also found that rats and hamsters fed a high-tannin diet showed a poor growth response compared to the control (Featherson & Rogler, 1975; Jambunathan & Mertz, 1973; Mehansho et al., 1985). In addition, Mitjavila and co-workers (1977) observed a significant increase in faecal calcium in rats fed a diet containing 1% tannic acid. An increased excretion of sodium was also noticed and this was considered to be caused by perturbed movement of sodium as a result of tannin ingestion. The effects of tea on iron absorption was reported by Disler et al. (1975). Compared to water, tea and coffee markedly inhibited iron absorption from a meal (Hallberg & Rossander, 1982). So tannins affect the digestion and absorption of food as well as the absorption of minerals.

### 1.2.2 Activities of Digestive Enzymes

The effect of tannins on the activities of $\alpha$-amylase, trypsin, and lipase has been studied *in vitro* and *in vivo*.

Feeding tannin to rats resulted in decreased activity of intestinal amylase and trypsin (Griffiths & Moseley, 1980, Horigome *et al.*, 1988). The same occurred in chickens, but in these animals a compensatory hypertrophy of the pancreas was also seen (Ahmed *et al.*, 1991). Furthermore, tannins extracted from bean and pea testas significantly inhibited the activities of trypsin and $\alpha$-amylase *in vitro* (Griffiths, 1981). Moreover Horigome *et al.* (1988) reported that tannins prepared from the leaves of fodder plants inhibited *in vitro* the activities of $\alpha$-amylase, trypsin, and lipase.

The inhibition of digestive enzymes by dietary tannins appears to be of a noncompetitive type and is believed to be a result of nonspecific binding of the enzymes to tannins (Goldstein & Swain, 1965; Horigome *et al.*, 1988).
Systemic toxic effects such as damage to liver and kidney after ingestion of tannic acid have been reported (Booth et al., 1961; Glick & Joslyn, 1970; Voha et al., 1966). Liver necrosis and at least eight fatalities were reported after tannic acid was used as an adjuvant in enemas (Kerzanoski, 1966; Singleton & Kratzer, 1969). Tannin has also been used in burn treatment of humans, applying it directly to the burnt surface, but this use has been discontinued since it was shown to cause liver necrosis (Singleton, 1981). Moreover, it is also interesting to notice experiments by Butler and Rogler (1986) which suggest that the major effect of dietary tannin is not to inhibit food consumption, but to decrease the efficiency whereby food is converted into new body substance. This suggests that the site affected by tannin is not in the intestine but elsewhere within the body.

Systemic effects, including liver and kidney damage, suggest that some components of tannins are absorbed from the alimentary canal. Korpassy et al. (1951) reported that tannic acid is detected in the blood of rabbits and dogs after oral administration. Moreover, using 125I labelled tannin, it could be demonstrated that the label was absorbed from the intestine, but it is not clear if tannin is degraded before absorption (Butler et al., 1986). Tannin can damage the mucosal lining of the gastrointestinal tract, and this may allow absorption to occur (Barcina et al., 1984). It has also been observed that in chickens oral administration of tannic acid led to the appearance of gallic acid derivatives in the urine, demonstrating degradation of tannic acid in the body (Kadirvel et al., 1969).

A recent study suggests that it is the low molecular weight polyphenols associated with tannin preparations rather than tannin itself that are adsorbed from the intestine and may be partly responsible for the toxic effects (Jiminez-Ramsey et al., 1994).
It was reported that condensed tannins are present in high quantities in aqueous extracts of cotton bracts and cotton mill dust (Bell & Stipanovic, 1983) and an acute pulmonary inflammatory response often occurs in cotton mill worker. Several studies demonstrated that inhalation of cotton mill dust or the purified condensed tannins isolated from this organic dust results in this inflammatory reaction (Mundie et al., 1985; Lauque et al., 1988).

It was found that this inflammatory reaction was associated with an increased amount of neutrophils in the airway and the accumulation of polymorphonuclear leucocytes in the bronchoalveolar lavage fluid. Lauque and his co-workers (1988, 1991) demonstrated that either tannin or cotton dust extract could lead to increase number of polymorphonuclear leucocytes and platelet aggregation, both of which play important roles in the inflammation. It was also found that the concentrations of several eicosanoids released from alveolar macrophages were increased in the airways of animals challenged by condensed tannin (Mundie et al., 1985; Lauque et al., 1988; Kreofsky et al., 1992). In addition, the results obtained by Vuk-Pavlovic et al. (1990) showed that tannin enhanced the production of peroxide from human neutrophils, and peroxide was suggested to be a factor in the inflammatory reaction. All of these studies indicate that condensed tannin in the cotton bracts play an important role in causing the acute pulmonary inflammation observed in cotton mill workers.

1.2.5 Relationship to Cancer

Tannins have also been implicated in carcinogenesis. An unusual high incidence of cancer of the upper alimentary canal and oesophageal cancer (Warner & Azen, 1988; Robbins et al., 1991) has
1972). Induction of tumors in rats and mice by subcutaneous injections of tannic acid and various
tannin extracts has also been reported (Kirby, 1960; Mosonyi and Korpasy, 1953). Nevertheless,
no firm cause-effect relationship has been established, and it remains to be demonstrated if tannins
have a role in these types of cancer.

In contrast to these studies recent observations indicate that flavonoids in vegetables and fruits
inhibit cancer development in vitro (Cody et al., 1988; Hertog et al., 1993, 1996). Boege and his co-
workers (1996) found that quercetin and related natural flavones can inhibit topoisomerase
1-catalyzed DNA religation. Inhibition of this enzyme would therefore in particular affect rapidly
growing malignant cells.

To sum up, it is clear that there is considerable evidence showing that tannin can interfere with
normal growth and metabolism and cause systemic toxic effects in many animals including humans.
Therefore, to fully take advantage of the consumption of plant foods, it is important to develop
defense mechanisms against tannin, especially mechanisms whereby tannin is neutralized in the mouth
when it enters the alimentary canal.
Humans consume many foods that contain considerable amounts of tannin (Hoff et al., 1977; Mehansho et al., 1987). High levels of tannin are found in cereals such as sorghum and barley, legumes including faba beans, pinto beans and cowpeas, and berries such as strawberries, red currants, blueberries and raspberries (Mehansho et al., 1987). Other foods with high tannin content include fruits such as bananas and apples and beverages such as red wine, tea, coffee, cocoa and beer (Mehansho et al., 1987). Sorghum, millet and food legumes are important food crops of several countries in Asia, the Near and Middle East and Africa. Sorghum contains up to 7.2% condensed tannin and 1.6% tannic acid (Subramanian et al., 1983). Polyphenols constitute as much as 43% of the solids in a cup of tea (Sanderson, 1972). The tannin content of instant tea is 16.8 mg/100 mg solid (Hoff and Singleton, 1977).

The daily intake of dietary tannin varies among people, depending on their dietary habits and food sources. In U.S.A., the daily consumption of flavonoids, a broad group of plant-derived natural flavouring components that includes tannin, has been estimated to be 1 g (Kuhnau, 1976). The daily intake of tannin by humans may be considerably higher in areas where high-tannin sorghum cultivars are grown and intensively consumed. A daily intake of 1.5 to 2.5 g of dietary tannin in different regions of India has been reported (Rao and Prabhavati, 1982).

The recommended maximal daily intake of hydrolyzable tannins is 0.6 mg/kg body weight (Bigwood, 1973), but there are no figures available on the acceptable maximum daily intake of condensed tannin.
2.1 **Size and Structure of Tannins**

The molecular size and structure of tannin are important factors that influence the protein-binding ability of tannins. It was reported that the capacity of tannin to precipitate protein was a function of its polymer chain length (Hagerman and Klucher, 1986; Horigome et al., 1988; Porter and Woodruffe, 1984). Hagerman & Klucher (1986) found that the purified condensed tannin and gallotannin with the same molecular weights precipitate similar amount of protein. Using purified tannins, it was found that condensed tannin is a more effective protein precipitating agent than is hydrolyzable tannins (Mehansho et al., 1987). When adding a basic deglycosylated proline-rich polypeptide to a tannic acid solution, Asquith et al., (1987) also found that the largest isoform of tannic acid was first precipitated.

2.2 **General Characteristics of Protein Binding to Tannin**

Hagerman and Butler (1981) using a competitive binding assay, found that the relative affinity of various proteins for condensed tannin differed by more than four orders of magnitude. Among the proteins and polypeptides examined, gelatin, polyproline, and a rat parotid proline-rich protein (PRP) showed the highest affinity for tannin. These proteins share characteristics of amino acid composition with tannin-associated protein purified from sorghum grain (Hagerman and Butler, 1980) and all have a high content of proline. They proposed that general characteristics of proteins with high affinity for tannin are large size, open loose structure and high proline content.
Hagerman & Klucher (1986) hypothesized four mechanisms for interaction between tannins and proteins. They are covalent interactions, ionic interactions, hydrogen bonding, and hydrophobic interactions. Covalent interactions (Pierpoint 1969a; Pierpoint 1969b) and ionic interactions (Hagerman & Klucher, 1986) may only happen at pH values greater than 11 (pK$_a$ of tannin hydroxyl groups = 9-11) and will not be considered. The most common mode of interaction between tannin and protein involves hydrogen bonding and hydrophobic interaction.

The hydrogen bond can form between the protein amide carbonyl and the phenolic hydroxyl groups (Hagerman & Butler, 1981). It is pH dependent, occurring at pH values less than the pK$_a$ of phenolic hydroxyl groups. Hydrophobic interactions can occur between the aromatic portion of tannin and non-polar amino acid side chains of the protein (Hagerman and Butler, 1980; Murray et al., 1994; Oh et al., 1980; Charlton et al., 1996). It is known that many proteins with high content of proline contain large regions of random coil structure (Muenzer et al., 1979). The carbonyl oxygen of the peptide bonds in these structures are more exposed and available for hydrogen bonding than those of a compactly folded protein. It is therefore possible that a large number of hydrogen bonds could form between carbonyl groups of the protein peptide bonds and phenolic hydroxyl groups of the tannins (Gustavson, 1954; Loomis and Battaile, 1966; Hagerman and Butler, 1981).

The formation of tannin-protein precipitate is pH dependent and each protein has an unique pH optimum for precipitation, usually near the pI value of the protein (Hagerman and Butler, 1978) and the reason for this is not totally understood. The non-covalent tannin-protein complexes can dissociate in the presence of suitable agents such as SDS (Hagerman and Butler, 1978) or in solvents such as acetone (Hestrin et al., 1955). The complexes may also be dissociated in the presence of
pH 11) \textit{in vitro}. It has been demonstrated that not all associations between tannin and protein result in precipitation, and the formation of soluble tannin-protein complexes may occur when protein to tannin ratios are larger than the optimum ratio for precipitation (Calderon \textit{et al.}, 1968; Buren and Robinson, 1969; Hagerman and Robbins, 1987). The non-polar side chains of proline residues in these proteins and the aromatic portion of tannin could contribute to hydrophobic interactions thus giving pro-rich proteins high affinity for tannin.

The nature of interaction at a molecular level was not clear until some recent studies (Murray \textit{et al.}, 1994; Charlton \textit{et al.}, 1996). Studying the interaction of pentagalloyl glucose (a representative hydrolysable tannin) and synthetic peptides containing a repeat sequence of a mouse salivary PRP (MPS) or a purified human BPRP (IB-5) by $^1$H-NMR spectroscopy, it was found that almost all of the major chemical shift changes involved proline resonance. This suggested that each proline residue form an independent and equivalent binding site because of its open, rigid, hydrophobic surface favourable for the association with the same flat, hydrophobic aromatic rings of polyphenols (Charlton \textit{et al.}, 1996). So the proline residues act not only to keep the structure extended, but also act as hydrophobic binding sites for polyphenols. The chemical shift changes indicate that the other residues which are involved in interaction with tannin are those preceding proline residues. So the binding site for the phenol ring extends beyond the pro side chain and as far as the preceding residue.

It was speculated that binding of salivary PRP to galloyl glucose is probably primarily driven by hydrophobic associations which may then be stabilized by hydrogen bond formation (Murray \textit{et al.}, 1994). The chemical shift data also indicated that the side chain of arginine took part in tannin-binding. The side chain of arginine possesses both hydrophobic nature and hydrogen-bonding
In contrast to the generally accepted features of proteins with high affinity for tannin, studies by Yan and Bennick (1995) have recently shown that salivary histatins (HRPs, histidine-rich proteins), which are small and practically devoid of proline, bind tannin with even greater capacity than macromolecules such as gelatin and salivary acidic PRPs (Yan and Bennick, 1995).

Histatins contain a high level of basic amino acids, especially histidine. In histatin 5, histidine, arginine and lysine together account for about 60% of total amino acids. Polyllysine shows an affinity for condensed tannins similar to that of polyproline. Several factors may contribute to the interaction of tannin with histatin, including hydrophobic interactions of the side chains of arginine and lysine with the aromatic part of tannin (Yan & Bennick, 1995) and hydrogen bonding interactions of the side chains of histidine and the hydroxyls of tannins. In addition, evidence that arginine takes part in tannin-protein interaction was reported in a NMR study (Murray et al., 1994). However, the nature of the interaction at the molecular level between tannin and protein is still not fully understood.

3. **Salivary Proteins**

Saliva contains a very complex mixture of secretory proteins. These proteins originate mainly from parotid, submandibular and sublingual glands. Salivary proteins can help to maintain a highly specialized biochemical environment in the mouth, and proteins such as α-amylases, mucins, immunoglobulins, lactoferrin, peroxidase and lysozyme have distinct functions in saliva which helps to protect and maintain the health of the oral cavity. Saliva also contains a few families of small proteins which are rich in certain amino acids, such as statherins (rich in tyrosine), cysteine-rich
proteins, histatins and proline-rich proteins show high capacity for tannin binding.

3.1 Salivary Histatins

Salivary histidine-rich proteins, or histatins, constitute a group of small proteins originating from submandibular and parotid salivary glands and are only found in human and certain monkeys (Sabatini et al., 1989). Twelve of these structurally related species named histatin 1 to 12 have been purified and their primary structures have been elucidated (Oppenheim et al., 1988 & 1989; Troxler et al., 1990). The three major proteins are histatin 1, 3 and 5, which constitute 85% to 90% of the total secretory histatin output. Histatin 1 is phosphorylated at the amino-terminal region and has no net charge at neutral pH. Histatins 3 and 5 are not phosphorylated and are basic at neutral pH. The levels of individual histatin species vary among the population. Dabbagh et al. (1994) reported levels of histatin 1, 3 and 5 in parotid saliva of 1.5-5.8 mg%, 0.4-11.3 mg% and 4.7-16.8 mg%, respectively.

It has been demonstrated that Histatin 1 and 3 are products of different genes (Sabatini and Azen, 1989) and the remaining histatins, with the possible exception of histatin 5, arise by proteolytic cleavage of the 3 major histatins (Troxler et al., 1990). Histatin 5 is identical to the N-terminal 24 residues of histatin 3, suggesting that histatin 5 is a proteolytic product of histatin 3 (Oppenheim et al., 1988). However, the results of a study (Van der Spek et al., 1990) indicate that histatin 5 might be derived from a distinct mRNA and not by proteolytic processing of histatin 3.

Histatins play an important role in the innate-immunity oral host defense system, although their concentrations in human saliva are not high. They have anti-microbial activity and have inhibitory
unusual and possibly more important biological function of histatins is their antifungal effect. They have been reported to exhibit remarkable inhibitory effects on growth and germination of the pathogenic yeast, *Candida albicans* (Lal *et al.*, 1992; Oppenheim *et al.*, 1986, 1989; Pollock *et al.*, 1984).

Histatin 1 also inhibits hydroxyapatite crystal formation and selectively adheres to hydroxyapatite and enamel powder (Hay, 1973; Oppenheim *et al.*, 1986), implicating that it may be important in maintaining enamel homeostasis in the mouth and in the formation of dental pellicle.

Recently, it has been demonstrated that the salivary histatins are readily precipitated by tannins and on a weight basis bind more tannin than proline-rich proteins in saliva (Yan and Bennick, 1995). It was found that HRP s have a much higher capacity for tannin binding than the acidic proline-rich proteins and gelatin. The latter is generally considered to have a high capacity for tannin binding. At neutral pH, HRP s showed the highest capacity to bind tannins among the three test proteins, HRP, PRP-1 and gelatin. In addition, HRP s also showed a protective role by preventing inhibition of α-amylase by tannic acid. 0.5 μg of tannic acid caused 75% inhibition of α-amylase, while preincubating equal amounts of amylase and histatin 5 before addition of tannic acid resulted in only 20% inhibition of α-amylase activity. This result showed that the presence of HRPs could prevent inactivation of α-amylase by tannic acid. It was therefore suggested that histatins play a role in combating the detrimental effects of dietary tannins.
Among all the proteins and polypeptides secreted by human salivary glands, PRPs are the most dominant proteins accounting for 70% of total proteins in human parotid saliva (Kauffman and Keller, 1979). PRPs are characterized by a proline content of 27% to 43% in individual members (Bennick, 1982).

More than 22 PRPs have been described in human saliva. On the basis of charge and structural properties, they are usually divided into acidic PRPs, basic PRPs, and glycosylated PRPs.

### 3.2.1 Acidic Proline-rich Proteins (APRPs)

APRPs, which are synthesized and secreted by both parotid and submandibular glands, constitute about 30% of human parotid salivary proteins (Kauffman & Keller, 1979). Many isoforms of APRPs including PRP-1, PRP-2, PIF-s, Db-s, Pa, As, At, Au, Av and Aw have been identified from different individuals and populations (Azen, 1978; Azen & Denniston, 1981; Minaguchi et al., 1980, 1986, 1988). Common isoforms are PRP-1, PRP-2, PIF-s, Db-s and Pa. There are slight differences in their primary structures. PRP-1, PRP-2 and PIF-s contain 150 residues, and they are almost identical except for residue 4 and 50; these are Asn₄ and Asp₅₀ in PIF-s, Asp₄ and Asn₅₀ in PRP-1 and Asp₄ and Asp₅₀ in PRP-2 (Hay et al., 1988). Even though the primary structures of Db-s and Pa are not available, their amino acid sequences can be deduced from the cDNA sequence data.

Pa is a 150 residue protein and exists as a dimer linked by a disulfide bond between the cysteines at position 103 of two Pa molecules (Azen and Maeda, 1988). The amino acid sequence of Db-s, derived from cDNA data, contains a 21 amino acid insertion at position 82, resulting in a protein of 171 residues (Azen & Maeda, 1988). The phosphorylation sites of Pa and Db-s have not been
APRPs also possess similar phosphorylation sites at positions 8 and 22.

With the exception of Pa, the isoforms undergo a single posttranslational proteolytic cleavage (Azen, 1978a; Azen and Denniston, 1981). Upon proteolytic modification of PRP-1, PRP-2 or PIF-s, two corresponding proteolytic products will be formed: 1) an N-terminal fragment PRP-3, PRP-4 and PIF-f, respectively, and 2) a C-terminal fragment IB-8b, which is a BPRP species. The N-terminal fragments generated from the parental polypeptides are all 106 residues in length (Hay et al., 1988). It was suggested that APRPs can be cleaved by the action of salivary kallikrein or other enzymes in vivo (Wong et al., 1983).

APRPs have many biological functions: they bind calcium, inhibit growth of hydroxyapatite crystals, and contribute to the acquisition of the dental pellicle (Bennick, 1982). The function and structure of the APRPs have also been extensively studied. By tryptic digestion, 3 peptides can be derived from PIF-s. They are Tx, the pro-poor N-terminal peptide (residues 1-30), Ty, the following peptide (residues 31-106) and Tz (also known as IB-8b), the C-terminal peptide. Both Ty and Tz are pro-rich peptides (Bennick et al., 1981) (Fig. 4a). The highly negatively charged amino-terminals contribute to calcium-binding (Bennick et al., 1981) and hydroxyapatite-binding (Bennick et al., 1979). The N-terminal segment contains almost all of the negatively charged amino acids in the protein, but the C-terminal segment contains several basic amino acid residues (Wong and Bennick, 1980). Studies on calcium binding to human acidic PRPs and on inhibition of hydroxyapatite formation by these proteins (Hay et al., 1979) suggest that there is an interaction of the N- and C-terminal regions of the proteins, possibly by electrostatic forces (Fig. 4b). The tryptic Tx peptides
Calcium and hydroxyapatite also when they are present in the tryptic digest (Bennick et al., 1981).

Based on these studies, it appears that the interactions between the N- and C-terminal regions impede Calcium-binding and inhibition of crystal growth.
Fig. 4 Structure of PIF-s. (a) Various domains of PIF-s based on its sequence
(b) Suggested interaction of N- and C-terminal regions of PIF-s

- Pro-poor region of PIF-s
- Pro-rich region of PIF-s
- Covalent linked phosphate

The numbers below the schematic primary structure indicates residue number. The numbers in bold follow by a + or - indicate the total number of charges in that region.
Ten BPRPs which constitute about 23% of parotid salivary proteins are designated IB-1, IB-4, IB-5, IB-6, IB-7, IB-8a, IB-8b, IB-8c, IB-9 and DEAE II-2. They have been isolated from human parotid saliva (Kauffman et al., 1991). Only IB-8b which is a proteolytic product of APRPs is present in submandibular saliva. It is identical to the Tz peptide.

Both IB-1 and DEAE II-2 are phosphorylated at residue 8 and have short acidic N-terminal segments similar to the corresponding region of APRP's. All the BPRP's have similar primary structure. They all contain repetitive regions of identical or very similar proline, glycine and glutamine rich sequences and the presence of lysine and/or arginine residues makes these regions basic (Kauffman et al., 1991). The different sizes and slight differences in amino acid residues make all these BPRP isoforms unique. However, they can be divided into 4 groups based on similarity in sequence and size (Kauffman et al., 1991) as shown in Fig.5.

Group I consists of three low MW BPRPs, IB-7, IB-8c and IB-9, which are similar in size (59-61 residues) and sequence. Group II is constituted of two large BPRP's, IB-6 and IB-8a. Their N-terminal regions are identical to IB-7 from the N-terminal to the 51st residue, but they have extensive carboxyl-terminal additions (total lengths of 118 and 170 amino acids respectively). Group III consists of two basic proline-rich phosphoproteins, IB-1 and II-2 (96 and 75 amino acids respectively). IB-1 contains the complete sequence of IB-9 within its C-terminal region. The amino-terminal ends of these proteins are blocked and contain less proline than the repeat sequence (20 mol% in the N-terminal region vs 40 mol% in the C-terminal region). These proteins are less basic than other BPRPs as a consequence of phosphorylation of the serine residues in position 8.
IB-8b, which have sequences that are different from IB-7 and IB-9. Protein IB-8b is a 44-residue polypeptide which has the same sequence as the C-terminal (Tz) segment of the major human parotid acidic PRPs (Wong & Bennick, 1980). IB-4 is a 56 amino acid polypeptide corresponding exactly to the C-terminal region of IB-6 (residues 63-118). IB-5 is a 70-residue polypeptide. All BPRPs except the phosphorylated BPRPs contain about 40 mol% of pro, 21 mol% Glx, 18 mol% Gly. For comparison IB-1 and II-2 contain 33 mol% pro, 17 mol% Glx, 17 mol% Gly.

Until the study presented in this thesis was undertaken the function of BPRPs and the reason for the existence of a vast number of isoforms was not well-understood.

3.2.3 Glycosylated Proline-rich Protein (GPRPs)

Like APRPs and BPRPs, GPRPs have a very high proline content, but unlike their relatives, GPRPs also contain about 40% to 50% carbohydrate by mass (Levine et al., 1987). Amino acid sequences deduced from the genomic DNA and cDNA sequences (Kim et al., 1993; Maeda et al., 1985) revealed that the tandemly repeated proline-rich regions are very similar to the BPRPs, but they contain the recognition sequence Asn-X-Ser of N-glycosylation sites. The GPRPs that have been identified include G1 and two other slow SDS-PAGE migrating variants, CON 1 and CON 2 (Levine et al., 1969; Mandel et al., 1965; Minaguchi et al., 1981; Minaguchi & Suzuki, 1981). PRG, a proline-rich glycoprotein (Levine et al., 1987; Reddy et al., 1982; Maeda et al., 1985), has been purified and characterized. It's secondary structure is comprised of 70% random coil (nated regions) and 30% β-turns (glycosylated domains) (Levine et al., 1987). It is 38.9 Kda in size and contains 40% carbohydrate consisting of 6 triantennary N-linked units. Generally, the serous cell produce glycoprotein containing N-glycans and the mucous cell secretes O-glycan-
3.2.4 PRP Genes

The assignment of gene loci to the human salivary PRP family has been a subject of intense study. Early investigations based on studies of salivary PRP polymorphism suggested that no fewer than 17 genes would be required (Azen et al., 1978, 1979, 1980). However, studies by Maeda et al. (1985), Azen et al. (1987) and Lyons et al. (1988), led to the replacement of the originally proposed 17-loci system with a 6-loci system. The 6-loci system is able to accommodate a large number of APRPs, BPRPs and GPRPs. The genes are named PRB1, PRB2, PRB3, PRB4, PRH1 and PRH2, and each of them has several alleles. APRPs are encoded by PRH1 and PRH2. BPRPs and GPRPs are encoded by PRB1, PRB2, PRB3, and PRB4 (Lyons et al., 1988).

The six human PRP genes are arranged on chromosome 12p (Mamula et al., 1985) in the order of 5' PRB2-PRB1-PRB4-PRH2-PRB3-PRH1 3' (Kim et al., 1990). This cluster spans approximately 600 kb with 70-180 kb separating adjacent genes.

Since the number of secreted PRPs exceed the number of genes it is likely that either differential splicing of mRNA occurs or that the PRPs are synthesized as proproteins that subsequently are cleaved.
In the case of BPRPs, all secreted forms are smaller in size than the BPRP precursor deduced from cDNA sequences, indicating that the secreted BPRPs could be generated from a larger BPRP precursor by postribosomal cleavage (Lyons et al., 1988). The proposed proteolytic cleavage site in the initially translated products of the BPRP and GPRP genes have the consensus sequence Arg-Ser-X-Arg/I where X can be Ser, Ala or Pro, and I indicates the cleavage position. Other studies in our laboratory have shown that a recombinant BPRP precursor protein, the product of PRBIM, is cleaved in vitro by furin, a proprotein convertase responsible for cleavage of various precursor proteins. While it is possible that differential mRNA splicing generating various BPRP transcripts could give rise to the secreted proteins there is no evidence for this mechanism at least in the case of acidic PRPs (Robinson et al., 1989).

Posttranslational cleavages may play a pivotal role in generating more than 22 secreted salivary PRPs from the precursors and also may alter the function of these proteins. This is well-demonstrated for APRPs. PRP-1, PRP-2, PIF-S and Db-s can bind calcium ions and inhibit the formation of hydroxyapatite due to their highly acidic N-terminal transitional regions. As PRP-1, PRP-2, PIF-s, Db-S become proteolytically modified into PRP-3, PRP-4, PIF-f, Db-f respectively, the ability of the proteins to inhibit crystal growth and bind calcium is dramatically increased (Moreno et al., 1979; Bennick et al., 1981).

The effect of proteolytic cleavages of BPRP and GPRP precursors on their biological functions has not been studied.

3.2.6 PRPs in Other Tissue and Animals

The unique multigene family of PRPs is not merely restricted to the oral cavity since small amounts of BPRPs have also been found in the respiratory tract (Warner & Azen, 1984, 1988).
mill dust which otherwise would cause an acute pulmonary inflammatory response in cotton mill workers. PRPs have also been isolated and characterized to a lesser extent in saliva or salivary glands of various animals including monkeys (Oppenheim et al., 1979), rabbits (Rajan and Bennick, 1983), rats (Fernandez-Sorenson & Carlson, 1974), mice (Mehansho et al., 1985), and hamsters (Mehansho et al., 1987).

4. **Defensive Response to Dietary Tannins**

In normal rat salivary glands there are only small amounts of PRPs (< 10% of the total soluble proteins) present (Muenzer et al., 1979). It has been observed that chronic isoproterenol treatment of rats results in an enlargement of the parotid gland and an increase in PRP synthesis (Robinovitch et al., 1977; Mehansho et al., 1983). An increase in PRP mRNAs was also reported (Robinovitch et al., 1977; Mehansho et al., 1992).

Interestingly, feeding rats high-tannin sorghum caused dramatic changes similar to those induced by isoproterenol (Mehansho et al., 1983). Within 3 days of the initiation of the feeding trial, the parotid glands were enlarged about 3-fold and PRP synthesis was increased about 12-fold. The amount of mRNAs coding for PRPs was also increased. Similar findings were obtained when rats were fed a diet containing tannin-rich extract of faba bean hulls (Jansman et al., 1994). There was a linear dose-response relationship between tannin level in the diet and increase in the size of parotid glands as well as quantity of PRPs in the gland (Jansman et al., 1994). It was demonstrated that tannin contained in the diet was responsible for the observed changes (Mehansho et al., 1992). Mice showed the same response to either isoproterenol or tannin as did rats (Mehansho et al., 1985). The induced rat and mouse PRPs have been isolated and characterized (Mehansho et al., 1984), and their high affinities for tannin have been determined.
Mehansho et al. (1992) reported that addition of propanolol (β-antagonist) to the high-tannin diet blocked both the induction of PRPs and the hypertrophy of the parotid gland. Therefore, it was proposed that tannin might mimic isoproterenol and have an effect on β-adrenergic receptors of the acinar cells.

Carlson and coworkers demonstrated that feeding rats and mice high-tannin sorghum led to an initial weight loss, but within 3 days there was a marked stimulation of PRP synthesis and at the same time weight gain was initiated (Mehansho et al., 1983, 1985). Hamsters also showed weight loss when placed on a diet high in tannin, but in contrast to rats and mice, there was no induction of PRP synthesis, and a growth inhibition was observed as long as the animals were kept on this diet (Mehansho et al., 1987). Inclusion of 4% tannin in the diet had no effect on rats and mice, but was fatal to hamsters, with most animals dying within 3 days (Mehansho et al., 1987).

The saliva of deer was found to contain a larger number of tannin-binding salivary proteins than the saliva of cow or sheep (Robbins et al., 1987). A major tannin-binding protein in deer saliva has been isolated and demonstrated to be a PRP-like protein. Mole and co-workers (1990) studied a wide range of animals and suggested that PRPs were the most likely proteins to act as a defense against tannins in the rodents and lagomorpha. These experiments provided powerful support for the protective role of PRPs against tannins and led to the novel suggestion that salivary PRPs may play an important role as a first line-defense against dietary tannins (Mehansho et al., 1987).

PRPs are the most abundant proteins in human saliva and many functions of APRPs and GPRPs have been delineated (Bennick, 1982), but the function of the human basic PRPs is more uncertain.
animals and that PRPs may protect them against this chemical. Thus, by inference, it would also be expected that tannin may be harmful to humans and that PRPs particularly BPRPs may play an important role in defence against tannin. Support for this suggestion can be found from observations on grain elevator and cotton mill workers who suffer from byssinosis, an acute inflammatory reaction of the lungs which can be traced to the presence of tannin in the dust inhaled by these workers (Lauque et al., 1988; Skea et al., 1988). Interestingly it has been demonstrated that salivary BPRPs are present in the respiratory tract (Warner et al., 1984). They may represent a defense system that has been overwhelmed in these workers.

Tannic acid has been used in humans for the treatment of burns, but this practice was discontinued because it was found to be hepatotoxic (Singleton, 1981). Moreover tannin has been used in enemas as an adjuvant to improve contrast in x-rays (Kerzanoski, 1966; Singleton and Kratzer, 1969), but it has been stopped because these procedures led to several fatalities caused by liver and renal necrosis due to the absorption of tannin (Lucke et al., 1963). These observations demonstrate the harmful effect of tannin once it has gained access to the human body. They also suggest that tannin in food must be neutralized before it reaches the intestines. However, except for the observations of Yan and Bennick (1995), there have been no studies on the interaction of tannins and human PRPs, the major proteins in saliva.

By studying the absorption of human saliva with tannin Yan and Bennick (1995) found that HRP's were the predominant proteins precipitated by tannin even when only small amounts of tannins were present, and that APRPs and BPRPs were also precipitated when the amount of tannin was increased. So HRP's and PRPs may act as a defence against tannin.
The study in this proposal is based on the hypothesis that human salivary proteins by complexing to food tannins neutralize their toxic ability and allow them to pass through the alimentary tract without causing any harmful effects. Therefore, the aim of this study was to test this hypothesis and the experiments included were as follows:

(1) Characterization of the interaction of tannins and human salivary proline-rich proteins. This includes:

a) Evaluation of the ability of acidic, basic and glycosylated PRPs to precipitate tannins;
b) Evaluation of the effect of the size of the proteins on the formation of insoluble tannin-PRPs complexes;
c) Evaluation of the effect of variation in the primary structure of BPRPs on their ability to bind tannin;
d) Evaluation of the effect of proteolytic cleavage of the acidic PRP PIF-s on its ability to precipitate tannin;
e) Evaluation of the effect of the carbohydrate moiety of glycosylated PRP on its tannin binding ability.

(2) Evaluation of the stability of tannin-PRP insoluble complexes under conditions prevailing in the alimentary canal.
Materials

Commercial tannic acid was purchased from Fisher Scientific Company, Fair Lawn, NJ, U.S.A. and crude quebracho tannin was a gift from Dr. A. E. Hagerman, University of Miami, Oxford, OH, U.S.A. Sephadex LH-20, G-200, SP Sephadex C-25 and DEAE Sephadex A-25 were obtained from Pharmacia LKB Biotechnology AB, Bä d’Urfé, PQ, Canada and Bio Gel P-10 from Bio Rad Laboratories, Richmond CA, U.S.A. Dialysis tubing was purchased from Spectrum Medical Industries, Houston, TX, U.S.A. and trypsin, chymotrypsin, elastase, carboxypeptidase A and B and peroxidase conjugated rabbit anti-goat IgG from Sigma Chemical Company, St. Louis, MO, U.S.A. N-glycosidase F was obtained from Boehringer Mannheim Canada, Laval, PQ, Canada, and glycodeoxycholic acid (sodium salt) from Calbiochem Corp., La Jolla, CA, U.S.A. Gelatin was the product of Anachemia Sci Inc., Montreal, PQ, Canada.

Tannic acid was purified by the method of Hagerman and Butler (1982). Condensed tannin was purified by the method of Asquith and Butler (1985) from the quebracho tannin obtained from Dr. A. Hagerman, Miami University, Oxford, OH, U.S.A. The basic PRP IB-9 was a gift from Dr. P. Keller, University of Washington, Seattle, WA, U.S.A.

Methods

1. Collection of Saliva for Purification

At least 200 ml of parotid saliva was collected at a time into a vessel kept on ice using Carlson-Crittenden Cups (Curby et al., 1953). The secretion was stimulated by the donor sucking sour lemon candy. A solution of 0.2 M EDTA (pH 7.2) was added into the collected saliva to a final concentration of 5 mM (Levine & Keller, 1977). The saliva was then dialyzed against 3
The dialysed salvia was freeze-dried. A total of 1,000 ml saliva was collected.

For genetic typing, 5 ml saliva was collected and dialyzed. Typing was done by Dr. K. Minaguchi, Tokyo Dental College, Tokyo, Japan.

2. **Identification of PRPs**

2.1 **SDS-PAGE**

Gel electrophoresis was done using Mini-protean II Electrophoresis Cells (Bio-Rad Lab, CA, U.S.A.). SDS-PAGE was performed according to Laemmli (1970). A 15% acrylamide separating gel and a 5% stacking gel were used, and the ratio of acrylamide : bisacrylamide was maintained at 30:8. Two x sample loading buffer containing glycerol, SDS, and bromophenol blue was mixed with an equal volume of sample and loaded on the gel. Gels were run at a constant voltage of 150 V at room temperature until the dye front ran down to the bottom of gel. The gel was stained in Coomassie Brilliant Blue R-250 for 2 min by heating in a microwave oven at 800 W. Destaining was done in 10% acetic acid for 2 min by heating in the same microwave oven, and destaining was continued at room temperature until the characteristic pink colour of PRPs was seen.

2.2 **Western Blots of Protein Fractions Containing PRPs**

Goat anti-PRP polyclonal IgG was used as the primary antibody. The second antibody was peroxidase conjugate rabbit anti-goat IgG. All protein electrotransblotting was performed in a Bio-Rad Mini Trans-Blot Cell at 4 °C, according to the manufactures instructions. The immunostaining was carried out following the protocol described in Spielman *et al.*, (1989) which was modified as follows:
solution, 0.5 M Tris-HCl pH 7.4 10 ml, HRP color reagent 60 mg, methanol 10 ml, H₂O 80 ml, 30% H₂O₂ 70 μl (added just before developing). After washing the neutral cellulose membrane containing transferred proteins it was incubated with 10 ml 0.25% gelatin in TBS containing 40 μl goat-anti PRP₁ serum at room temperature over night. After rinsing the membrane 3x for 45 min. it was incubated with 5 ml 0.25% gelatin in TBS containing 2.5 mg peroxidase conjugate rabbit anti-goat Ig G for 3 hrs. The membrane was washed 3x before adding the developing solution. The membrane was incubated in developing solution for 10 min and then washed with H₂O.

3. Purification of GPRP and BPRPs

All procedures were carried out at 4 °C. Procedures leading to pure BPRPs and glycosylated PRPs were those described by Kauffman et al. (1991) with various modification as described in the following sections.

3.1 Ammonium Sulfate Fractionation

700 mg of freeze-dried saliva was redissolved in 100 ml of 50 mM potassium phosphate buffer, pH 6.8 and solid ammonium sulfate (25.8 g) was very slowly added under gentle stirring to a final concentration of 45%. This solution was left overnight, and the resulting suspension was centrifuged at 12,000 g at 4 °C for 15 min. 100 ml of supernatant was recovered and extensively dialyzed against H₂O using dialysis tubing with MW cut off 6,000 - 8,000 for 20 hrs at 4 °C. This was followed by dialysis against the 50 mM Tris-HCl pH 8.5, until the conductivity of the sample was the same as that of the buffer (24 hrs).
Half the amount of the prepared saliva sample was applied to a column of DEAE Sephadex A-25 (2.6 x 92 cm, bed volume 450 ml), which had been equilibrated with the starting buffer (50 mM Tris-HCl pH 8.5). The column was developed with 600 ml of the starting buffer followed by a linear gradient of 250 ml of the starting buffer and 250 ml of the same buffer containing 1 M NaCl. Elution was monitored by measuring $A_{230}$ in a 1 cm cell. The flow rate was 9.6 ml/h and 4.8 ml fractions were collected.

3.3 Sephadex G-200 Chromatography

Peak I (DEAE I) from the DEAE Sephadex A-25 column, which contains BPRPs and GPRPs, was dialyzed against H$_2$O and freeze-dried. The dried sample was dissolved in 50 mM Tris-HCl buffer pH 8.5 and one fifth of the redissolved sample (25 ml) was loaded on a column of Sephadex G-200 (2.6 x 92 cm, bed volume 450 ml), which had been equilibrated in 50 mM Tris-HCl buffer pH 8.5. The column was developed at a flow rate of 6.0 ml/h, and 5 ml fractions were collected. The elution was monitored by measuring $A_{230}$. Peak A (IA) of Sephadex G-200, which contained large slowly migrated PRPs, was concentrated and refractionated on G-200 to get a pure glycosylated PRP.

3.4 Chromatography in SP Sephadex C-25

Fractions constituting peak B (IB) of Sephadex G-200, which contained smaller BPRPs, were pooled, freeze-dried, redissolved in 30 ml H$_2$O, and immediately dialyzed against 0.14 M acetic acid/sodium acetate buffer containing 50 mM NaCl, pH 3.6 using dialysis tubing with MW cut off 2,000 until the conductivity and pH of the sample was the same as that of the starting buffer (4 hrs). One half of the sample was loaded on a column of SP Sephadex C-25 (2.6 x 53
50 mM NaCl (starting buffer). The column was developed at a flow rate of 18 ml/h and 6 ml fractions were collected. After application of 150 ml of starting buffer, a linear gradient which consisted of 750 ml starting buffer and 750 ml of 0.14 M acetate buffer containing 0.3 M NaCl pH 3.6 was initiated. Identification of BPRPs was performed by comparing them with standard BPRPs on SDS-PAGE and western blotting.

3.5 Purification on Bio Gel P-10

To eliminate minor contamination, the BPRPs needed for this study (IB-1, IB-4, and IB-8b) were freeze-dried and further purified on a column of Bio Gel P-10 (2.6 x 90 cm, bed volume 420 ml) with 50 mM ammonium bicarbonate as the eluent. The column was monitored by measuring $A_{230}$.

3.6 Purification of IB-6

IB-6 was purified from 200 ml parotid saliva from a donor of IB-6 genotype. The procedure was performed as described by Kauffman et al., (1991) except that all chromatography was carried out on an FPLC system (Fast protein liquid chromatography, Pharmacia Co.) using Mono Q HR 10/10, Superose 12, and Mono S HR 5/10 columns (Pharmacia).

The saliva sample was precipitated with ammonium sulfate as described in section 3.1 and one tenth of the supernatant applied to an anion exchange column (Mono Q HR 10/10). To develop the column, two buffers were used, Buffer A: 50 mM Tris-HCl pH 8.5; Buffer B: Buffer A contain 1 M NaCl.

The method used was as follows: flow rate, 1 ml/min; fraction volume, 1 min; 0% B for 30 min; 0-100% B for 30 min; 100% B for 15 min; 0% B for 20 min. The unbound peak eluted
(Superose 12) using 50 mM ammonium bicarbonate at a flow rate 0.5 ml/min. Fractions were collected every 2 min. Those fractions containing IB-6 were further purified on a Mono S cation exchange column with two buffers, Buffer A, 0.014 M acetic acid/sodium acetate pH 3.6 containing 0.05 M NaCl and Buffer B, 0.014 M acetic acid/sodium acetate pH 3.6 containing 0.3 M NaCl. The column was developed at a flow rate 0.5 ml/min and fractions were collected every 1 min. The gradient used was 0% B for 5 min; 0-100% B for 30 min; 100% B for 5 min; and 0% B for 5 min.

The purified protein (IB-6) was identified by SDS-PAGE.

4. Purification of PIF-s. Tryptic Peptides of PIF-s and Dephosphorylated PIF-s

PIF-s was separated from a mixture of PIF-s and PRP-1 prepared in our laboratory, on a Mono Q column using FPLC as described by Bennick et al. (1985). To avoid bacterial contamination, the buffer which was made with autoclaved water contained 5% chloroform and 0.1% PMSF. Some contaminating peptides were eliminated as described by Bennick et al. (1981). To further obtain a pure PIF-s, a column of superose 12 equilibrated with 5 mM ammonium bicarbonate was used. Tryptic digestion of PIF-s was done for 7 min. as described by Bennick et al. (1981). SDS-PAGE was performed to identify the tryptic products of PIF-s. PIF-s, standard Ty, standard Tz, trypsin and pancreatic trypsin inhibitor, and tryptic product of PIF-s were loaded on a 15% SDS-gel.

Tx (N-terminal peptide of PIF-s, residues 1-30) had previously been purified in our laboratory, and Ty (constituting residues, 31-106) was purified as described (Bennick et al., 1989) by fractionation of the tryptic digest on a Mono Q column. Tz (the C-terminal peptide, residues 101-150), which is identical to IB-8b (one of the BPRPs), was purified during purification of

36
dephosphorylated PRP1, which previously had been dephosphorylated as described (Bennick et al., 1989). It was separated by the same procedure as used for purifying PIF-s.

5. **Protein Analysis**

To identify the purified proteins, the following methods were employed:

5.1 **Amino Acid Analysis**

Amino acid analysis of purified proteins and peptides was performed at the Biotech Centre (University of Toronto). Samples were hydrolysed under vacuum in 6 N HCl containing 1% phenol for 24 hours at 110 °C and analyzed on a Waters PICO-TAG system (Waters Associates, MA, U.S.A.).

5.2 **Mass Spectroscopic Analysis**

The purified samples were sent to the Mass Spectrometry Laboratory, Faculty of Medicine, University of Toronto and the analysis was done employing a PE SCIEX API-III mass spectrometer (Perkin Elmer, ON, Canada) using electrospray ionization.

5.3 **Carbohydrate Analysis**

The procedure was performed following the method described by Dr. I. Brockhausen, Dept. of Biochemistry, University of Toronto at the Carbohydrate Centre, Hospital for Sick Children (personal communication). For analysis of neutral sugar, 100 µl (1 µg/µl) of GPRP was freeze-dried and then hydrolysed under nitrogen in 0.6 ml 6 N HCl for 1 hour at 100 °C. The sample was evaporated by blowing nitrogen into the tube. It was redissolved in 0.1 ml water and loaded
column). The same procedure was used for analysis of sialic acid, except that hydrolysis was done by incubating GPRP in 0.1 ml 33.3% trifluoroacetic acid for 1 hour at 80 °C.

5.4 Enzymatic Deglycosylation of GPRP

The result of the sugar analysis of GPRPs suggested that GPRP is an N-glycan so N-Glycosidase F (PNGase F) digestion was used to release the carbohydrate sidechains. GPRP was incubated with the N-Glycosidase F at a ratio of 20 μg GPRP to 50 mU of PNGase F for 3 days according to the manufacturers instructions. A sample of 800 μl (1 μg/μl) GPRP dissolved in H₂O was mixed with 800 μl 50 mM phosphate buffer pH 8.6, and 10 μl PNGase F (200 mU/μl) was added. The mixture was incubated at 37 °C for 3 days, and stored at -20 °C. GPRP and deglycosylated polypeptide cores of GPRP (de-GPRP) were loaded on a 12.5% separating SDS-gel to monitor deglycosylation. Western Blotting of de-GPRP was performed as described in section 2.2.

De-GPRP was purified on a FPLC system using a column of Superose 12 equilibrated in 50 mM ammonium bicarbonate. Protein content of the fractions was monitored by measuring A₂₃₀ and the carbohydrate was determined by PAS assay (Zanetta et al., 1972).

6. Assay for Insoluble PRP-Tannin Complexes of Various PRPs and Tannins

The assay was performed as described by Hagerman and Butler (1978) modified as described below. Stock solutions included a colour reagent containing 0.01 M FeCl in 0.01 N HCl and an alkaline solution containing 1% SDS in 5% (V/V) triethanolamine. A 50 mM phosphate buffer containing 250 mM NaCl pH 7.4 was used as incubation buffer.
of tannic acid and $A_{281}$ in the case of condensed tannin. Tannins were weighted and dissolved. The extinction coefficient of tannin was 32 cm$^{-1}$·(mg/ml)$^{-1}$ at OD 275 for tannic acid and 14 cm$^{-1}$·(mg/ml)$^{-1}$ at OD 281 for condensed tannin which were obtained from the mean values of 4 individual assays of tannin solution on the spectrophotometer.

6.1 Incubation of PRPs and Tannin

Purified tannins were dissolved in the incubation buffer, centrifuged at 15,000 g for 15 min to remove a small undissolved pellet, and adjusted to a final concentration of 4 mg/ml using the appropriate extinction coefficient. Various amounts of tannin 0 to 160 µg were added to microcentrifuge tubes and dried down in a speed vac. These samples were used as standards to determine the amount of tannin in the samples assayed. Freshly prepared tannin solutions were used due to the sensitivity of tannin to oxidation.

Proteins assayed for their ability to form insoluble complexes with tannin were PIF-s (an acidic PRP), tryptic digest of PIF-s, purified tryptic peptides obtained from PIF-s (Tx, Ty, Tz), dephosphorylated PIF-s, GPRP (glycosylated PRP), de-GPRP (deglycosylated GPRP), gelatin, and purified BPRPs (IB-1, IB-4, IB-9, IB-6).

Volumes of 10 µl of a solution containing 1 µg/µl protein in H$_2$O were placed in microcentrifuge tubes and 50 mM phosphate buffer, pH 7.4 containing 250 mM NaCl, and 0 to 80 µl of 4 µg/µl tannin dissolved in the 50 mM phosphate buffer were added. 50 mM phosphate pH 7.4 containing 250 mM NaCl was added to a final volume of 120 µl. The mixture was incubated at 37 °C for 1 hour and the insoluble protein-tannin complexes were obtained by centrifugation at 15 000 g for 15 min. The supernatant of each tube was removed and the pellet was washed twice by adding 120 µl of the reaction buffer and centrifuging at 15 000 g for 5 min.
After the final wash, the pellet was dissolved by adding 600 μl of the alkaline solution followed by addition of 100 μl of the colour reagent and mixing. The samples were incubated at room temperature for 20 min and A512 was read.

7. Identification of Tryptic Peptides Present in the Insoluble Complexes

After incubation of 10 μg tryptic digest of PIF-s and tannic acid, the insoluble pellet and supernatant were separated and loaded onto 15% separating SDS-gel to identify the Ty and Tz peptides in the pellet and supernatant. Native electrophoresis on 35% gels was performed as described by Minaguchi et al. (1988) for visualisation of the Tx peptide in pellet or supernatant.

8. Physiological Stability of Insoluble PRP-tannin Complexes

The stock solutions included: 1 mg/ml IB-8b in dH2O; solution A, 50 mM phosphate buffer containing 250 mM NaCl pH 7.4; solution B, 0.01 M HCl/0.09 M NaCl pH 2.0; solution C, solution B containing 1 mg/ml pepsin; solution D, 29 mM phosphate buffer containing 76.5 mM NaCl, pH 7.4; solution E, solution D containing 1 mg/ml trypsin, 1 mg/ml chymotrypsin, 0.6 mg/ml elastase, 0.14 mg/ml carboxypeptidase B, and 0.05 mg/ml carboxypeptidase A; solution F, solution D containing 9.5 mg/ml glycodeoxycholic acid; solution G, solution E containing 9.5 mg/ml glycodeoxycholic acid; solution H, 1 mg/ml pepsin in dH2O; solution I, solution E but replacing the buffer with water. The procedure is outlined in the flow chart in Fig. 6.
Fig 6. Procedure for Studying the Stability of Insoluble Complexes under the Conditions (solutions B, C, D, E, F, and G) Prevailing in the Alimentary Canal
Stomach.

IB-8b was incubated with tannin at a concentration of 160 μg/120 μl in solution A (120 μl, final volume) for 1 hour and the pellet was isolated and incubated at 37 °C for 210 min either in solution B which had ionic composition and pH similar to gastric juice or in solution C which contains pepsin. This time period is twice that of the "half time" for food in the stomach (Hunt and MacDonald, 1954). The pellet and supernatant were separated and the amount of tannin in each sample determined by the colorimetric assay. The stability of the complexes was determined by comparing the amount of tannins remaining in the pellet with that in the pellet before incubation in solution B or C.

8.2 Stability of Insoluble PRP-tannin Complexes under Conditions Prevailing in the Small Intestine.

After incubation of the protein-tannin pellet in solution C, insoluble tannin-protein complexes were sedimented by centrifugation at 15,000 g for 15 min. and the pellet was incubated in solution D which had ionic composition and pH similar to duodenal juice or solutions E, F, or G which had concentrations of proteolytic enzymes or bile similar to those found in duodenal juice. The samples were incubated for 150 min. which is twice that of the "half time" of food in the small intestine (Caride et al, 1984) at 37 °C, and the stability was determined by comparing the amount of tannin left in the pellet with that which was present in the pellet at the start of the incubation.
The procedure used is outlined in Fig. 7. The pellet was incubated at pH 2.0 containing pepsin (solution C) and the pellet (1) isolated. Another pellet was incubated at pH 2.0 (solution B) and the pellet (2) was also isolated. After removing pellet (2) the supernatant was transferred to a microcentrifuge tube containing the dried down pepsin solution (solution H) incubated for 210 min and the pellet (3) was isolated. The amount of tannin was determined by the colorimetric assay as described in II 6.2.

The amount of tannin in pellet (1) was compared to the sum of amounts of tannin in pellets (2) and (3). Similarly, the amount of tannin in the pellet obtained after incubation in solution pH 7.4 containing pancreatic enzymes (solution E) or pH 7.4 containing pancreatic enzymes and GDC (solution G) was compared with the sum of tannin in the pellet obtained after incubation in solution D (pH 7.4) or F (pH 7.4 containing GDC) and the pellet obtained by incubating the supernatant derived from incubation of solution D or F with the dried down pancreatic enzymes in dH$_2$O.

### 8.4 Effect of Time on the Stability of Pellet

To study the effect of time on the stability of insoluble complexes, the incubation period of pellet in the solutions D (pH 7.4), E (pH 7.4 containing pancreatic enzymes), F (pH 7.4 containing GDC, or G (pH 7.4 containing pancreatic enzymes and GDC) was increased up to 4 days since the transient time for food in the colon has been estimated to 2-5 days (Caride et al., 1984).
Fig. 7 Procedure for determining the precipitation of dissociated tannins by enzymes in the alimentary canal
1. **Tannic Acid Purification**

HPLC of purified tannic acid on a μ Porasil column showed that components with low MW were removed from the gallotannins. The main components were tetragalloyl glucose, pentagalloyl-glucose, hexagalloyl-glucose, heptagalloyl-glucose, and octagalloyl-glucose. Only a small amount of gallic acid remained in the mixture. (Results not shown).

2. **Purification of PRPs**

2.1 **Determination of Donor YL's Phenotype**

On the basis of gel electrophoresis of YL's parotid saliva as performed by Dr. Minaguchi, the following proteins were found to be present:

<table>
<thead>
<tr>
<th>Acidic PRPs</th>
<th>Basic PRPs</th>
<th>Glycosylated PRPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIF-s +</td>
<td>IB-6 -</td>
<td>Gl₁ -</td>
</tr>
<tr>
<td>PIF-f +</td>
<td>IB-8a +</td>
<td>PS -</td>
</tr>
<tr>
<td></td>
<td>IB-5 +</td>
<td>CON₁ -</td>
</tr>
<tr>
<td></td>
<td>IB-4 +</td>
<td>PC₂ +</td>
</tr>
<tr>
<td></td>
<td>IB-7 +</td>
<td>PC₁ -</td>
</tr>
<tr>
<td></td>
<td>IB-8c +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IB-9 -</td>
<td></td>
</tr>
</tbody>
</table>

- Denotes absence of protein. + Indicates that the protein is present.
to be able to assay PRPs of different sizes and amino acid sequences for tannin binding the acidic PRP, PIF-s, the basic PRPs, IB-1, IB-4, IB-8b (a product of proteolytic processing of PIF-s) and glycosylated PRP (GPRP) were selected for purification.

Additionally IB-6 (MW, 11513) was purified from a second donor (AB).

2.2 Purification of IB-1, IB-4, IB-8b and GPRP

Salivary proteins remaining in solution in 45% (NH₄)₂SO₄ were fractionated on a column of DEAE Sephadex A-25, equilibrated with Tris-HCl, pH 8.5. Five peaks were generated, DEAE I to V (Fig.8).

An initial peak I (DEAE I), which contained the basic and glycosylated PRPs, was eluted with the starting buffer before initiating a salt gradient.

Fig. 9A shows the result of SDS-PAGE of fractions from DEAE I. DEAE I contained pink-stained bands which is a characteristic colour for proline-rich proteins.

Fig. 9B shows the result of western blotting of fractions from DEAE I. The gel shows that fractions #46 to #70 reacted with the goat anti-PRP IgG but they did not react with normal goat IgG. These results indicate that the DEAE I contains basic proline-rich proteins.

Fraction DEAE I, which contained basic and glycosylated proline-rich proteins, was concentrated and loaded on a Sephadex G-200 column. Two major peaks (IA and IB) were obtained (Fig.10). Fig. 11 show the results of SDS-PAGE of fractions from Sephadex G-200 chromatography. The large molecules, glycosylated PRPs, were eluted in IA, while small pink-staining non-glycosylated PRPs eluted mainly in the second peak (IB). Some low MW proteins that stained blue eluted in the fractions following IB (fractions 88-98). Western blotting of the same fractions confirmed that PRPs were contained in the peak IA and peak IB while the small
IA was refractionated on the Sephadex G200 column to eliminate contaminants from the glycosylated PRPs (GPRP). Fig. 12 shows SDS-PAGE of samples obtained from refractionation on G-200. Fractions #55 to #65 were pooled as purified GPRP.

Peak IB, containing basic proline-rich proteins (BPRPs), was further fractionated on a column of Sp Sephadex C-25. The BPRPs were separated into 5 peaks by a very shallow linear gradient of NaCl from 0.05 M to 0.3 M in 750 ml buffer (Fig. 13). SDS-PAGE showed that the major proteins in these peaks had the same migration rate as standard IB-1, IB-4, IB-5, IB-7 and IB-8b. As well, western blotting showed that they reacted well with the primary antibody (goat anti-PRP IgG) and that normal IgG could not detect these proteins (results not shown).

To eliminate minor components, peaks containing IB-1, IB-4, and IB-8b were applied separately to a Bio Gel P10 column. This gave rise to pure IB-1, IB-4, and IB-8b as shown by SDS-PAGE of these and other purified PRPs (Fig. 20).
Fig. 8 Chromatography of saliva soluble in 45\% (NH_4)_2SO_4 on DEAE-A 25. Fractions from \#45 to \#90 contained the basic and glycosylated proline-rich proteins were pooled and freeze-dried as DEAE I. It was further fractionated on Sephadex G-200.

---

Fraction run on gel

NaCl Concentration
Fig. 9  SDS-PAGE (15 % gel) (A) and Western Blotting (B) of Fractions obtained by Anion Exchange Chromatography on a DEAE Sephadex A-25 Column. Goat anti-human PRP1 Ig G was used as primary antibody and peroxidase conjugated rabbit anti-goat Ig G was the second antibody.
Fig 10. Chromatography of DEAE-I on Sephadex G-200 Column. Peak IA which contained large glycosylated PRPs was refractionated on the Sephadex G-200 and fraction IB was subjected to purification on Sp-Sephadex C-25.

---

**A230**

- Fraction run on gel.
Fig. 11 SDS-PAGE (15% gels) of Fractions from G-200. Fractions #45 to #62 (Peak IA) were enriched in GPRP and fractions #64 to #80 (Peak IB) contained predominantly small BPRPs.
Fig. 12 SDS-PAGE (12.5% gel) of GPRP obtained by refractionation on Sephadex G-200.
Fig. 13 Chromatography of IB on Sp-Sephadex C-25. BPRPs were eluted when a gradient of NaCl from 0.05 M to 0.3 M in 750 ml buffer was initiated. The peaks are labeled IB-1, IB-4, IB-5, IB-7 and IB-8b according to their content of BPRPs.
IB-6 was purified using a number of Pharmacia columns, Mono Q HR 10/10, Superose 12 HR 10/26, and Mono S HR 5/5 on an FPLC system. A sample of 200 ml parotid saliva from a second donor (A.B) was fractionated by addition of ammonium sulphate to a concentration of 45% and the supernatant was loaded on a Mono Q column. The first peak was eluted by buffer A (starting buffer) before initiating a salt gradient (Fig. 14). Fig. 15 shows the result of SDS-PAGE of fractions from the first peak eluted from the Mono Q column. This peak contained IB-6. Fractions #4 to #8, which contained IB-6, were pooled, concentrated and loaded on a Superose 12 column (Fig. 16). IB-6 was present in fractions #14 and #15 (Fig. 17) and these fractions were combined and further purified on a Mono S column (Fig. 18). SDS-PAGE showed that the protein in peak II had the same migration rate as standard IB-6 (Fig. 19). Fractions #18 and #19 were pooled as pure IB-6.
Fig 14. Chromatography of Saliva soluble in 45% (NH₄)₂SO₄ on Mono Q. Proteins in the first peak were eluted before initiating a gradient buffer and fractions #3 to #8 were pooled and subjected to further fractionation on a gel filtration column.

---

A230

Diamond symbol: Fraction run on gel.

Dashed line: NaCl Concentration
Fig. 15  SDS-PAGE (15% gel) Analysis of Fractions Obtained from a Mono Q HR 10/10 Column. Fractions were from peak I which was eluted from the Mono Q column before initiating a gradient buffer. Fractions #3 to #8 were subjected to further fractionation on a gel filtration column.
Fig 16. Chromatography of Mono Q Peak I on Superose 12

- A230
- Fraction run on gel
Fig. 17  SDS-PAGE (15% gel) Analysis of Fractions obtained from the Superose 12 Column. Standard IB-6 was loaded on the second lane to the left as a indicator.
Fig 18. Chromatography of Superose 12 IB-6-rich fractions (#14, 15) on a Mono S Column

- A_{230}
- Fraction run on gel
- NaCl Concentration
Fig 19. SDS-PAGE (15% gel) Analysis of Fractions obtained by Chromatography on a Mono S Column
Dephosphorylation of PIF-s was monitored by staining the dephosphorylated PIF-s with Stains All which is a very sensitive stain for phosphoproteins. The result of SDS-PAGE showed that 10 µg dephosphorylated PIF-s could not be detected while 0.3 µg of PIF-s showed a strong blue staining band with Stains All (result not shown).

PIF-s, dephosphorylated PIF-s and Ty were purified on columns of Mono Q and Superose 12 using FPLC and the results of SDS-PAGE of these purified APRPs and Ty are shown in Fig. 20. Tz (IB-8b) was purified during fractionation of BPRPs.

Fig. 20 shows SDS-PAGE of purified IB-1, IB-4, IB-6, IB-8b, GPRP, PIF-s, dephosphorylated PIF-s, Ty.
Fig 20. SDS-PAGE (15% gels) of Purified PRPs

Gel (1):
Lane 1: MW Markers, 66K, 45K, 36K, 29K, 24K, 20K and 14.2K
Lane 2: Standard IB-1, 3 μg
Lane 3: Purified IB-1, 3 μg
Lane 4: Standard IB-4, 3 μg
Lane 5: Purified IB-4, 6 μg
Lane 6: Standard IB-6, 3 μg
Lane 7: Purified IB-6, 3 μg
Lane 8: Standard IB-8b, 2 μg
Lane 9: Purified IB-8b, 3 μg

Gel (2):
Lane 1: MW Markers, 66K, 45K, 36K, 29K, 24K, 20K and 14.2K
Lane 2: Standard PIF-s, 2 μg
Lane 3: Purified PIF-s, 3 μg
Lane 4: Purified dephosphorylated PIF-s, 4 μg
Lane 5: Standard Ty, 2 μg
Lane 6: Purified Ty, 2 μg
Lane 7: Purified GPRP, 3 μg
The results of the amino acid analysis of purified IB-1, IB-4, IB-8b, GPRP, IB-6 and Ty are shown in tables 2-8 together with published data. There is good agreement between values obtained in this study and published data (Kauffman et al., 1991; Levine et al., 1969; Bennick, 1982). The amino acid analyses also allow determination of protein concentration and extinction coefficient. These values were used for concentration determination in the tannin binding assays.

The results of mass spectroscopy for IB-4, IB-6, IB-8b, PIF-s and dephosphorylated PIF-s are in good agreement with the molecular weight (MW) calculated from published data (Kauffman et al., 1991; Bennick et al., 1989). Attempts to determine the MW of GPRP by mass spectroscopy were unsuccessful.

The difference between the MW (9595) of purified IB-1 and MW (9628) of IB-1 calculated from the amino acid sequence (Kauffman et al., 1991) may be due to mutation of an amino acid residue mutation such as leu to ala or lys to val.

The major component of purified IB-1 is compound A with MW 9595 and minor compounds constituting of B, C and D with MW 9615, 9633, and 9647 respectively. B is probably the sodium salt of A, and C the potassium salt of A, while D is not known. Because the related proteins are similarly ionized during determination of the mass of protein, the amount of proteins in the peaks is correlated to the intensity of protein peak from the result of mass spectroscopy. However this is only a rough comparison and can not be stated precisely. Compounds A, B and C together account for 88% of total according to the intensity of peaks. The major components determined in purified IB-4 are compounds B, C and D which account for 84% of total. The mass of Compound B is 5590 and a mass of 5583 can be calculated from published data (Kauffman et al., 1992). Compound C has a mass of 5611 and D has a mass of 5629. They may be sodium and potassium salts of compound B. The major components of
has a mass of 11,516 which is very close to a mass 11,513 calculated from the composition of IB-6 (Kauffman et al., 1991). Compound B has a mass of 11,536, compound C has a mass of 11,559 and compound D has a mass of 11,580. Their masses are equal to those expected of the sodium, disodium sand trisodium salts respectively of A. Compound B of purified PIF-s has a mass of 15,510. The mass of PIF-s as calculated from its sequence (Hay et al., 1989) is 15,510. Compound B is the major one and accounts for 85% of total, and the minor ones are compound A with a mass 15,428 and compound C with a mass 15,681 accounting for 15% in total. The major component of purified dephosphorylated PIF-s is compound A which has a mass of 15,351, accounting for 66% of total mass. This value is equal to the mass of completely dephosphorylated PIF-s [15,352=15,510 (a mass of PIF-s) - 2 x 79 (a mass of a PO$_3^2$)]. The minor compound B with a mass 15,521 may be the native PIF-s. The major compound A of purified IB-8b with a mass of 4371 is close to an expected mass of 4369 calculated from published data (Kauffman et al., 1992). The other compounds may be sodium salts of compound A.

Carbohydrate analysis of GPRP (Table 14) showed the presence of neutral sugars including fucose, mannose, galactose, galactosamine and glucosamine. No sialic acid was found and together the carbohydrate accounted for 45% of total mass of the protein. The presence of mannose indicates that GPRP is an N-glycan. No sugar was detected in IB-1, IB-4, IB-8b, and IB-6.
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* Adapted from Kauffman et al., 1991.

**Table 3. Amino Acid Analysis of IB-4**

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* Adapted from Kauffman et al., 1991.
### Table 4. Amino Acid Analysis of IR6

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** Adapted from Levine et al., 1976.

### Table 5. Amino Acid Analysis of IB-6

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* Adapted from Kauffman et al., 1991.
### Table 6. Amino Acid Analysis of T25-35

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* Adapted from Kauffman et al., 1991.

### Table 7. Amino Acid Analysis of Ty

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*** Bennick et al., 1982
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<td>A</td>
<td>9594.71±0.94</td>
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<td>B</td>
<td>9615.73±0.58</td>
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<td>C</td>
<td>9633.13±0.79</td>
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<td>D</td>
<td>9647.84±0.65</td>
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Table 9. Mass Spectroscopy of IB-4

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<tr>
<td>A</td>
<td>5461.64±0.62</td>
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<td>B</td>
<td>5590.32±0.46</td>
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<td>C</td>
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<td>D</td>
<td>5628.52±0.44</td>
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<td>E</td>
<td>5650.95±0.82</td>
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Table 10. Mass Spectroscopy of IB-6

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<tr>
<td>A</td>
<td>11,516.24±0.67</td>
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<td>B</td>
<td>11,536.01±0.51</td>
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<td>C</td>
<td>11,558.55±0.52</td>
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<td>11,580.30±0.39</td>
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<td>12,566.48±0.35</td>
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### Table 11. Mass Spectroscopy of PIF-s

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<td>B</td>
<td>15,509.60±0.70</td>
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<td>C</td>
<td>15,681.61±0.71</td>
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### Table 12. Mass Spectroscopy of Dephosphorylated PIF-s

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### Table 13. Mass Spectroscopy of IB-8b

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<td>C</td>
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2.6 PRPs Used in Tannin Binding Studies

Fig. 21 illustrates the relationship of the various PRPs used in the tannin binding studies.

PIF-s is an APRP which has an acidic pro-poor N-terminal region (Tx) and a basic pro-rich region with tandem repeats that are slightly different from those in BPRPs.

Of the basic PRPs, IB-9 has an amino acid sequence that is almost identical to the N-terminal half of IB-6 and the sequence of IB-4 is identical to the C-terminal part of IB-6. While IB-9 and IB-4 have very similar length, their sequences show a number of differences.

IB-1 shares a pro-rich repeat region with IB-9 except for one amino acid difference. In addition it contains a short acidic pro-poor N-terminal region.

The primary structures of basic and glycosylated PRPs are very similar except for the presence of carbohydrate in GPRP. Based on data presented in section 5, the MW of the polypeptide backbone is 19,310 while MW of IB-6 is 15,513 so their sizes are comparable.
Basic PRPs

- IB-9: basic Pro-rich region, 61 A.A.
- IB-4: basic Pro-rich region, 56 A.A.
- IB-6: basic Pro-rich region, 118 A.A.
- IB-1: basic Pro-rich region, 91 A.A.

Glycosylated PRP

- Basic Pro-rich polypeptide back bone
- Sugar Chain

GPRP

Fig. 21 Representative PRPs used in Tannins Binding Studies
The results of the binding assay for BPRPs, acidic PRP and GPRP are shown in Fig. 22 and 23. Fig. 22 lists the result obtained with condensed tannin and Fig. 23 those obtained with tannic acid.

BPRPs (IB-6, IB-9, IB-4, and IB-1) show higher ability to bind condensed tannin as well as tannic acid than gelatin which is generally considered to be a good tannin binder. GPRP does not bind to condensed tannin and only binds to tannic acid at concentration higher than 1.2 µg/µl. These characteristics were also displayed by APRP (PIF-s). There was no binding of tannic acid to APRP unless the concentration of tannic acid was higher than 0.7 µg/µl. Under no conditions did tannin binding to GPRP and APRP exceed that of gelatin. Although the four isoforms of BPRPs have different sizes or sequences, there is little or no difference in the capacity of these BPRPs to bind tannin. IB-9 and IB-4 are very similar in size but although the primary structures are different there is no difference in tannin binding. IB-6 is approximately double the length of IB-9 and a IB-4, but all three proteins bound almost the same amount of tannin. IB-1 has an acidic N-terminal not present in the other BPRPs. It bound less condensed tannin than the other BPRPs and less tannic acid at all except the highest tannic acid concentration.
Fig. 22 Condensed Tannin Binding To PRPs. The curves are based on mean values of three individual assays and error bars were shown (otherwise the error bars are too small to see). 10 µg of each protein was used.
Fig. 23 Tannic Acid Binding To PRPs. The curves are based on mean values of three individual assays and error bars were shown (otherwise the error bars are too small to see). 10 μg of each protein was used.
**PRP-tannin**

Fig. 24 shows SDS-PAGE of a tryptic digest of PIF-s. Three major peptides Tx, Ty, and Tz were obtained. Tx corresponds to the N-terminal region (residues 1-30), Ty to the peptide including residues 31-106, and Tz (IB-8b) to the C-terminal peptide (residues 101-150). In addition a small amount of PIF-f was present. It is derived from PIF-s by removing the C-terminal Tz peptide. Tx migrates at the dye front, it stains poorly with Coomassie blue and cannot be seen on the gel.

Figs. 25 and 26 show the result of tannin binding-assays of dephosphorylated PIF-s, a digest of PIF-s as well as purified Ty, Tz and Tx peptides, and native PIF-s. Digestion of PIF-s resulted in a marked increase in tannin binding ability. Whereas PIF-s showed practically no condensed tannin binding, the amount of tannin bound to the digest was similar to that which bound to BPRPs. Similar results were obtained with tannic acid, although PIF-s does bind tannin at higher tannin concentrations. The tannin binding occurred only to Ty and Tz since assays of individual tryptic peptides showed no tannin binding to Tx but tannin binding to Ty and Tz which was comparable to that of the digest. The dephosphorylated PIF-s could not bind condensed tannin indicating that dephosphorylation of PIF-s does not change its ability to bind tannin.

Fig. 27 shows the results of SDS-PAGE and Fig. 28 the results of native gel electrophoresis of pellets and supernatant obtained after incubation of digested PIF-s and tannin. Ty and Tz were present in the protein-tannin pellet but not in the supernatant (Fig. 27). Tx remained in the supernatant and was not found in the insoluble complexes (Fig. 28).
Fig. 24  SDS-PAGE (15 % gel) of digested PIF-s by trypsin

Lane 1: Mw Markers, from top to bottom are 66 KD, 45 KD, 36 KD, 29 KD, 24 KD, 20 KD, and 14.2 KD.

Lane 2: PIF-s, 10 μg
Lane 3: Ty, 5 μg
Lane 4: Tz, 2 μg
Lane 5: Tryptic digest of PIF-s, 10 μg
Lane 6: Trypsin (1μg) and inhibitor (1μg)
Fig. 25 Condensed Tannin Binding to modified PIF-s. The curves are based on mean values of three individual assays and error bars were shown (otherwise the error bars are too small to see). 10 μg of each protein was used.
Fig. 26  Tannic Acid Binding To modified PIF-s. The curves are based on mean values of three individual assays and error bars were shown (otherwise the error bars are too small to see). 10 μg of each protein was used.
Fig. 27  SDS-PAGE (15% gel) of Supernatant and Pellet obtained after Incubation of Digested PIF-s Precipitated with Tannic Acid and stained with coomassie brillian blue

Lane 1: MW Markers, from top to bottom, 66 KD, 45 KD, 36 KD, 29 KD, 24 KD, 20 KD and 14.2 KD
Lane 2: PIF-s, 10 μg
Lane 3: Ty, 5 μg
Lane 4: Tz, 2 μg
Lane 5: Tryptic Digest of PIF-s, 10 μg
Lane 6: Trypsin and inhibitor, 1 μg each
Lane 7: Pellet
Lane 8: Supernatant
Fig. 28  Electrophoresis of Supernatant and Pellet obtained after Incubation of Digested PIF-s on a 35 % Native Gel stain with Stains All

Lane 1: Dye alone
Lane 2: Tx,
Lane 3: Tryptic Digest of PIF-s,
Lane 4: Tannic Acid alone
Lane 5: Pellet
Lane 6: Supernatant

Tannic acid run just behind the dye front and Tx was identified in the supernatant but cannot be found in the pellet.
GPRP which had been digested by PNGase F for 3 days was subjected to electrophoresis on a 12.5% SDS-gel (Fig. 29). The result shows that the deglycosylated polypeptide cores of GPRP (de-GPRP) have a much faster migration rate than native GPRP. The de-GPRP were purified on a gel filtration column (Superose 12) using an FPLC system and a PAS assay (Zanetta et al., 1972) was used to locate sugars eluted from the column (Fig. 30). The result shows that polypeptide cores were separated from the carbohydrates. The result of carbohydrate analysis of purified polypeptide cores showed that there was no carbohydrate left on the polypeptide backbone. On SDS-PAGE it was found that the deglycosylated polypeptide cores contained a minor band (Fig. 29). A western blotting was performed to identify the minor band. The result showed that both bands reacted with anti-PRP IgG, but they did not react with normal IgG (result not shown). Amino acid analysis of de-GPRP showed that it had a composition similar to that of GPRP (Table 15) and this indicates that the polypeptide backbone is still intact after digestion. The result of mass spectroscopy revealed that the MW of de-GPRP cores are 19,310 and 18,651 (Table 16). This indicates that my GPRPs are a mixture of glycosylated PRPs. All these results indicate that deglycosylated polypeptide cores do not contain any sugar and that they are basic proline-rich polypeptides. The de-GPRP was used in binding assay with tannins. The results (Fig. 31 and 32) showed that the deglycosylated GPRP had a much higher capacity for tannin binding than native GPRP and it was comparable to that of BPRPs. This suggests that the carbohydrate sidechains inhibit interaction of tannin with GPRP.
Fig. 29  SDS-PAGE (12.5% gel) of deglycosylated GPRP and GPRP. Polypeptides deglycosylated by PNGase F (de-GPRP) and GPRP were loaded on a 12.5% gel. Note the faster migration of de-GPRP.
Fig. 30 Chromatography of deglycosylated GPRP on Superose 12. After incubation of GPRPs with PNGase F for 3 days the sample was loaded on a Superose 12 column equilibrated in 50 mM ammonium bicarbonate. The flow rate was 0.5 ml/min, 1 ml fractions was collected and A_{230} monitored. The PAS assay (----) was used to locate carbohydrate.

A_{230}

A_{555}
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>de-GPRP (%)</th>
<th>GPRP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>5.04</td>
<td>5.1</td>
</tr>
<tr>
<td>Glu</td>
<td>18.63</td>
<td>18.71</td>
</tr>
<tr>
<td>Ser</td>
<td>5.47</td>
<td>7.3</td>
</tr>
<tr>
<td>Gly</td>
<td>19.93</td>
<td>19.0</td>
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<tr>
<td>His</td>
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<td>1.8</td>
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<tr>
<td>Arg</td>
<td>5.64</td>
<td>5.1</td>
</tr>
<tr>
<td>Thr</td>
<td>0.68</td>
<td>0.8</td>
</tr>
<tr>
<td>Ala</td>
<td>0.45</td>
<td>0.6</td>
</tr>
<tr>
<td>Pro</td>
<td>31.97</td>
<td>32.6</td>
</tr>
<tr>
<td>Val</td>
<td>0.82</td>
<td>0.7</td>
</tr>
<tr>
<td>Ile</td>
<td>0.77</td>
<td>0.7</td>
</tr>
<tr>
<td>Leu</td>
<td>1.38</td>
<td>1.2</td>
</tr>
<tr>
<td>Phe</td>
<td>0.64</td>
<td>0.6</td>
</tr>
<tr>
<td>Lys</td>
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<td>5.3</td>
</tr>
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</table>

**Table 16. The mass of de-GPRP**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass</th>
<th>Intensity</th>
<th>% of Total Intensity</th>
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<tbody>
<tr>
<td>A</td>
<td>19,309.72±2.03</td>
<td>1,620,000</td>
<td>62</td>
</tr>
<tr>
<td>B</td>
<td>18,650.20±2.90</td>
<td>830,000</td>
<td>38</td>
</tr>
</tbody>
</table>
Fig. 31 Condensed Tannin Binding To Deglycosylated Polypeptide Core of GPRP

The curves are based on mean values of three individual assays (error bars are too small to see). 10 μg of each protein was used.
Fig. 32  Tannic Acid Binding To Deglycosylated Polypeptide Core of GPRP

The curves are based on mean values of three individual assays (error bars are too small to see). 10 μg of each protein was used.
Alimentary Canal

Figs. 33 and 34 show the results of the stability of insoluble complexes under conditions prevailing in the alimentary canal. The insoluble condensed tannin-PRP complexes were very stable under condition prevailing in the stomach since 96% of the complexes remained insoluble when pepsin was present and 93% in the absence of pepsin (Fig. 33 columns IA and IB). The stability of insoluble PRP-tannic acid complexes in the solution with low pH was less than that of condensed tannin-PRP complexes but still 82% of tannin remained insoluble when pepsin was present and 71% in the absence of pepsin (Fig. 34 column: IA and IB). Thus there were small differences in the amount of insoluble tannin with and without pepsin being present. In the presence of pepsin 3% more condensed tannin remained insoluble and 9% more tannic acid was retained in the insoluble complexes.

The insoluble condensed tannin-PRP complexes were less stable when they subsequently were incubated under conditions prevailing in the intestine without glycodeoxycholic acid (GDC), a representative bile salt. A total of 93% remained insoluble in the presence of pancreatic enzymes and 82% when enzymes were absent (Fig. 33, columns IIB and IIA). Addition of GDC decreased the amount of insoluble tannin by about 20%, from 82% to 63%, when enzymes were absence and from 93% to 72% when enzymes were present (Fig. 34, columns IIA and IIB, IIB and IIIB).

When the tannic acid-PRP complexes were incubated under conditions prevailing in the small intestine without GDC, about 82% remained insoluble, but in the absence of pancreatic enzymes, the amount of tannin remaining insoluble decreased to 69% (Fig. 34, columns IIB and IIA). The addition of GDC decreased the stability so that only 53% remained insoluble in the
enzymes (Fig. 34, columns IIIb and IIIa).

To resolve why there were more insoluble complexes in the presence of enzymes, the experiments illustrated in Fig. 35 - 40 were performed. Fig. 35 shows that condensed tannin (column IV) present in the pellet obtained by incubating at pH 2.0 with pepsin is equal to the sum (column III) of the condensed tannin (column I) found in the pellet obtained by incubation at pH 2.0 in the absence of pepsin and the condensed tannin (column II) precipitated by the addition of pepsin to the supernatant (see the procedure in Fig. 7). Similar results were obtained when incubations were done at neutral pH in the absence of GDC (Fig. 36) and when GDC was present (Fig. 37). The condensed tannin (Fig. 36 and 37, column IV) present in the pellet obtained by incubation in 29 mM phosphate buffer pH 7.4 containing pancreatic enzymes was equal to the sum (Fig. 36 and 37, column III) of the condensed tannin (Figs. 36 and 37, column I) present in the pellet obtained by incubation in 29 mM phosphate buffer, pH 7.4 and the condensed tannin (Figs. 36 and 37, column II) precipitated by addition of pancreatic enzymes to the supernatant from that incubation.

Fig. 38 shows that tannic acid present in the pellet formed by incubation of pH 2.0 with pepsin (column IV) equals to the sum (column III) of the tannic acid found in the pellet (column I) obtained by incubation at pH 2.0 in the absence of pepsin and the tannic acid (column II) precipitated by the addition of pepsin to the supernatant. When incubations were done at neutral pH in the absence of GDC (Fig. 39) and when GDC was present (Fig. 40), the results were similar to that described in the case of condensed tannin.

The effect of time on the stability of the insoluble complexes is shown in Fig. 41 and 42. The stability of insoluble condensed tannin-PRP complexes decreased by 22% in 29 mM phosphate pH 7.4, 11% in 29 mM phosphate pH 7.4 containing pancreatic enzymes, 25% in
pancreatic enzymes and GDC by increasing the incubation time from 2.5 hours to four days (Fig. 41). Under the same conditions the stability of insoluble tannic acid-PRP complexes decreased by 40% in 29 mM phosphate pH 7.4, 30% in 29 mM phosphate pH 7.4 containing pancreatic enzymes, 49% in 29 mM phosphate pH 7.4 containing GDC, and 26% in 29 mM phosphate pH 7.4 containing pancreatic enzymes and GDC (Fig. 42).
Fig. 33 Stability of condensed tannin-IB-8b complexes under conditions prevailing in the stomach and small intestine. The bar diagram shows % tannic acid remaining in the insoluble tannin-protein complexes after incubation. The condensed tannin-IB-8b complexes was formed at neutral pH as same as described in section II. 6.1. The pellet was seperated and incubated as in the following conditions:

IA. Incubation in 0.01 M HCl:0.09M NaCl, pH 2.0.
IB. Incubation in 0.01 M HCl:0.09M NaCl, pH 2.0 containing 1mg/ml pepsin.
IIA. Incubation in 29 mM phosphate, pH 7.4.
IIB. Incubation in 29 mM phosphate, pH 7.4 containing trypsin, chymotrypsin, elastase, carboxypeptidase A and B.
IIIA. Incubation under the same conditions as in IIA, but with addition of glycodeoxycholic acid.
IIB. Incubation under same conditions as in IIB but with addition of glycodeoxycholic acid.

The values are based on mean values of three individual assays and error bars are shown (otherwise the error bars are too small to illustrate).
Fig. 34 Stability of tannic acid-IB-8b complexes under conditions prevailing in the stomach and small intestine. The bar diagram shows % tannic acid remaining in the insoluble tannin-protein complexes after incubation. The tannic acid-IB-8b complexes was formed at neutral pH as same as described in section II. 6.1. The pellet was separated and incubated in the following conditions:

IA. Incubation in 0.01 M HCl/0.09M NaCl, pH 2.0.
IB. Incubation in 0.01 M HCl/0.09M NaCl, pH 2.0 containing 1mg/ml pepsin.
IIA. Incubation in 29 mM phosphate, pH 7.4.
IIB. Incubation in 29 mM phosphate, pH 7.4 containing trypsin, chymotrypsin, elastase, carboxypeptidase A and B.
IIIA. Incubation under the same conditions as in IIA, but with addition of glycodeloxcholic acid
IIIB. Incubation under same conditions as in IIB but with addition of glycodeloxcholic acid.

The values are based on mean values of three individual assays and error bars are shown (otherwise the error bars are too small to illustrate).
Fig. 35 The role of pepsin in precipitation of condensed tannin at pH 2.0

I: Incub. of IB-8b-tannin pellet at pH 2.0

II: Incub. of supernatant from I with pepsin

III: Sum of I + II

IV: Incub. of IB-8b-tannin pellet at pH 2.0 with pepsin

The columns are based on mean values of three individual assays and error bars are too small to illustrate.
Fig. 36 The role of pancreatic enzymes in precipitation of condensed tannin

I: Incub. of IB-8b-tannin pellet at pH 7.4

II: Incub. of supernatant from I with pancreatic enzymes

III: Sum of I + II

IV: Incub. of IB-8b-tannin pellet at pH 7.4 with pancreatic E.

The columns are based on mean values of three individual assays and error bars are too small to illustrate.
Fig. 37 The role of pancreas enzymes and GDC in precipitation of condensed tannin

I: Incub. of IB-8b-tannin pellet at pH 7.4 with GDC

II: Incub. of supernatant from I with pancreatic enzymes

III: Sum of I + II

IV: Incub. of IB-8b-tannin pellet at pH 7.4 with (GDC + pan. E.)

The columns are based on mean values of three individual assays and error bars are too small to illustrate.
Fig. 38 The role of pepsin in precipitation of Tannic Acid at pH 2.0

I: Incub. of IB-8b-tannin pellet at pH 2.0
II: Incub. of supernatant from I with pepsin
III: Sum of II + II
IV: Incub. of IB-8b-tannin pellet at pH 2.0 with pepsin

The columns are based on mean values of three individual assays and error bars are shown or are too small to illustrate.
The role of pancreatic enzymes in precipitation of tannic acid

I: Incub. of IB-8b-tannin pellet at pH 7.4

II: Incub. of supernatant from (1) with pancreatic enzymes

III: Sum of (1) + (2)

IV: Incub. of IB-8b-tannin pellet at pH 7.4 with pancreatic E.

The columns are based on mean values of three individual assays and error bars are shown or are too small to illustrate.
Fig. 40 The role of pancreatic enzymes and GDC in precipitation of tannic acid

I: Incub. of IB-8b-tannin pellet at pH 7.4 with GDC

II: Incub. of supernatant from I with pancreatic enzymes

III: Sum of I + II

IV: Incub. of IB-8b-tannin pellet at pH 7.4 with (GDC + panc. E.)

The columns are based on mean values of three individual assays and error bars are shown or are too small to illustrate.
Fig 41. Effect of time on the stability of condensed tannin-IB-8b complexes. Percent tannic acid found in pellet after incubation 150 min. (A) or 4 days (B) in solution I, (29 mM phosphate buffer pH 7.4); II, (29 mM phosphate, pH 7.4 containing pancreatic enzymes); III, (29 mM phosphate, pH 7.4 containing GDC); IV, (29 mM phosphate, pH 7.4 containing GDC and pancreatic enzymes. The columns are based on mean values of three individual assays and error bars are shown or are too small to illustrate.

- A: Incub. 150min.
- B: Incub. 4 days
Fig 42. Effect of time on the stability of tannic acid-IB-8b complexes. Percent tannic acid found in pellet after incubation 150 min. (A) or 4 days (B) in solution I, (29 mM phosphate buffer pH 7.4); II, (29mM phosphate, pH 7.4 containing pancreatic enzymes); III, (29mM phosphate, pH 7.4 containing GDC); IV, (29mM phosphate, pH 7.4 containing GDC and pancreatic enzymes. The columns are based on mean values of three individual assays and error bars are shown or are too small to illustrate.

- A: Incub. 150 min.
- B: Incub. 4 days
Since there are a large number of different human PRPs, it is not practical to test all of the isoforms for interaction with tannin. Because of this, representative members of the acidic, basic and glycosylated PRPs were investigated. They include PIF-s (an acidic PRP), GPRP (a glycosylated PRP), and IB-1, IB-4, IB-6, and IB-9 which are all basic PRPs. For a linear of the structure of the proteins used in this study see Fig 21. IB-1 was chosen because it contains the entire structure of IB-9 in addition to a 35 residue acidic extension at the N-terminal end with a sequence that differs from that of the typical repeat region. IB-6 was used because it is almost double the length (118 residues) of IB-9 (61 residues) and consists of repeat regions that are very similar to those found in IB-9. IB-4 consisting of 56 amino acids is about the same size as IB-9, but it has a somewhat different primary structure. The mol % of pro in the chosen BPRPs varies between 39% to 42%, except IB-1 which contains 33% pro. Thus evaluation of tannin binding to these proteins allows a comparison of tannin binding to acidic, basic, and glycosylated PRPs as well as determining the effect of variation in size and sequence of BPRPs on tannin binding.

Analysis of the purified PRPs gave results including MW in agreement with published data. The only exception was IB-1 which had a MW as determined by mass spectroscopy that was slightly different from a value calculated from amino acid sequence. All other analyses of purified IB-1 such as elution position on column chromatography, migration rate on SDS-PAGE and amino acid composition agreed with published data. The result of mass spectroscopy showing a mass difference of 33 from the mass of 9628 calculated from the amino acid sequence could be due to a mutation of leu to ala or lys to val.

Of the three types of PRPs (acidic, basic, and glycosylated), the BPRPs by far have the greatest capacity to precipitate tannin and they also exceed the tannin binding ability of gelatin,
among the BPRPs for tannin binding, Wilcoxon's rank test was applied (Snedecor and Cochran, 1967). The result of the rank test showed that IB-1 bound significantly less condensed tannin than IB-6, IB-4 and IB-9. IB-6 bound significantly less condensed tannin than either IB-4 or IB-9 and there was no difference in condensed tannin binding ability of IB-4 and IB-9. The similar result was seen for tannic acid binding, IB-1 bound significantly less tannic acid than IB-4 and IB-6. Moreover there was no difference in tannic acid binding of IB-4 and IB-6. However these differences are very small indicating that the size and sequence of secreted BPRPs have little or no effect on the amount of tannin precipitated. Thus phenotypic variations in BPRPs have little effect on a person's ability to precipitate tannin.

The mol percent of pro in IB-4, IB-6 and IB-9 varies from 39% to 42%. In contrast IB-1 contains 33 mol percent pro. NMR studies have shown that the pyrrolidine rings in PRPs interact with the aromatic rings in tannins (Charlton et al., 1996), suggesting that the amount of tannin bound to BPRPs may be related to the amount of pro. Moreover, IB-1 is the only BPRP assayed which has a short N-terminal acidic region in common with APRPs. As shown in this study the acidic region in APRPs impedes tannin binding and the same may be the case for IB-1.

In contrast to BPRPs, native APRPs and GPRPs had little ability to bind tannin. There was practically no binding of condensed tannin to APRP and GPRP and tannic acid bound only to these proteins when it was present in high concentrations. This implies that the functions of these proteins such as calcium binding, inhibition of hydroxyapatite crystal growth and lubrication are little affected by the presence of tannin.

PIF-s, a representative APRP, has an acidic pro-poor (1-30) N-terminal region followed by the pro-rich region (31-150) which is similar to the repeat region of BPRPs. The GPRPs and BPRPs have similar amino acid sequences but GPRPs contain 45% carbohydrate. It was therefore
proteins.

Studies on the ability of APRPs to bind calcium and inhibit hydroxyapatite crystal growth suggest that there is an interaction between the highly negatively charged N-terminal and the positive charged C-terminal region possibly due to electrostatic interaction (Bennick et al., 1981; Hay et al., 1979). Such an interaction could make the proline-rich region less accessible and lower the tannin binding ability.

To test this hypothesis, proteolytic cleavage of PIF-s by trypsin and dephosphorylation of PIF-s by alkaline phosphatase were performed. The proteolytic cleavage of PIF-s resulted in digestion of PIF-s giving rise to the N-terminal proline-poor Tx peptide and the proline-rich Ty and Tz peptides. Proteolytic cleavage of PIF-s caused a dramatic increase in tannin binding and on a weight basis the ability of the digest to bind tannin was similar to that of basic proline-rich proteins.

Tannin binding to the digest can be completely accounted for by its interaction with Ty and Tz. Thus the Tx region only prevent tannin binding when it is covalently bound in APRP. The absence of interaction of Tx with the tannin-Ty and Tannin-Tz complexes is also shown by the absence of Tx from the pellet and its recovery from the supernatant. This result supports previous studies indicating that there is an interaction of the proline-poor and proline-rich regions of APRPs which interferes with their biological activities.

The result of mass spectroscopy of dephosphorylated PIF-s indicate 66% of protein was dephosphorylated resulting in the removal of a total of 4 negative charges but this had no effect on binding of condensed tannin. It implies that if there is a charge interaction it does not involve phosphoserine.
composition of GPRP was first determined. The result of sugar analysis showed that mannose was present in GPRP; thus it is expected to be an N-glycan. Digestion of GPRP by N-glycosidase F resulted in complete removal of carbohydrate and confirmed that it is strictly an N-glycan.

Although a minor component was present as shown by SDS-PAGE of de-GPRP, western blotting indicated that it belongs to the BPRP family so the results are not affected by the presence of other non-PRP components. The result of mass spectroscopy of de-GPRP shows two components. So the minor component shown by SDS-PAGE of de-GPRP is probably an isoform of de-GPRP. This therefore indicates that my GPRP consists of a mixture of two glycosylated PRPs with a slight difference in size.

The de-GPRP has a much higher tannin-binding ability than GPRP and it is comparable to BPRPs. This indicates that the carbohydrate side chains of GPRP inhibit the interaction of GPRP and tannin.

Once the PRPs have been secreted in the mouth, they may undergo degradation by bacterial enzymes and this could affect their biological activities. Indeed such degradation of APRPs has been demonstrated (Bennick et al., 1982) and it results in the formation of N-terminal proline-poor peptides with an increased ability to inhibit hydroxyapatite crystal growth. It is likely that the proline-rich peptides which also would be formed as a result of this degradation would have enhanced ability to bind tannin. Thus proteolytic cleavage of APRPs after secretion enhances their biological activities. Similarly glycosidases in the mouth may remove carbohydrate sidechains of GPRPs and enhance their tannin binding ability.

In contrast to my result, Asquith et al. (1987) using a competitive binding assay reported that a mouse salivary proline-rich glycoprotein (GP 66sm) had 8-fold greater affinity for the condensed tannin than did the deglycosylated protein, which had been deglycosylated with
between the intact protein and deglycosylated protein. No detectable affinity of the oligosaccharides isolated from the glycoprotein for tannin was observed. It was also shown that the glycoprotein-tannin complexes were more soluble than tannin complexed to deglycosylated protein.

In my assay system, only insoluble complexes were detected, and it is not clear if there are soluble complexes of tannin and GPRP. In contrast, the competitive binding assay which was used by Asquith et al. (1987) cannot distinguish between soluble and insoluble complexes. The deglycosylation in my study was performed by N-glycosidase F which would leave the polypeptide backbone of GPRP intact, while deglycosylation of GP 66sm by TFMA (trifluoromethanesulfonic acid) may cause damage to the polypeptide backbone of GP 66sm by cleavage of peptide bonds to generate peptides and/or the structure of polypeptide may be denatured by concentrated TFMA at 25 °C. It was reported that 76% of protein was recovered from the deglycosylation of glycoprotein by TFMA (Edge et al., 1981). Thus deglycosylation of GPRP by TFMA may decrease the ability of deglycosylated protein to bind tannin.

Carbohydrate can interfere in the interaction of tannin and protein, and it is believed to act as a barrier against tannin binding to the polypeptide backbone (Mehansho et al., 1987). This was also reported by other investigators. The catalytic activities of two glycoprotein enzymes, yeast invertase and Aspergillus flavus tannase, are quite resistant to inhibition by condensed and hydrolyzable tannins, as compared to the situation with non glycosylated enzymes (Strumeyer and Malin, 1970). Unfortunately, they did not test the effect of deglycosylation of these enzyme on the inhibition of tannin. The failure of condensed tannin to precipitate bovine submaxillary mucin has been ascribed to the carbohydrate moiety (Jones and Mangan, 1977).
tannin-binding proteins in human parotid and submandibular/sublingual saliva. Even at very low concentration of tannins, where there is little tannin binding to PRPs, extensive binding to HRP is seen. Unstimulated saliva is mainly constituted of submandibular/sublingual saliva while stimulated saliva consists mainly of parotid saliva. BPRPs are only present in parotid saliva (Bennick, 1982), so HRP may be particularly important in unstimulated saliva. Stimulation of salivary flow, such as food intake, would lead to secretion of a large amount of BPRPs. Thus the tannin binding of PRPs and HRP may complement each other in the mouth.

During fasting, there are only HRP present in the unstimulated saliva. Although their concentration is low, they would be effective precipitators of tannin due to their high capacity to bind tannin. During eating, there is a large amount of BPRPs in the stimulated saliva and this could insure precipitation of all the food tannin. Thus the HRP present in the submandibular/sublingual saliva and BPRPs present in the parotid saliva constitute an effective defense system against tannin over a wide range of tannin concentration.

Furthermore, there is evidence indicating that the presence of HRP in certain species of monkey is related to a fruit-based diet (Azen et al., 1978) and implies that HRP play the role in defense against tannin.

No estimate was made of the strength of tannin-protein interaction, but it is interesting to note that Charlton et al. (1996) found that the dissociation constant for IB-5 with tannin was smaller than that of a synthetic peptide. Thus it is possible that for naturally occurring BPRPs the affinity for tannin may increase with increased size of the BPRPs.

To evaluate if salivary PRPs can act as a defense against tannin in vivo, the stability of the protein-tannin complexes during passage in the alimentary canal must be evaluated. Many factors may affect the stability of the insoluble complexes, such as the low pH and presence of pepsin in
evaluate these factors the stability of tannin-protein complexes were tested *in vitro* under conditions similar to those that exist *in vivo*.

The complexes of condensed tannin-IB-8b were very stable when incubated at pH 2.0 without pepsin. Only 7% of the complexes dissociated. If pepsin was also present in the solution with pH 2.0, an additional 3% tannin was precipitated. This indicates that a small amount of pepsin was also precipitated.

It was similarly found that under conditions simulating those of the intestine, 82% condensed tannin remained bound to IB-8b in a solution with pH 7.4 that did not contain glycodeoxycholic acid (GDC) and enzymes. Eleven percent more condensed tannin remained in the pellet if the incubation was done in the presence of enzymes and this could be ascribed to the formation of complexes of condensed tannin and pancreatic enzymes. When GDC was present in the incubation solution at pH 7.4, or in the solution at pH 7.4 containing pancreatic enzymes, the stability of complexes decreased about 20%. This can ascribe to the detergent properties of GDC (Hagerman and Klucher, 1986). The apolar part of GDC might interact with the insoluble complexes causing increased dissociation of the insoluble complexes and may bind the tannin to form soluble complexes.

The stability of tannic acid-IB-8b complexes under conditions prevailing in the alimentary canal showed a trend similar to that of condensed tannin-IB-8b complexes. The tannic acid-IB-8b complexes partly dissociated under these conditions, GDC decreased the stability of the insoluble tannin-IB-8b complexes and there are more insoluble complexes in the presence of enzymes. The stability of tannic acid-IB-8b complexes is lower than that of condensed tannin complexes but there may be soluble tannic acid-GDC complexes present in the solution, which may prevent adsorption of tannin.
Subramanian et al., 1983) the higher solubility of the tannic acid-PRP complexes may not have serious physiological implications.

These studies have shown that a large part of the tannin-protein complexes remain insoluble under conditions similar to those that exist in the digestive tract. Moreover it is possible that tannin also is present as soluble complexes with PRPs. Such complexes may still provide protection as shown by the ability of PRPs to prevent inactivation of amylase by tannin (Yan and Bennick, 1995).

In conclusion, this work clearly supports the hypothesis that human salivary PRPs can bind tannins to make harmless protein-tannin complexes that could be excreted.
1. **Summary**

1.1 BPRPs are the PRPs which are able to precipitate most tannin at neutral pH. They have higher capacity for tannin-binding than gelatin which is considered to have high tannin binding ability.

1.2 The size and sequence of BPRPs have little or no effect on tannin binding.

1.3 Proteolytic cleavage of APRPs enhances their ability to bind tannin via the pro-rich region.

1.4 Carbohydrate interferes with the interaction of tannin and PRPs. Since there is little tannin binding to GPRPs, in the presence of tannin these proteins will largely retain their other functions which depend on the carbohydrate sidechains such as lubrication.

1.5 PRPs can be expected to still be effective tannin binders after degradation and deglycosylation in the mouth. Indeed the activity of PRPs is increased.

1.6 The tannin-PRP complexes are very stable under conditions prevailing in the stomach.

1.7 More than half of the condensed tannin-PRP complexes are stable under conditions prevailing in small intestine.
Food digestion and absorption occur in the stomach and small intestine whereas water, mineral matter *etc.* are absorbed in the colon. The stability of the insoluble PRP-tannin complexes may be affected by the enzymes produced by bacteria in the colon, so it is also important to evaluate the stability of insoluble complexes under the condition prevailing in this segment of the digestive system.

In a preliminary experiment (Yan and Bennick, 1995), it was found that HRP and PRP can prevent inhibition of amylase by tannin but the BPRPs have not been tested for this role. Using the method described by Yan and Bennick (1995), it would be useful to find the protective role of BPRPs to prevent inhibition of pepsin, amylase, trypsin *etc.* from tannin under the conditions prevailing in the alimentary canal.

These experiments will help to clarify the manner in which PRPs play a protective role against tannin in human beings.


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