1. INTRODUCTION

1.1 Statement of the problem

Sorghum (*Sorghum bicolor* (L.) Moench) is an indigenous cereal crop to Africa. It is the fifth most important cereal after wheat, rice, maize and barley (reviewed by Taylor and Belton, 2002). Sorghum has played an important role in the development of and food security in Southern Africa. Traditionally, sorghum is prepared and consumed mainly as a porridge, a staple diet of the African people. It is also malted and mashed with porridge to produce sorghum beer by fermentation. At the commercial level, in South Africa, sorghum is sold as decorticated and milled flour, malted sorghum flour and sorghum beer. The commercial processing of sorghum grain has generated large quantities of by-products such as sorghum bran from milling and spent grain from beer production. Sorghum bran (Jones and Beckwith, 1970) and spent grain (Van Heerden, 1987) as by-products are rich in protein and are generally sold at a cheap price for animal feed. As an alternative, the protein could be extracted and could be used for its functional properties such as the use as a biopolymer for food packaging (Cuq et al., 1998). This alternative use of the local by-products would add value to the sorghum processing industry.

Proteins are biopolymers made up of amino acid subunits. They can be made into edible and biodegradable films and coatings. Progress towards a sustainable society in the 21st century and the exploration of new packaging materials for the food industry have supported research and development in edible and biodegradable coatings and films (reviewed by Debeaufort et al., 1998). Environmental issues are becoming increasingly important because consumers want to live in a pollution-free environment. Current packaging systems consist mainly of synthetic plastics. The disposal of these plastics is a huge environmental problem because of their low biodegradability. Plastic packaging is generally disposed in landfills and by incineration. Both methods cause environmental problems as landfills cause land pollution and incineration causes high emission of green house gases like carbon dioxide and also toxic gases (Anonymous, 2002a). Thus, edible and biodegradable coatings and films

can be an environment-friendly alternative to synthetic plastic packaging systems.

Edible and biodegradable films and coatings as packaging systems can act as selective barriers against gases, water vapour and other solutes (Kester and Fennema, 1986). They can also be carriers for food additives such as antimicrobials to extend the shelf life of food products (Baldwin et al., 1995). The use of edible coatings and films can create modified atmosphere to extend the shelf life of fruits and vegetables (Baldwin et al., 1995). Thus, edible coatings and films could enhance the quality of Southern African agricultural produce for the local and export markets (Enviropak, 2002).

Edible and biodegradable films and coatings can be prepared from three natural sources, namely, polysaccharides, lipids and proteins, either singly or in combination. Unlike the polysaccharide-based films that consist of a repeated monomer, proteins are heteropolymers which give better film making properties with good functional properties (Cuq et al., 1998). Kafirin, the prolamin protein of sorghum, has shown potential to be produced into films by the casting method (Buffo et al., 1997).

The commercial use of edible and biodegradable films/coatings has been hampered due to their properties being inferior to that of synthetic plastic packaging (Petersen et al., 1999). Synthetic plastics are considered to have better mechanical and barrier properties. Modification can alter the properties of protein-based films. Chemical modification with aldehydes has shown promising results to enhance zein (maize protein) (Parris et al, 1998), gluten (wheat protein) (Lens et al., 1999) and sunflower protein films (Orliac et al., 2002). However, some cross-linking agents such as glutaraldehyde are not food compatible. Orliac *et al.* (2002) also found that food compatible phenolic compounds such as gallic acid, and chestnut and tara tannins, can be an alternative to chemically modify sunflower protein films. Thus, phenolic compounds may be potentially used as chemical agents to modify kafirin films.

1.2 Literature review

The following chapter reviews current literature on sorghum kafirin protein; protein-based films; the limitations and modification of protein-based films; phenolic compounds; and the interaction of proteins with phenolic compounds. The literature review will show that there is a gap in our scientific knowledge of prolamin-phenolic compound interactions and about the possible effects of phenolic compounds on prolamin films.

1.2.1 Sorghum kafirin

The protein content of sorghum grain ranges from about 7.3 to 15.6% (Hulse et al., 1980) depending on the genotype and the abiotic conditions during grain development (Serna-Saldivar and Rooney, 1995). Sorghum proteins, like those of other cereals, can be fractionated based on their extractability according to the Osborne method into albumins (water soluble), globulins (saline soluble), glutenins (base and acid soluble) and prolamin (aqueous alcohol soluble). The prolamin of sorghum has been named kafirin when it was first isolated from the sorghum variety Dwarf kafir by Johns and Brewster (1916). Kafirin is the major protein in sorghum grain, and its proportion depends on the cultivar and the amount isolated depends on its efficiency of extraction (Taylor et al., 1984a).

1.2.1.1 Chemistry of kafirin

The synthesis of prolamin in sorghum is believed to be the same as the synthesis of maize prolamin, zein, as they are extensively homologous (DeRose et al., 1989). Prolamins are synthesized on membrane-bound polyribosomes of the rough endoplasmic reticulum (Miflin et al., 1981) and can self associate to form deposits known as protein bodies (Larkins et al., 1984). The protein bodies are mostly located in the outer vitreous endosperm, rather than in the central opaque endosperm of the sorghum grain (Watterson et al., 1993). Transmission electron microscopy of the sorghum protein bodies showed that they are circular in section, varying between 0.4-2.0 μm in diameter and are mainly embedded in a glutelin matrix (Taylor et al., 1984b).

Kafirin can be extracted using various aqueous alcoholic solvent systems. Johns and Brewster (1916) used 64% (w/w) ethanol at 80 °C to extract kafirin. Since then several other alcoholic solvents such as 60% (w/w)tertiary butanol and 70% (w/w) isopropanol are often used with a reducing agent and the most effective solvent is 60% (w/w) tertiary butanol plus a reducing agent (Taylor *et al.*, 1984a). Reducing agents such as mercaptoethanol and dithiothreitol are added to break disulphide bonds to solubilise the cross-linked kafirins (El Nour et al., 1998). Taylor *et al.* (1984a) found that at room temperature the use of 60% tertiary butanol with 0.05% dithiothreitol as reducing agent to extract kafirin gave twice the yield than without a reducing agent.

Kafirins are classified into α, β, and γ, which are analogous to α, β and γ-zein, respectively (Shull et al., 1991). The amino acid composition of total kafirin as well as its polypeptides is shown in Table 1.1. Kafirin proteins are rich in glutamine and low in lysine. The high content of hydrophobic amino acids suggests why kafirin is considered to be hydrophobic (Belton et al., 1997). Comparing the classes of kafirin, it is notable that β and γ-kafirin have a high content of cysteine and γ-kafirin has the highest amount of proline.

Table 1.1 Partial amino acid composition of total, α-, β-, and γ- kafirin.

Amino acid	Total kafirin (g 100 g^{-1})[a]	α-kafirin (mole%)[b]	β-kafirin (mole%)[b]	γ-kafirin (mole%)[c]
Glutamine	22.0	21.8	17.7	13.8
Proline	9.8	8.9	9.7	22.6
Alanine	10.6	15.2	13.4	6.30
Leucine	14.7	15.1	12.0	9.0
Cysteine	0.5	1.1	4.9	6.9
Methionine	2.4	0.6	5.7	1.0
Lysine	0.2	0.4	0.5	0.4

[a] from endosperm, adapted from Taylor and Schüssler (1986)

[b] adapted from Shull et al. (1992)

[c] adapted from Watterson et al. (1990)

When kafirins are separated according to their molecular size using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, α- kafirin gives 2 bands, α_1 of M_r 24-28 k and α_2 of M_r 22 k; β-kafirin is M_r 19 k; and γ-kafirin has M_r 27 k (Shull et al., 1991). SDS-PAGE, under non-reducing conditions, shows kafirin to exist as dimers, trimers and polymers because of the extensive cross-linking behaviour of the β- and γ-kafirin (El Nour *et al.*, 1998). This may suggest the potential of kafirin as biopolymers for film making.

There are no X-ray structures of kafirin. However, the amino acid compositions of kafirin and zein are very similar (Coleman et al., 1990) and detailed sequence analysis has shown extensive homology between them (DeRose et al., 1989). Thus, it is likely that the structure of kafirin is similar to zein. A structural model of zein (α-zein) has been produced using circular dichroism and a structure prediction approach (Argos et al., 1982). The model is shown schematically in Figure 1.1.

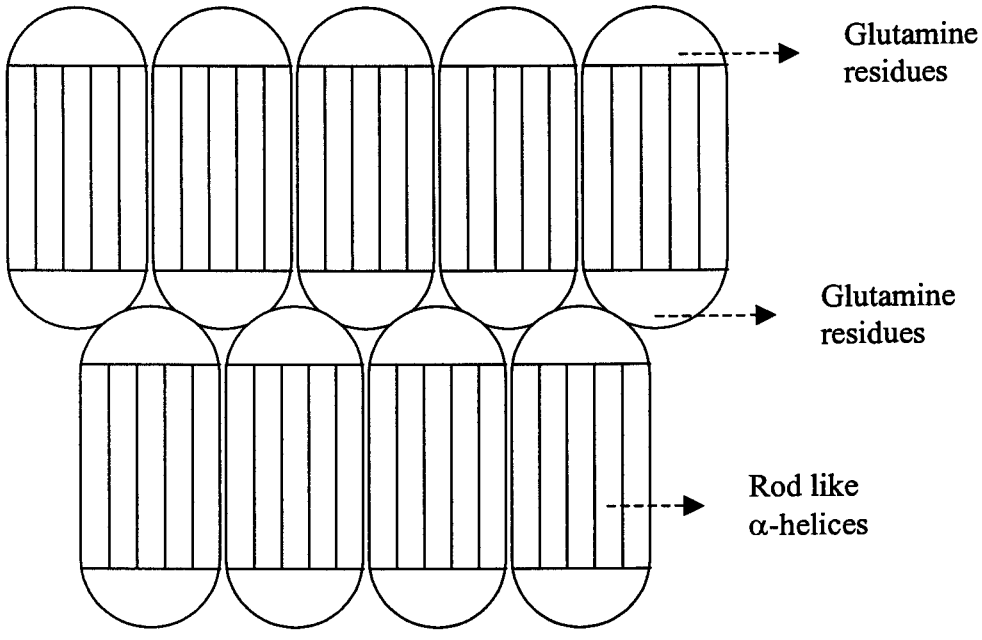


Figure 1.1 A structural model of zein (redrawn from Argos et al., 1982).

One zein protein molecule is thought to consist of nine topologically antiparallel and adjacent rod like α-helices in a cylindrical form. The top and bottom part of the cylinder consist mostly of glutamine residues that allow hydrogen bond

interaction between molecules in neighbouring planes. Polar and hydrophobic amino acid residues distributed along the α-helical rod surfaces allow intra and inter molecular hydrogen bonding and Van der Waals forces between neighbouring helices. As a result, zein molecules can aggregate in molecular planes which then stack through inter glutamine-glutamine interactions at the cylindrical caps. During wet heat processing, it appears that the α-helical structures can unravel somewhat to form intermolecular β-sheet structures (Duodu et al., 2001).

1.2.2 Protein-based films

Protein-based films are desirable for packaging and coating applications as they are renewable, biodegradable and can be edible. Protein-based films can be defined as thin sheets that are formed around or within a product to offer a selective barrier against transmission of water vapour, gases and solutes together with mechanical properties (Gennadios and Weller, 1990). Edible films are also referred as edible coatings when the film casting solution is applied directly onto the product. The possible functional properties of edible films according to Kester and Fennema (1986) are to:

Retard moisture, oil and fat migration
Retard gas and solute transport
Impart added structural integrity to food
Retain volatile flavour compounds
Carrier of food additives

Edible films can be polysaccharide, lipid, protein based or composite, with added plasticizers such as polyhydric alcohols to improve the extensibility and practicability of biopolymeric substances (reviewed by Gennadios and Weller, 1990). Polysaccharide-based films can be produced from materials such as alginates, chitosan, starch, carrageenan and cellulose. They are good gas barriers, but are poor moisture barriers as they are hydrophilic in nature (Kester and Fennema, 1986). Lipid-based films can be made from substances such as beeswax, vegetable oil, mineral oil, acetylated monoglycerides, stearic acid, lauric acid and sucrose esters of fatty acids. Lipid-based films are good

moisture barriers, but poor gas barriers (Hagenmaier and Shaw, 1992). Protein-based films can be produced for example from maize zein, wheat gluten, soy protein, egg albumen, collagen and sunflower protein. They are generally good film formers (Baldwin et al., 1995), adhere well to hydrophilic surfaces and can have good barrier properties, depending on the type of protein (Gennadios and Weller, 1990).

Although protein-based films have advantages, the use of protein of animal origin to make edible films and coatings may raise concerns among certain religious groups and vegetarian consumers. Moreover, some proteins such as wheat gluten are known to be allergenic to some people. Zein, the maize prolamin has shown potential as edible films (reviewed by Lawton, 2002) and is not known to be allergenic (Skerritt, 1988). Kafirin can also be made into films (Buffo et al., 1997). Compared to zein, kafirin is probably more hydrophobic (Belton et al., 1997), more cross-linked (El Nour et al., 1998) and less digestible when wet cooked (Duodu et al., 2002). This may suggest that kafirin has a better potential for film making than zein.

Protein-based films have been applied to many food products at the experimental scale. They have shown potential to enhance the shelf life of fresh and minimally processed fruits and vegetables (Baldwin and Baker, 2002), as carriers of antimicrobials agents (Baldwin et al., 1995) and to decrease oil absorption for deep fat fried food (Rayner et al., 2000). A few products for making protein-based films are commercially available. Those from zein are marketed under the names zcoat® or cozeen® and can be used for coating of food products such as nuts (Druchta and Johnston, 2002).

1.2.2.1 Formation of protein-based films

Proteins can be made into films generally by two processes, wet or dry, as illustrated in Figure 1.2. The wet process is also known as the casting method. It involves the dissolution of the protein material with other additives, pouring the solution on a flat surface and evaporation of the solvent to produce a film. The dry process utilises the thermoplastic properties of protein. The protein is

generally melted by high temperature with shear force and additives at a low moisture content. The melted protein material is then shaped into a film.

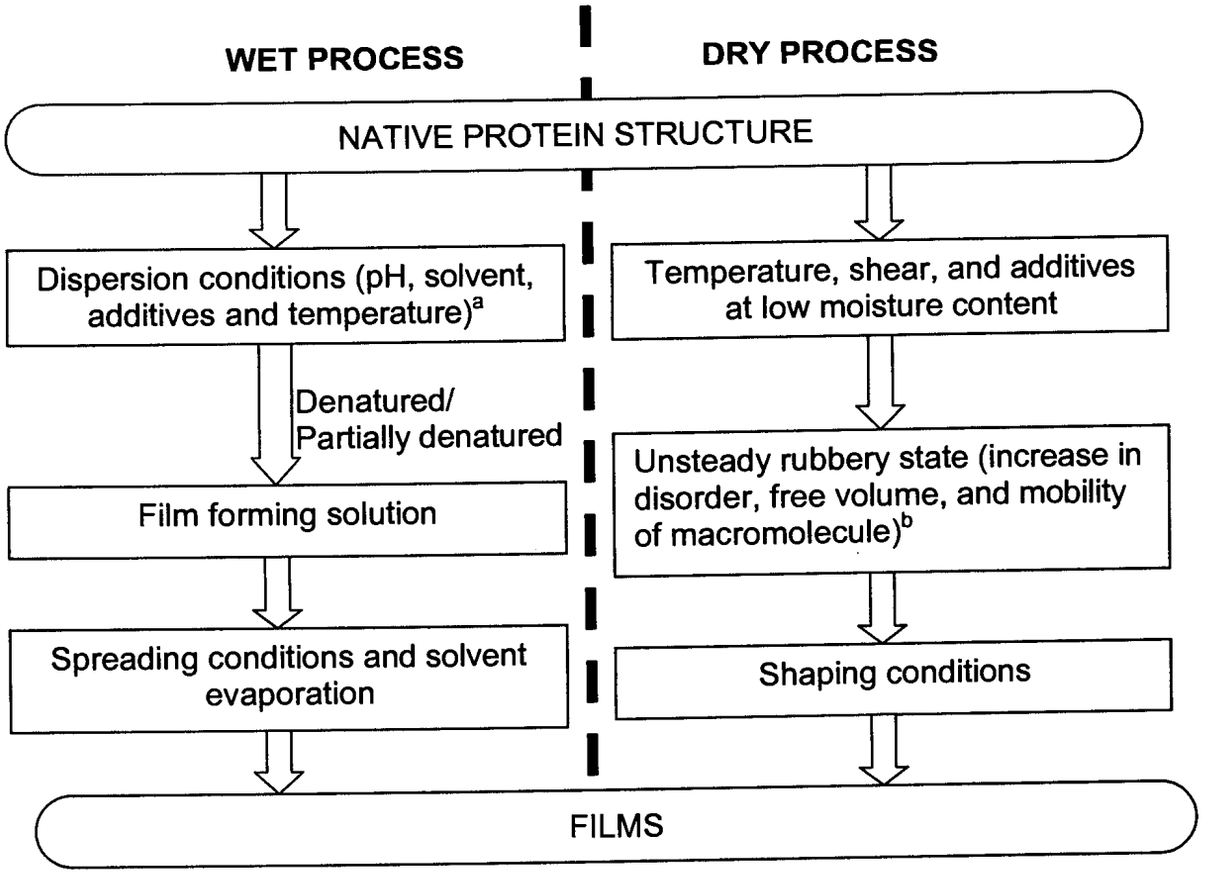


Figure 1.2 Schematic representation of film formation from protein by the wet and dry process (modified; [a]Gontard et al., 1992, and Donhowe and Fennema, 1993; [b]Cherian and Chinachoti, 1996, Cuq et al., 1998)

The mechanisms of formation of protein-based films can be explained as follows (Cuq et al., 1998; De Graaf , 2000):

Rupture of low energy intermolecular bonds that stabilize the biopolymers in the native state through a certain degree of protein denaturation

Additional bond formation (example covalent disulphide bonds between cysteine molecules) and new orientation of the protein molecules

Formation of a 3-dimensional network stabilised by new interactions and bonds (example ionic, hydrogen and hydrophobic) after solvents or agents that disrupted intermolecular forces are removed.

1.2.2.2 Mechanical and barrier properties of protein-based films

Among the functional properties of protein-based films, mechanical and barrier properties are the most important. The mechanical properties give an indication of the integrity and strength of the films in terms of tensile strength and elongation. The tensile properties of protein-based films are expressed as stress, strain and Young's modulus. According to the American Society for Testing and Materials (1988), stress at maximum tensile force (σ_y) expresses the maximum force (the internal resistance to an external load) developed in a film during tensile test. It is expressed as the force per unit area. Strain at break (ε_b) is a representation of the film's ability to stretch and is expressed as the percentage extended compared to the original length. Young's modulus (*E*) is the ratio of stress to strain in the linear range of the stress-strain graph. It is expressed as force per unit area and it measures the stiffness of the film.

The tensile strength (stress) and the elongation (strain) of some protein-based films are compared to synthetic films in Table 1.2. Different protein-based films have different mechanical properties. It can be seen that protein-based films have lower tensile strength and percentage elongation when compared to synthetic films. Thus, this may suggest a limitation to the use of protein-based films in heavy duty applications, for example plastic shopping bags.

Water vapour and oxygen barrier properties can be considered as two important factors for food quality. Water present in food can affect the a_w (water activity), which in turn can affect the microbial stability of the product (Kester and Fennema, 1986). The presence of oxygen can enhance oxidative processes such as rancidity in high fat foods (Coultate, 1996) and lack of oxygen can cause anaerobic respiration in fruits and vegetables (Baldwin and Baker, 2002). It can be seen that protein-based films have higher water vapour permeability when compared to synthetic films (Table 1.3). Protein-based films have low oxygen permeability (OP), but it may not be low enough to act as an oxygen barrier for products such as a coating for nuts to prevent oxidative rancidity (reviewed by Trezza and Krochta, 2002).

Table 1.2 Mechanical properties of protein-based and synthetic films

Films	Plasticizer	Tensile stress (MPa)	Strain (%)
Protein based films			
Zein[a]	None	10.9	3
Zein[a]	30% glycerol: PEG* (1:3)	5.1	117
Zein[b]	46% glycerol:PEG*(1.1:1	2.6	85
Kafirin[b]	46% glycerol:PEG*(1.1:1)	2.1	106
Deamidated gluten[c]	30% glycerol	4.5	175
Wheat Glutenin[d]	25%glycerol	2.1	384
Whey[e]	40% glycerol	4.0	80
Soy protein[f]	60% glycerol	3.3	67
Synthetic packaging films			
Low density polyethylene[g]		8.6-17	500
High density polyethylene[g]		17-35	300
Oriented polypropylene[g]		165-170	50-75
Ethylene-vinyl alcohol[g]		14	650-800

*PEG is polyethylene glycol; [a]Parris and Coffin (1997);[b]Buffo et al. (1997); [c]Larre et al. (2000a); [d]Micard et al. (2000); [e]Fang et al. (2002); [f]Brandenburg et al. (1993);[g]Biddle (1986)

The mechanical and barrier properties of polymeric materials are affected by the glass transition temperature (T_g). Gas and water vapour permeability (WVP) tend to be low below T_g and high above T_g (Cherian et al., 1995). Below the T_g, polymers exist mostly in a 'glassy' state which limits chain mobility. Thus polymers exhibit low barrier properties and a high tensile stress. However, at temperatures above T_g, polymers exist in a 'rubbery' state where polypeptide chain mobility is increased and this can lead to an increase in gas and WVP, and low tensile stress.

The inferior mechanical and barrier properties of protein-based films indicate that modification is necessary to improve their properties so that they can compete with synthetic films. From Tables 1.2 and 1.3, it also seems that the physical properties of protein-based films from the literature are dependent on types and amount of plasticizers present (discussed in section 1.2.2.3.1).

Table 1.3 Water vapour permeability (WVP) and oxygen permeability (OP) of protein-based films and synthetic films

Films	Plasticizer	Test conditions[#]	WVP (g mm m^{-2} d^{-1} kPa^{-1})
Protein-based films			
Zein[a]	No plasticizer	100/0% RH @ 25 °C	15
Zein[b]	46% glycerol:PEG*(1:1)	100/50% RH @ 25 °C	5.7
Kafirin[b]	46% glycerol:PEG*(1:1)	100/50% RH @ 25 °C	5.5
Gluten[c]	25% glycerol	85/0% RH @ 26 °C	53
β-casein[d]	50% glycerol	53/76% @ 22.5 °C	45.2
Soy[e]	33% glycerol	100/50% @ 30 °C	11.3
Synthetic packaging films			
Low density polyethylene[f]		90/0% RH @ 38 °C	0.08
High density polyethylene[f]		90/0% RH @ 38 °C	0.02
Ethylene-vinyl alcohol[g]		90/0% RH @ 38 °C	0.25
Protein based films			OP (cm^3 μm m^{-2} d^{-1} kPa^{-1})
Peanut[h]	30% glycerol	0% RH @ 30 °C	16.8
Soy[e]	60% glycerol	0% RH @ 25 °C	3.75
Maize zein[d]	20% glycerol	0% RH @ 30 °C	13
Wheat gluten[i]	30% glycerol	0% RH @ 25 °C	6
Collagen[j]	None	63% RH @ 20 °C	23
Whey protein[k]	50% sorbitol	50% RH @ 23 °C	8
Synthetic packaging films			
Low density polyethylene[l]		50% RH @ 23 °C	1870
High density polyethylene[l]		50% RH @ 23 °C	427
Ethylene-vinyl alcohol[l]		0% RH @ 23 °C	0.1

*PEG is polyethylene glycol; [#]100/0% means RH gradient from inside to outside of the cup for the WVP; [a]Parris and Coffin (1997); [b]Buffo et al. (1997); [c]Park and Chinnan (1990), [d]Mauer et al. (2000), [e]Brandenburg et al.(1993), [f]Smith (1986); [g]Foster (1986); [h]Jangchud and Chinnan (1999); [l]Gennedios et al. (1993); [j]Lieberman and Guilbert (1973); [k]McHugh and Krochta (1994), [l]Salame (1986)

1.2.2.3 Modification of protein-based films

Protein-based films can be modified to change their functional properties. Modifications can be by plasticization; compositing with other types of biopolymers; physical treatments including heat, γ-irradiation and ultra violet; enzymatic; and chemical.

1.2.2.3.1 Plasticization

A plasticizer is a low molecular weight compound added to polymeric material during production to make the film more plastic. Plasticizers improve the extensibility of films. Plasticization is believed to interrupt polymer chain to chain interaction (Sears and Darby, 1982) and thus can lower T_g (Arvanitoyannis et al., 1998). Plasticizers added to polymeric materials include glycerol, sorbitol, propylene glycol, polyethylene glycol, fatty acids and monoglycerides (Krochta, 1986).

Hydrophilic and hydrophobic plasticizers at different concentration affect the functional properties of protein-based films differently. Hydrophilic plasticizers such as sorbitol, when added to gelatin films at increasing concentration decreased the puncture strength and increased the puncture deformation (Sobral et al., 2001). Similarly, the tensile strength, the stiffness, T_g and WVP of whey protein isolate films were decreased and the% strain increased when glycerol and sorbitol was increased from 60 to 80% by weight of protein (Shaw et al., 2002). Hydrophilic plasticizers generally increase the WVP of protein-based films because of their ability to absorb water from an environment of high relative humidity (high partial pressure of water) and thereafter lose it to an atmosphere of low relative humidity (low partial pressure of water) (Gontard et al., 1993).

Water can also act as a hydrophilic plasticizer (Madeka and Kokini, 1996). The presence of water in biopolymeric films, such as cassava starch can reduce the T_g, tensile strength and increase WVP (Chang et al., 2000). Similarly, water can act as a plasticizer for gluten protein to decrease the T_g (Slade et al., 1989).

When the surrounding relative humidity (RH) increased from 40 to 60% the oxygen permeability and WVP increased exponentially with coatings of fruit puree (McHugh et al., 1996), probably because of plasticization by water. When water is absorbed by edible films due to increased RH, it can decrease the storage modulus drastically, indicating a drop in the T_g (Stading et al., 2001). The decrease in T_g and increase in oxygen and water barrier properties due to increased RH support the concept of the plasticization effects of water.

Hydrophobic plasticizers, such as fatty acids, have also been used in protein-based films. A series of saturated fatty acids with an even number of carbons from 6-18 have been investigated for gluten film plasticization and compared with glycerol plasticization (Pommet et al., 2003). The substitution of glycerol by fatty acids greatly reduced the WVP of the films, and the decrease was greater with increase in the length of carbon chain of the fatty acids.

1.2.3.3.2 Compositing films

Composite protein-based films involve the combination of the protein polymer with compatible polysaccharides, lipids or other proteins. Composite films can be prepared in laminated or emulsion form. Zein films have been coated with sorghum wax/medium chain triglycerides and carnauba wax/medium chain triglycerides at different ratios (Weller et al., 1998). The addition of the wax layer significantly decreased the WVP, without greatly affecting the tensile properties and slightly increased the% strain. The decrease in WVP was attributed to a more homogeneous coating and a more hydrophobic film. Composite films made from an emulsion of casein and wax have also resulted in a decrease in WVP when compared to casein films (Chick and Hernandez, 2002).

Lamination of hydrophilic protein biopolymers like whey and casein (Cho et al., 2002) and soy protein isolate (Pol et al., 2002) with zein results in a significant decrease in WVP because of the increase in hydrophobicity due to zein.

Xylan, a polysaccharide, can be composited with gluten during film production (Kayserilioglu et al., 2003). Xylan can have positive effects, decreasing water

solubility and WVP probably because of the hydrophobic and covalent bonding that may happen during film processing.

1.2.2.3.3 Enzymatic modification

The enzyme transglutaminase can catalyze the self-polymerization of proteins through the creation of ε-(γ-glutamyl) lysine bonds, and also introduce cross-links between added primary amine and glutamine residues of protein (Larre et al., 2000a). This enzymatic modification when applied to gluten films resulted in an increase in tensile strength and elongation at break (Larre et al., 2000a). Transglutaminase modification caused the formation of high molecular weight insoluble polymers as shown by electrophoretic bands that did not enter the separating gel in an SDS-PAGE gel under non reducing conditions (Larre et al., 2000b).

Casein films have also been modified by transglutaminase (Faergemand et al., 1999). This modification increased the apparent surface viscosity as a result of formation of cross-links. The action of transglutaminase also gave an increase in surface pressure relaxation, indicating that the modified films were stiffer in character because of the occurrence of more cross-linking covalent bonds.

1.2.2.3.4 Physical treatments

The physical treatment of heat can modify the properties of protein-based films. When gluten films were cured at different temperatures, the functional properties changed depending on the temperature (Cuq et al., 2000; Micard et al., 2000). When curing temperature increased, the tensile strength increased, but the% strain decreased. Heat curing does not affect the WVP (Ali et al., 1997; Micard et al., 2000). Heat treatments can also result in a decrease in protein solubility of the films in different solvents. The decrease in protein solubility has been attributed to network formation by protein–protein aggregation or to cross-linking between the protein polypeptides (Jeanjean et al., 1980). This aggregation is believed to occur by disulphide bond formation and hydrophobic interaction. These are probably facilitated by thermally induced

unfolding of the protein structure resulting in a partially denatured state (Cuq et al., 2000).

Heat has also been found to modify other protein-based films, for example whey and peanut. When peanut protein films were dried at different temperatures, ranging from 70 to 90 °C, both the tensile strength and the% strain increased and there was a decrease in OP (Jangchud and Chinnan, 1999). Similarly, an increase in tensile strength has been found when whey protein was heated before casting (Vachon et al., 2000). An increase in high molecular weight proteins occurred with heating. These workers attributed high molecular weight protein formation to cross-linking of protein molecules, probably by inter molecular disulphide bond formation.

Other physical ways to modify protein-based films include ultra violet (UV) and γ-irradiation. When gluten films were UV and γ-irradiated, there was an increase in tensile strength and Young's modulus, but a decrease in% strain (Micard et al., 2000). However, there was no significant change in WVP, and only a slight decrease in film solubility in water. Similar effects on mechanical properties have been reported when other protein-based films like zein, egg albumen and casein were UV irradiated (Rhim et al., 1999). Irradiation is believed to cause the formation of some high molecular weight polymers through cross-linking between the polypeptide chains. Vachon et al. (2000) found an increase of more than 60 fold in M_r as determined by size exclusion chromatography, when calcium caseinate was γ-irradiated. They suggested that bityrosine cross-linking between two protein chains was the cause during irradiation.

Another useful effect of γ-irradiation is the decrease in biodegradability of protein-based films. When casein films were treated with γ-irradiation from 4 to 64 kGy, a delay of about 8 days in the biodegradablility of the films by the bacterium *Pseudomonas aeruginosa* at the highest dose was found (Mezgheni et al., 1998). The decrease in biodegradability was suggested to be because of an increase in cross-links between casein molecules changing the protein conformation.

1.2.2.3.5 Chemical modification

Chemical modification involves the reaction and/or interaction of proteins with a chemical with the objective of changing the protein structure, which in turn will lead to a change in film properties. Chemical modification can make a protein more hydrophilic by introducing charged groups or can make a protein more hydrophobic by attaching hydrophobic groups or long non-polar chains (reviewed by Lens et al., 1999). Protein polypeptide chains can also be linked to each other by chemical modification and this is generally referred as cross-linking. Acetylation can modify proteins. It involves activated acyl groups from compounds like carboxylic acid anhydrides, carboxylic acid and acid chlorides interacting with the nucleophilic amino group of the protein (reviewed by Lens et al., 1999). Acetylation has limitations as the reaction can be reversible and the acetylating agent can be hydrolysed by water in aqueous systems. Other carbonyl compounds such as aldehydes have shown promising results for modifying proteins (reviewed by Lens, et al., 1999). The products of the reaction between aldehydes and protein depend on the additives present. For example, the presence of a reducing agent like sodium cyanoborohydride (a reductive alkylation compound) can yield a more stable akylamine bond compared to an akyl bond without any additive (Fig. 1.3). When proteins are modified with aldehydes, the reaction may involve the addition of hydrophobic groups or cross-linking, depending on the side chain groups of the aldehyde. When the side group R in Figure 1.3 is methylene groups as in caproaldehyde and lauraldehyde (5 and 11 methylene groups respectively), the reaction involves the addition of a hydrophobic group. When the side group is hydrogen as in formaldehyde, or dialdehyde, the reaction is cross-linking of the protein polypeptide chain, probably by the addition of methylene bridges between adjacent lysine groups of the polypeptide chains.

Aldehydes have been used to change the functional properties of protein-based films. When gluten based films were treated with formaldehyde vapour, the elongation decreased by 62%, and Young's modulus and tensile strength increased by almost 4 fold (Micard et al., 2000). The gluten film was also less soluble in SDS solution. It was suggested that this showed reinforcement of the

gluten network, probably by covalent linkages with lysine residues. Other aldehydes such as glutaraldehyde also gave an increase in tensile strength and Young's modulus; and a decrease in strain and water uptake when used to modify sunflower protein films (Orliac et al., 2002). Similarly glutaraldehyde modification of gelatin films increased stress, Young's modulus, and decreased the strain and swelling in water (Bigi et al., 2001). The swelling of the gelatin films in water decreased from 950% for the control to 100% with a 1% (w/v) glutaraldehyde solution after 5 h. A gelatin hydrogel crosslinked with dextran dialdehyde also had altered rheological properties such as an increase in viscosity, increase in storage modulus, as well as an increase in the melting temperature (Schacht et al., 1997).

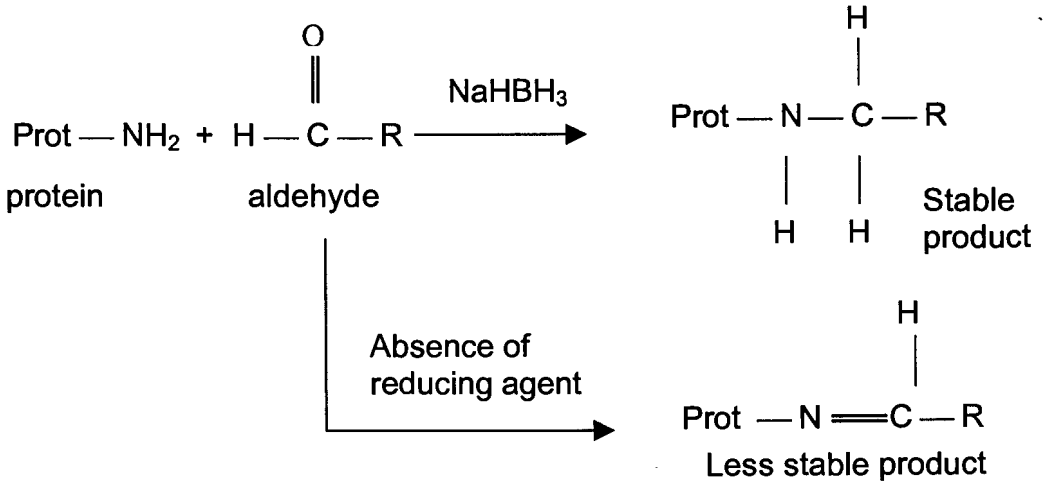


Figure 1.3 Possible reaction of an aldehyde with an amino group in the presence or absence of a reducing agent. The side R can be an aliphatic chain (attaching hydrophobic groups) or an aliphatic chain end capped by another functional group (cross-linking) (Lens et al., 1999)

Although the use of aldehydes as chemical modifying agents for protein-based films has shown promising results, the main limitation is that they are not food compatible. Compounds like cysteine, genipin and tannins can be food compatible as they are natural components of food materials. Thus they can potentially be used to modify protein-based films for food use. When cysteine

was added to soy/gluten composite films an increase in tensile strength, but no effect on WVP and OP was observed (Were et al., 1999). Protein modification by cysteine is believed to be due to polymerization via sulphydryl-disulphide interchange reactions during heating to form a stronger network upon cooling (Lindsay, 1985).

Genipin has been used to modify gelatin films (Bigi et al., 2002). Chemically, genipin has two carbon cyclic rings similar to the phenol aromatic ring, and has the chemical formula $C_{17}H_{14}O_5$ (Ozaki et al., 2002). The modification of gelatin film by genipin resulted in an increase in stress and Young's modulus; and a decrease in strain (Bigi et al., 2002). A decrease in swelling of the gelatin in water and an increase in denaturation temperature and T_g was also noted. The change in the properties of gelatin films was considered to be by cross-linking of the gelatin polypeptide chains by genipin.

Hydrolysable tannins, extracted from tara and chestnut, have been found to be alternatives to aldehydes as agents to modify sunflower protein films (Orliac et al., 2002). The addition of the tannins resulted in an increase in tensile strength, decrease in elongation and decrease water uptake of films. However, the mechanical properties of these films were inferior to those modified with glutaraldehyde and formaldehyde. Thus, it was suggested that tannins interact through weak bonds with the sunflower protein, unlike the covalent bonds for the aldehydes. The tannic acid modification was referred to as cross-linking (Orliac et al., 2002). Similar results have been reported when dermal sheep collagen protein was modified with tannic acid or glutaraldehyde (Heijman et al., 1997). The tensile strength increased more when the collagen was treated with glutaraldehyde than with the tannic acid. However, when the collagen strips were degraded with a bacterial collagenase, the tannic acid treated protein showed better stability than the glutaraldehyde treated collagen. In fact, the tannic acid treated collagen was not degraded by the enzyme as revealed by the fact that there was no change in its the tensile strength. This is probably because of indigestible tannin-collagen complexes formed by tannin protein interaction.

1.2.3 Phenolic compounds

Phenolic compounds such as tannins are secondary plant metabolites. They can range from simple molecules like phenolic acids to highly polymerized polyphenolic compounds for example sorghum condensed tannins with M_r higher than 30 k (Bravo, 1998). The importance of phenolic compounds to scientists is several fold, including:

Contribution to plant pigmentation,

Acting as phytoalexins to provide plants with resistance to pathogens,

Astringency, anti-nutrient and anti-oxidant activity,

Phenolic and protein interaction.

1.2.3.1 Chemistry of phenolic compounds

There is no common classification of phenolic compounds. Bravo (1998) in his review on polyphenols has suggested 16 different classes of phenolic/polyphenolic compounds that include simple phenols, benzoquinones, phenolic acids, acetophenones, phenylacetic acids, hydroxycinnamic acids, phenylpropenes, coumarins/isocoumarins, chromones, naftoquinones, xanthones, stilbenes, anthraquinones, flavonoids, lignans/neolignans and lignin. He further classified the food flavonoids into 13 different classes, including the calcones, dihydrochalcones, aurones, flavones, flavonols, dihydroflavonol, flavanones, flavanols, leucoanthocyanidins, anthocyanidins, isoflavonoids, biflavonoids and proanthocyanidins/condensed tannins. According to Hahn et al. (1984), phenolic compounds can be classified into three basic groups: phenolic acids, flavonoids and tannins. For simplicity, this classification will be used to describe the chemistry of phenolic compounds.

Phenolic acids

Phenolic acids generally have one phenolic group as the basic structure. They are derivatives of benzoic or cinnamic acid (Fig. 1.4) with hydroxyl (OH) and methoxy (OCH_3) groups substituted at various places on the aromatic ring. For example, when there is a hydroxyl group at position 4 and a methoxy group at position 3 of cinnamic acid and benzoic acid, the phenolic acid is ferulic acid

and vanillic acid, respectively. Phenolic acids are found mainly in the pericarp of cereal grains such as sorghum (Waniska et al., 1989).

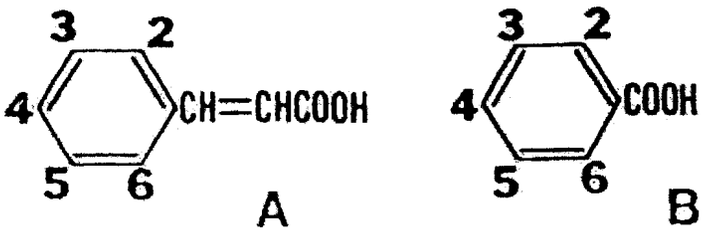


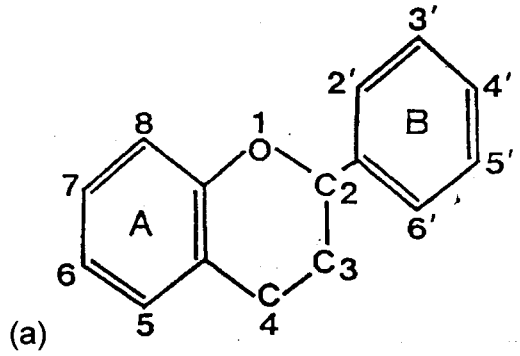
Figure 1.4 Basic structure of phenolic acids: A is cinnamic acid and B is benzoic acid (Hahn et al., 1984)

Flavonoids

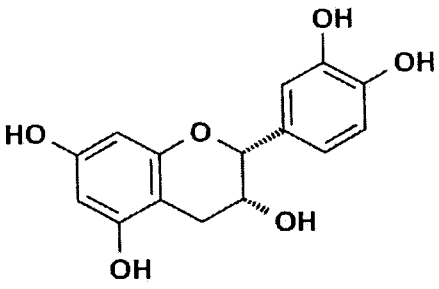
Flavonoids have a basic structure of diphenyl propane (C_6-C_3-C_6) and consist of two aromatic rings linked through three carbons (Fig. 1.5). The aromatic rings are named A and B, and they are believed to be produced by different pathways (reviewed by Bravo, 1998). Some examples of the flavonoid type phenolics are given in Figure 1.5. Flavonoids can be of different types such as flavanols, flavonols, flavones and flavans. The flavans found in plants are the anthocyanidins. They exist as an ionized anthocyanidin form, and can give an intense red pigmentation in acid medium. Anthocyanins are generally the glycosides of anthocyanidin. Examples are pelargonidin, malvidin and cyanidin. The anthocyanins contribute to the major pigmentation of plants (Chen and Hrazdina, 1981). The other flavans are the catechins and their derivatives and leucoanthocyanidins (Hahn et al., 1984). In sorghum grain, the pericarp colour appears to be due to a combination of anthocyanin pigments (Hahn et al, 1984).

Tannins

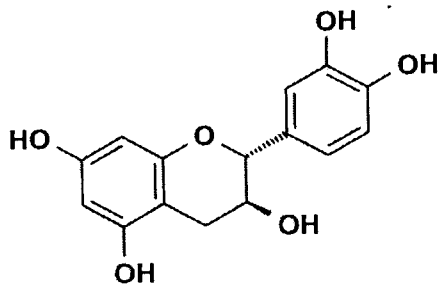
The term tannin comes from the tanning capacity which transforms animal hides into leather by forming stable tannin-protein complexes. Plant tannins can be divided into two chemically distinct types: hydrolysable and condensed tannins.



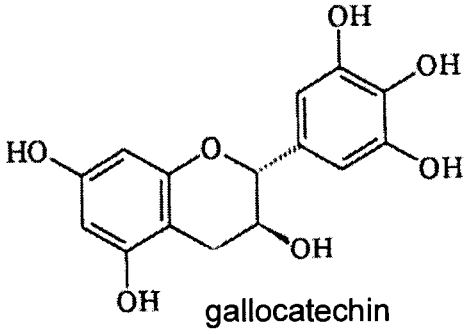
(a)



(-)-epicatechin



(+)-catechin



gallocatechin

(b) some flavanols

Figure 1.5 Flavonoid type phenolics (a) The basic structure of flavonoids and (b) flavanol (catechin) type (Hahn et al., 1984, Hagerman, 2002).

The hydrolysable tannins are derivatives of gallic acid (3,4,5-trihydroxyl benzoic acid)and elagic acid. Gallic acid is esterified to a core polyol, generally glucose (Fig. 1.6) and the galloyl group may be further esterified or oxidatively cross-

linked to yield more complex hydrolysable tannins (Fig. 1.6) (Hagerman, 2002). The simple hydrolysable tannin, known as gallotannin, comprises several polygalloyl esters of glucose (Fig 1.6), generally known as pentagalloyl glucose. Other gallotannins can have up to 12 esterified galloyl groups and a core glucose. The commercially available hydrolysable tannin, known as tannic acid is a mixture of gallotannins from sumac (*Rhus semialata*), galls (Chinese gallotannin) and Aleppo oak (*Quercus infectoria*) (Hagerman, 2002).

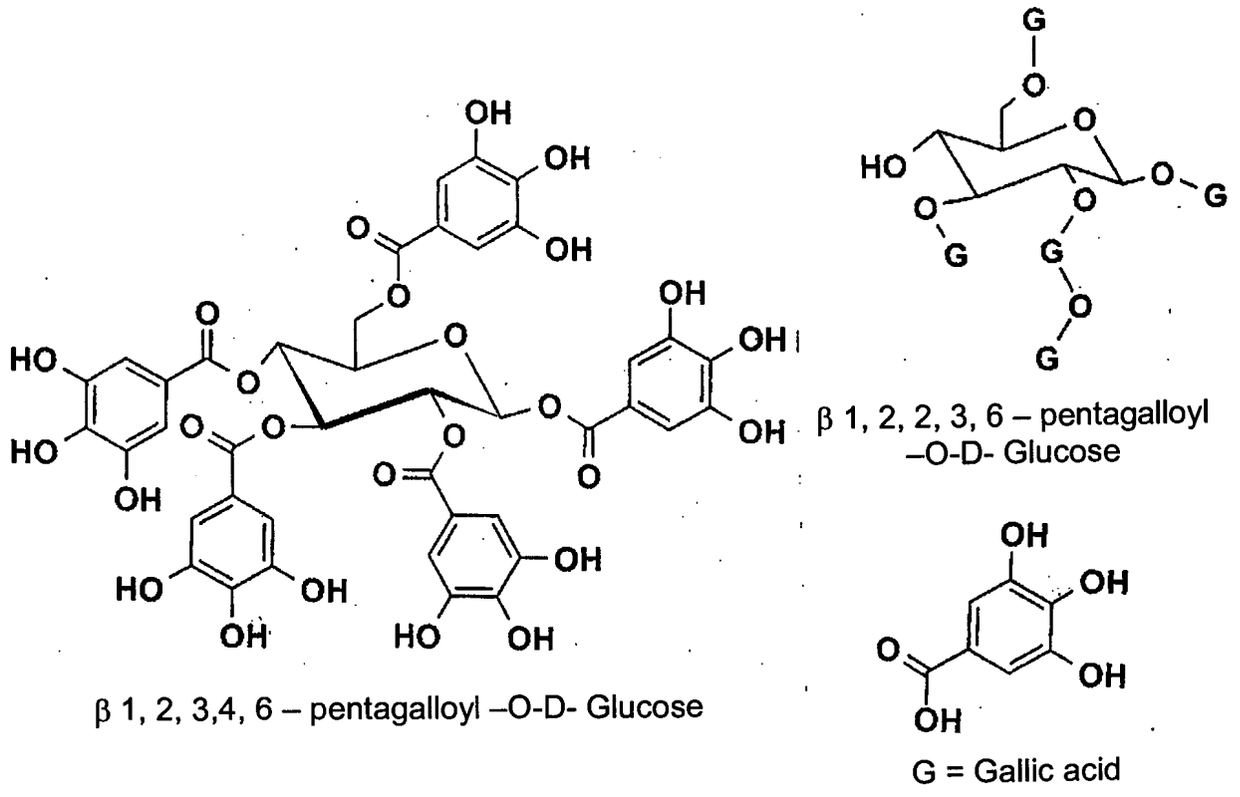
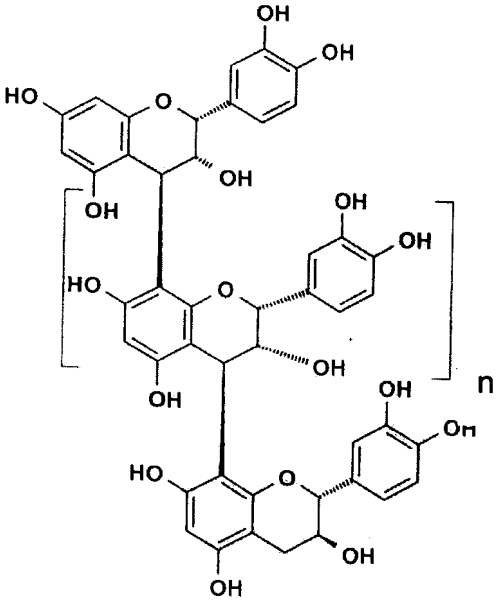


Figure 1.6 The chemical structure of gallotannins (Hagerman, 2002)

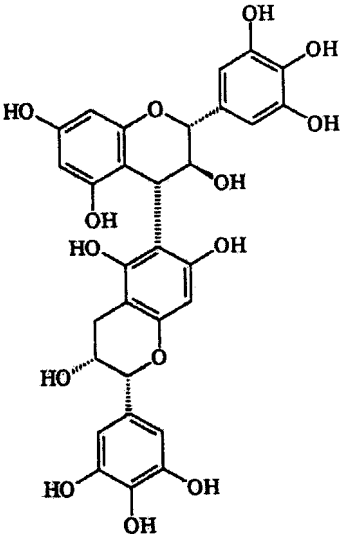
Condensed tannins

Condensed tannins are polymers of the flavan-3-ol unit (example catechin) connected by interflavan bonds (Fig. 1.7) (Hagerman, 2002). The interflavan bond can be C_4-C_8 or sometimes C_4-C_6 as indicated in Figures 1.7a and b, respectively. They are generally referred as proanthocyanidins or procyanidins, because anthocyanidins are released when the condensed tannins are hydrolysed with mineral acids. Condensed tannins have a M_r of approximately 5 k but molecular weights up to 50 k have been reported (McGrath and Smith, 1990). The type of interflavan bond and the degree of polymerization can be

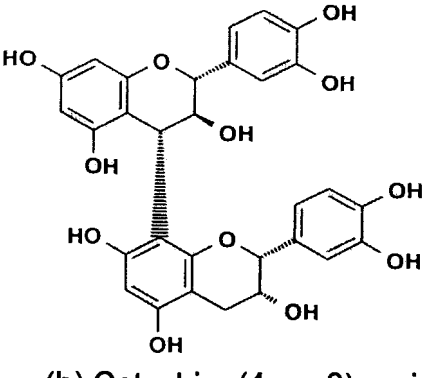
diverse in condensed tannins. The condensed tannin of tannin (high tannin) sorghum is quite complex. It has been shown to have different types of interflavan bonds as well as high structural heterogeneity of the monomeric unit (Krueger et al., 2003). But Hagerman (2002) suggested that the interflavan bonding is mostly C_4-C_8 and a degree of polymerization uo to 15 (Hagerman, 2002). Other condensed tannins are also complex. Mimosa condensed tannin contains both the C_4-C_8 and C_4-C_6 interflavan bonds and has a degree of polymerization of 5 (Pasch et al., 2001).



(a) Basic structure



(c) Gallocatechin- (4 α→6)- epigallocatechin



(b) Catechin- (4 α→8)- epicatechin
(procyanidin B_4)

Figure 1.7 Chemical structure of condensed tannins, (a) basic structure with n>2, (b) C_4-C_8 interflavan bond and (c) C_4-C_6 interflavan bond (Hagerman, 2002)

1.2.3.2 Phenolic-protein interaction

Protein-phenolic interactions are involved in several chemical and biological processes such as haze formation in beverages, astringency sensation, tanning of leather and reduction in protein digestibility. The protein-phenolic interaction is a complex phenomenon. Various mechanisms and types of chemical interaction have been proposed; and several factors affect the interaction.

1.2.3.2.1 Mechanisms of phenolic-protein interaction

Two mechanisms of phenolic-protein interaction have been proposed. The first involves complexation and subsequent precipitation and the second one involves cross-linking.

The first mechanism has two stages, namely the complexation of the phenolic compounds with the protein and subsequent precipitation of the complex (Fig. 1.8). Kawamota et al. (1996) have provided some evidence to support this proposed mechanism. They used different types of galloyl glucose molecules (hydrolysable tannins) with up to five galloyl substitution and bovine serum albumin (BSA) in a model system. They argued that one galloyl group from one molecule of galloyl glucose should bind to more than two BSA molecules simultaneously for cross-linking. However, the binding between each binding sites between of the galloyl group and BSA molecule is considered to be very weak because of very low relative affinities of mono and di-galloyl glucose, so no cross-linking can occur. Thus it is unlikely that cross-linking of the BSA polypeptides can occur for a molecule like galloyl glucose with five or less galloyl groups, as at least three galloyl groups have to be complexed by each BSA molecule. Effective complexation requires more than three galloyl groups The precipitation is directly related to the number of galloyl groups bound to a BSA molecule. According to these authors, under experimental conditions 30 galloyl groups are needed to complex a BSA molecule before precipitation can occur.

Haslam and co-workers (1992) suggested that the complexation mechanism can be reversible or irreversible. Reversible complexation involves non-covalent forces, but irreversible complexation involves covalent bonds. Irreversible complexation occurs when reactive intermediates such as ortho-quinones (formed from the phenolics) chemically interact with the nucleophilic groups (-NH_2, -SH groups) of proteins to form covalently linked polyphenol-protein complexes. Reactive phenolic intermediates such as ortho-quinones can be produced through oxidation of phenolic compounds by the polyphenol oxidase enzyme, metal ions or autocatalysis in mildly basic media. This type of irreversible phenolic-protein complexation is responsible for enzymatic browning of fruits and juices and the tanning of hides for leather manufacture.

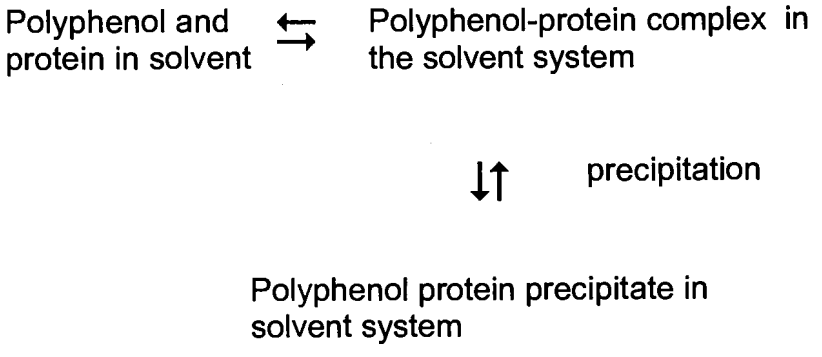


Figure 1.8 Schematic representation of polyphenol complexation and co-precipitation mechanism (Haslam et al., 1992)

Siebert et al. (1996) proposed the cross-linking mechanism because phenolic compounds like tannic acid can bind simultaneously to two gelatin molecules to cross-link the polypeptide chains and form haze (Fig. 1.9). When there is an excess amount of protein relative to polyphenol, each polyphenol molecule can bridge between two protein molecules resulting in dimers and small protein aggregates. With an excess of polyphenols relative to protein, all the protein binding sites will be occupied, but bridging ability may still be low as the polyphenol will have little chance to find a free binding sites. So even at high polyphenol concentration, small aggregates will still be formed. This proposed mechanism is based on *in vitro* data of tannin interaction with gelatin. When

tannic acid was added at increasing concentration to gelatin in a model system, an increase in haze was observed. However, further addition of tannic acid did not result in further haze formation.

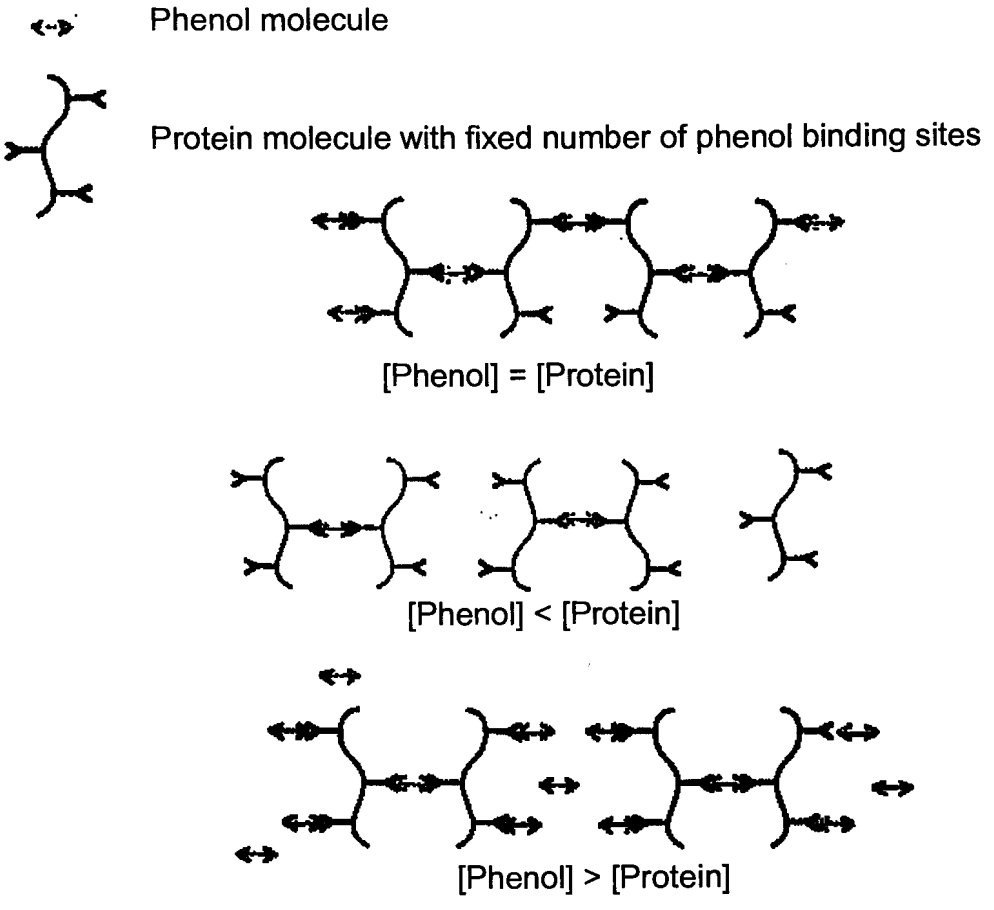


Figure 1.9 Cross-linking mechanism of the interaction between polyphenol and gelatin at different ratios (Siebert et al., 1996)

A cross-linking mechanism to explain the interaction between phenolics and proteins is also supported by experiment work of Charlton et al. (2002). These authors used the dynamic laser light scattering technique, which measures the relaxation rate of particles in solution to estimate the particle size diameter of complexes formed between salivary proline rich protein PRP and various phenolic compounds. The phenolic compounds were epigallocatechin gallate and pentagalloyl glucose (a hydrolysable tannin) prepared from tannic acid. They proposed that the interaction between polyphenol and peptide could be divided into three stages (Fig. 1.10).

At first, the added polyphenol binds to the polypeptide, and several polyphenol molecules can bind to the same polypeptide (Fig. 1.10a). The binding is reversible at this stage and is believed to be mostly by hydrophobic interaction. The first stage basically involves the complexation of one polypeptide by the phenolic compounds to form soluble aggregates, i.e. to coat the polypeptide with the phenolic compounds. The second stage requires a second polyphenol coated polypeptide molecule. Because of their multiple binding sites, the polyphenols already bound to one polypeptide can interact with other polypeptides or can interact with other polyphenols which are bound to other polypeptides. At this stage (Fig. 1.10b), the interaction can result in intermolecular bridging action through forces like hydrogen bonds. This causes dimerization of the polypeptides, leading to a doubling in size of the complex. The dimerization makes the protein and phenolic compounds complex insoluble. In the third stage (Fig. 1.10c), these polypeptide dimers can aggregate together. As a result, spontaneous aggregation of these insoluble aggregates by dimerization into larger complexes occurs to give a bimodal distribution of particle sizes, as observed by the laser light scattering analysis.

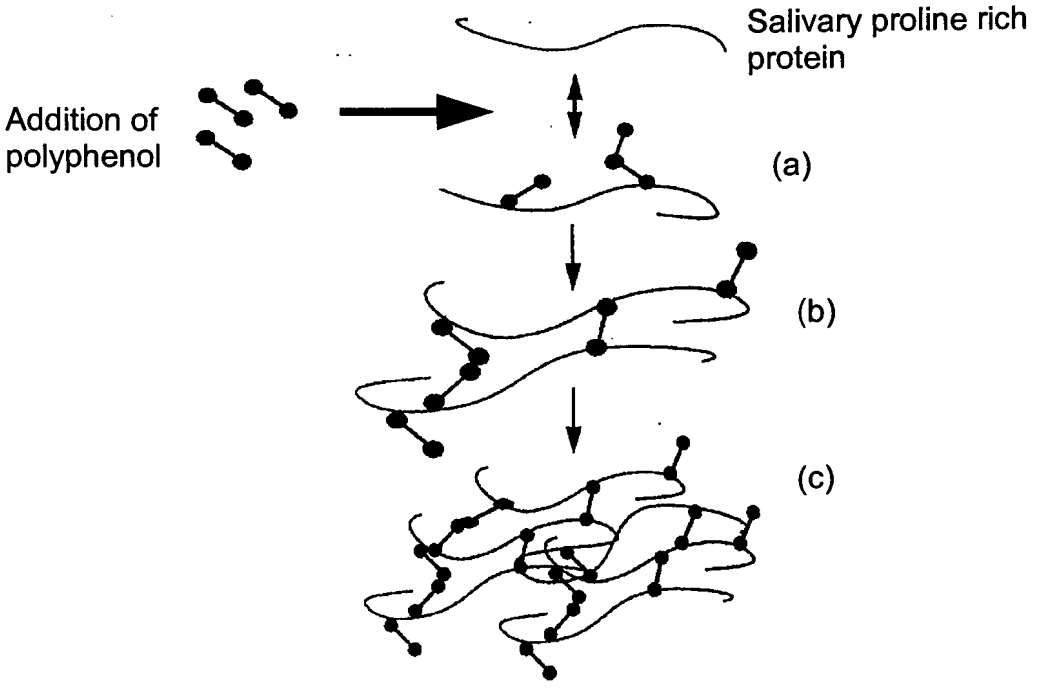


Figure 1.10 Three stages in the cross-linking mechanism between salivary proline rich protein and phenolic compounds (Charlton et al., 2002)

Spencer et al. (1988) proposed that the interaction between polyphenols and PRPs is a surface phenomenon. They suggested that both the complexation and co-precipitation, as well as the cross-linking mechanism are possible, depending on the type of phenolic compounds and their concentration. Three mechanisms were postulated (Fig. 1.11). Polyphenols can bind at different protein binding sites. When the polyphenols are in excess and the protein concentration is low, the polyphenols can form a monolayer around the protein molecule. This monolayer is more hydrophobic than the protein, so there is precipitation (mechanism a, Fig 2.11). At high protein to polyphenol ratios, precipitation can occur because the relatively high hydrophobic surface layer of the complexes, as well as the cross-linking of different protein molecules (mechanism b, Fig 2.11). Simple phenolic compounds, for example phenolic acids can also interact with the proteins by forming a coat around the protein, but precipitation is limited because of their high solubility in water (mechanism c, Fig 2.11).

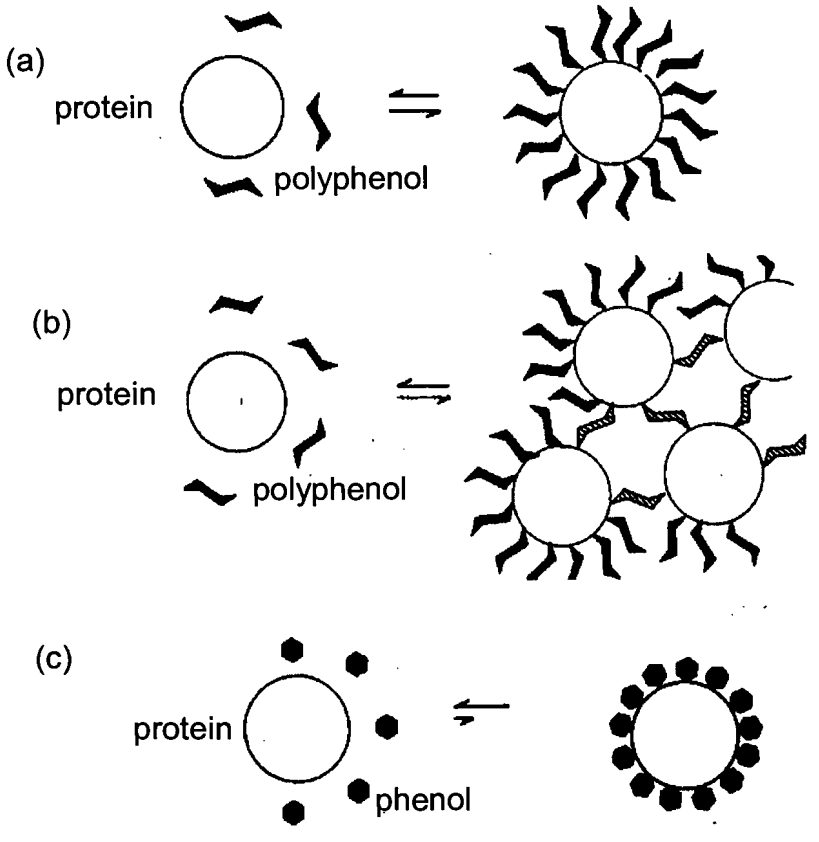


Figure 1.11 Proposed mechanisms of phenolic and protein interaction (Spencer et al., 1988)

1.2.3.2.2 Chemical interactions between protein and phenolic compounds

There may be four potential types of chemical interactions between phenolics and protein: covalent, ionic, hydrogen bonding and hydrophobic interaction. The last two have been found to be most common modes of interaction during the formation of phenolic-protein complexes (reviewed by Butler et al., 1984).

The phenolic hydroxyl group is an excellent hydrogen bond donor and forms hydrogen bonds with the carbonyl groups of the polypeptide chains. Spectroscopic studies using ^{1}H-NMR (nuclear magnetic resonance) have shown that hydrogen bonds as a mode of interaction can stabilise the complex between salivary PRP and pentagalloyl glucose, a hydrolysable tannin (Murray et al., 1994). Hagerman et al. (1998) have shown that the hydroxyl groups of the polyphenols have a high affinity to form hydrogen bonds with proline residues in BSA. The carbonyl function of tertiary amides like in proline are much more effective in forming hydrogen bonds than primary and secondary amides (reviewed by Haslam, 1996). In addtition, the methylene groups substituents of the tertiary amide nitrogen can 'donate' electrons into the peptide bond, causing it to be electron rich (reviewed by Haslam, 1996). This enhances the capability of the carbonyl group to be more electro-negative to form hydrogen bonds with the hydroxyl groups of the phenolic compounds.

Hydrophobic forces are difficult to define because they arise from the presence of regions in molecules that are not polar. The non-polar region and groups move away from water and seek to coalesce by hydrophobic interaction. Van der Waals forces may make a substantial contribution to the stability of the interaction (reviewed by Haslam, 1996). Phenolic compounds contain several non-polar groups such as the aromatic ring, and the carbon-hydrogen skeleton of sugars in the hydrolysable tannins (reviewed by Haslam, 1996). Hydrophobic interaction between the galloyl ring of the pentagalloyl glucose and the pyrrolidine ring face from the PRPs has been interpreted by ^{1}H-NMR spectroscopy (Murray et al., 1994). Similarly, Hagerman et al., (1998) have supporting data to show that phenolics such as pentagalloyl glucose can form a hydrophobic coat around BSA to cause precipitation. Possible hydrophobic

interaction between condensed tannins and gelatin or poly-L-proline has been demonstrated by the effective absorption ability of the tannins on an uncharged polystyrene resin (Oh et al., 1980).

Covalent bonds have not been widely investigated in phenolic-protein interaction. However, such interactions can happen depending on the conditions. Covalent interactions may result from nucleophilic attack of amino acid side groups by the orthoquinoid oxidation products of the phenolic compound (Pierpont, 1969), as already discussed above. Such reactions can occur at high pH where oxidation of phenolic compounds is most likely to happen. Phenolic compounds are most unlikely to interact through ionic bonds. At high pH values (alkaline), phenolate anions are formed, so there is no complex formation as these phenolate anions cannot form hydrogen bonds (Hagerman and Butler, 1978).

1.2.3.2.3 Factors affecting protein-phenolic interaction

Intrinsic factors of the interacting species may influence the protein and phenolic interaction. Type of protein, type of phenolic compound, the solvent system and additives are factors to be considered in protein-phenolic interaction.

The presence of proline in the protein can play a crucial role in phenolic-protein interaction. When the amino acids in a PRP were examined by NMR, it was found that the proline residues show the largest shift when proline residues were bound to the tannins (Luck et al., 1994). It has been suggested that the conformation of the proline imino acid can provide a pyrrolidine ring to hydrophobically interact with the phenolic aromatic ring (Luck et al., 1994). As stated earlier, the carbonyl function in proline can effectively form a hydrogen bond with the hydroxyl group of polyphenols. PRPs are believed to have an extended, rather than a compact globular structure (Murray and Williamson, 1994). This may increase the ability of the protein to unfold and to expose binding sites for polyphenols (Hagerman and Butler, 1981). The type of PRP has also been found to affect the protein-phenolic compound interaction. Basic

PRP can be precipitated by tannins, but glycosylated PRP and acidic PRP do not form insoluble complexes with tannins (Lu and Bennick, 1998).

Although proline residues of salivary PRP have been shown to interact with polyphenols, a low M_r protein, known as histatin, characterized as being rich in histidine residues, can be bound and precipitated by hydrolysable tannins (Yan and Bennick, 1995). Besides proline, other amino acids like arginine, glycine and glutamine have also been reported to interact with hydrolysable tannins during complexation (Luck et al., 1994).

Different types of phenolic compounds can affect the protein-phenolic complexation because they can have different numbers of aromatic rings. To explain the effect of different types of phenolics, Siebert and Lynn (1998) put forward the concept of 'single ended' or 'double ended' or 'multiple ended' phenolics. They used PVPP (polyvinyl polypyrrolidone) and several phenolic compounds in a model system. It was suggested that simple phenolic acids such as gallic acid and methyl gallate can be considered as 'single-ended' phenolics. These 'single-ended' phenolics could interact with PVPP, but as they have only one aromatic ring, they could not cross-link another PVPP molecule to cause precipitation. Siebert and Lynn (1998) considered flavonoid type phenolic compounds like epicatechin as 'double-ended' because of its two aromatic rings. However, both aromatic rings are not strong binding sites. Theoretically, flavonoid type phenolics like epicatechin can bind to two molecules of PVPP at one time, but haze formation is low (Siebert and Lynn, 1998) because one binding site of the flavonoid is considered to be strong, and the other is weak. It has been suggested (McManus et al., 1985) that a phenolic ring should have at least two hydroxyl groups for binding and if the hydroxyl groups are close together, the binding is stronger. Thus in flavonoid type phenolics such as catechin, epicatechin and gallocatechin (Fig. 1.5), they will have a strong binding site (the aromatic ring B) as they have vicinal hydroxyl groups at position 3′ and 4′. The weak binding site is the aromatic ring A because the hydroxyl groups are not close as they are at positions 5 and 7.

Hydrolysable and condensed tannins have several aromatic rings, so several binding sites. They can bind and cross-link to cause haze with PVPP (Siebert and Lynn, 1998). Depending on the degree of polymerization, different tannins have different binding ability to proteins (Baxter et al., 1997). For example, tannic acid (a hydrolysable tannin) has a lower haze forming ability with BSA than sorghum condensed tannin because the latter is generally of higher degree of polymerization (Hagerman and Klucher, 1986). Similarly, the order of binding affinity to salivary PRP is procyanidin dimer B2> pentagalloyl glucose> trigalloylglucose> proanthocyanidin (-)-epicatechin (Baxter et al., 1997). These workers proposed that larger polyphenols occupy two or more proline residues from different salivary PRP molecules for stronger binding and cross-linking. In contrast, the low M_r or less complex polyphenols will not occupy several proline residues, so a weaker binding will result. The conformation of condensed tannin can also result in different binding ability to salivary PRP. A C(4)-C(8) interflavan bond dimer of the condensed tannin type phenolic has higher binding ability with salivary PRP than one with a C(4)-C(6) interflavan bond (Freitas and Mateus, 2001).

Other factors like pH, temperature and ionic strength of a system can affect protein-phenolic complexation (Kawamoto and Nakatsubo, 1997). The binding pH of galloyl-α-D-glucoside (a hydrolysable tannin) with proteins like lysozyme, BSA and myoglobin occurs within a range of pH 2.2 to 7, but the optimum binding occurs at the pI of the specific protein. Above pH 7, little or no binding occurs, as phenolate ions produced are not active in hydrogen bonding. Low temperature is favourable for the complexation of protein and tannin. At low ionic strength, induced by the addition of sodium chloride, haze is promoted. This can be explained by the disruption of some electrostatic forces to cause more open protein structure for binding to occur (Rubino et al., 1996).

1.2.4 Phenolic compounds and sorghum proteins

Research in the area of phenolic compound interaction with sorghum proteins is mostly limited to the anti-nutritional issue. The phenolic compounds may also contaminate isolated sorghum protein during extraction.

Tannin sorghum has been shown to have lower *in vitro* protein digestibility than condensed tannin free sorghum (Nguz and Huyghebaert, 1998). The increase in amount of tannin in sorghum was linearly correlated with a decrease in *in vitro* protein digestibility. The low protein digestibility of high tannin sorghum has been attributed to enzyme-tannin (Nguz et al., 1998) and dietary protein-tannin complexes (Butler et al., 1984). Butler et al. (1984) showed that the indigestible protein fractions in tannin sorghum are mostly the prolamin fractions of sorghum protein. It should be noted that these studies suggested that the condensed tannins can bind to sorghum proteins, but not specifically to the prolamin fraction, kafirin.

In sorghum, the extraction of protein fractions is affected by phenolic compounds. This suggests protein and phenolic interaction. When formaldehyde was used in dilute concentration to inactivate tannins in tannin sorghum, the yield of extracted albumins, globulins and prolamin proteins showed a significant increase (Daiber and Taylor, 1982). Thus, these authors suggested the possiblility of tannin-kafirin complex formation, because the tannins decreased the prolamin yield during extraction. The protein removed from the tannin associated proteins is quite hydrophobic and rich in proline residues (Hagerman and Butler, 1980). This suggests that the tannins have the potential to interact with kafirin.

.

1.2.5 Conclusions

Kafirin films like other protein-based films, have inferior mechanical and barrier properties when compared to synthetic plastic packaging. Chemical modification, for example with phenolic compounds from natural sources, has shown potential to change the functional properties of protein-based films. Phenolic compounds like hydrolysable and condensed tannins can interact with proteins like salivary PRP and other proteins rich in proline, for example gelatin. Since kafirin is rich in proline residues, this may suggest the potential of phenolic compounds to modify kafirin films. However, the interaction of kafirin with phenolic compounds is not well elucidated.

1.3 Objectives and hypotheses

1.3.1 Objectives

The objectives of the research were as follows:

1) To determine and compare the complexing ability of different phenolic compounds like phenolic acids; flavonoids; hydrolysable and condensed tannins with kafirin in terms of haze formation and phenolic bound to the protein.

2) To determine whether phenolic compounds (most interacting ones from objective 1) can modify kafirin films in terms of their tensile, barrier and rheological properties.

3) To determine the possible mode and/or the possible mechanism involved in the interaction between phenolic compounds (most interacting ones from objective 1) and kafirin.

1.3.2 Hypotheses

Salivary PRP can interact with hydrolysable and condensed tannins by hydrogen bonding and hydrophobic interaction. As kafirin is rich in proline residues, kafirin will interact with phenolic compounds. Phenolic compounds can be of different types. Different types of phenolics will have different binding ability because of different amounts and arrangement of the aromatic rings, and types,numder and arrangement of substituted hydroxyl groups on the aromatic rings.

Because phenolic compounds contain numerous hydroxyl groups and some contain numerous aromatic rings, it has been hypothesized that these phenolics can interact with more than one protein polypeptide chain simultaneously to cross-link protein to form haze. Thus, if these phenolics can be added during production of kafirin film, they will bind and cross-link the kafirin to change the film's mechanical and barrier properties.

Heat processing can lead to changes in the secondary structure of kafirin. The α-helical structures can unravel to form intermolecular β-sheets. If phenolic compounds are added during kafirin dissolution under heat treatment, the presence of tannins may affect the secondary structure of kafirin in the film.

2. RESEARCH

The research Chapter is divided into 3 parts to address the objectives stated in section 1.3. The three parts are as follows:

2.1: Sorghum kafirin interaction with various phenolic compounds

2.2: Sorghum kafirin film property modification with hydrolysable and condensed tannins

2.3: Effects of tannins on the secondary structure of kafirin and kafirin films

The relationships between the three parts of the research are illustrated in Figure 2.1

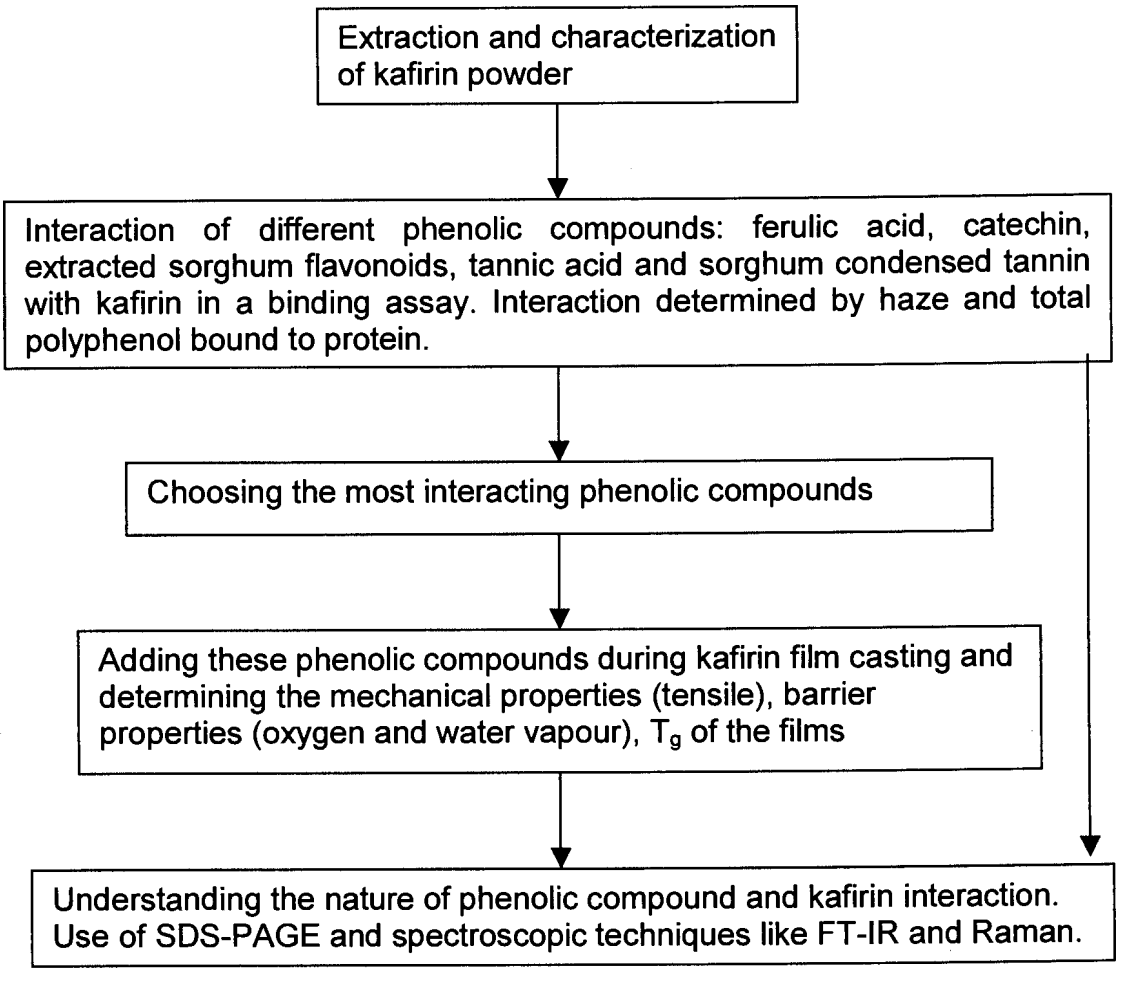


Figure 2.1 Schematic representation of the research parts

2.1 Sorghum kafirin interaction with various phenolic compounds

2.1.1 Abstract

The ability of various phenolic compounds to bind and precipitate kafirin, the prolamin protein of sorghum, was investigated with regard to effects on its nutritional and functional uses. The protein-phenolic compound interaction was quantified by haze formation and colorimetric determination of total polyphenol bound. Ferulic acid, catechin and extracted phenolics (flavonoids) from condensed tannin-free sorghum did not complex kafirin. Only tannic acid and sorghum condensed tannins had the ability to complex kafirin. The sorghum condensed tannins interacted more strongly than tannic acid. The haze of sorghum condensed tannins-kafirin was permanent, indicating a strong interaction. The fact that sorghum condensed tannins can bind irreversibly to kafirin indicates that this may play a role in decreasing protein digestibility in tannin sorghum and can cause haze formation in lager beer made from tannin sorghum. However, in condensed tannin-free sorghum, phenolic compounds may not appear to play a major role in decreasing protein digestibility, as these endogenous phenolics may not bind with kafirin.

Key words: sorghum prolamin, kafirin, phenolic compounds, tannin, haze

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2.1.2 Introduction

The cereal sorghum, *Sorghum bicolor* (L. Moench) is indigenous to the semi-arid tropics of Africa. The protein content of sorghum grain is in the range 7.3-15.6% (dry matter basis) (Hulse et al., 1980) and the main protein is a prolamin, kafirin, representing approximately 50-60% of total protein (Taylor and Schüssler, 1986). Sorghum protein has numerous nutritional and functional properties. In terms of nutrition, sorghum is a major dietary source of protein for many African people where the staple food is porridge made from the cereal (Murty and Kumar, 1995). Concerning functionality, the protein is also important in malting (Taylor, 1983) and beer brewing (Taylor and Boyd, 1986). However, various factors can affect the properties of sorghum protein in food, one of which is the presence of phenolic compounds.

Phenolic compounds are secondary plant metabolites and occur in sorghum as phenolic acids, flavonoids and tannins (Hahn et al., 1984). All sorghums contain phenolic acids and flavonoids; several sorghum varieties also contain anthocyanidins or procyanidins that give rise to the red pigmented pericarp (Nip and Burns, 1969). Some sorghum varieties also contain condensed tannins, *syn*. proanthocyanidins (Gupta and Haslam, 1978). Phenolic compounds can interact with food proteins to influence their physical and biochemical properties, for example the astringency sensation, lower nutritional value and haze formation in beverages (reviewed by Haslam et al., 1992).

Knowledge about sorghum protein-polyphenol interactions is scarce and is mainly limited to their nutritional significance. The presence of condensed tannins was found to decrease *in vitro* (Nguz and Huyghebaert, 1998) and *in vivo* (Nyambi et al., 2000) protein digestibility in sorghum. The low digestibility can be attributed to complex formation of tannins with both the dietary protein to render it indigestible (Butler et al., 1984), and digestive enzymes to inhibit their action (Nguz et al., 1998). It is notable that negative effects of phenolic compounds on sorghum food quality have been investigated on the protein of sorghum in general but not specifically on kafirin. In this respect, it is known that the proline rich protein (PRP) from saliva can associate with polyphenols such

as hydrolysable and condensed tannins (Lu and Bennick, 1998). The PRP complexation with polyphenols suggests that they may have a high affinity for sorghum kafirin, which is rich in proline residues (Taylor and Schüssler, 1986).

This study investigated sorghum kafirin-phenolic compound interaction. The objective was to determine and compare the binding ability of different phenolic compounds to kafirin.

2.1.3 Experimental

2.1.3.1 Materials

Protein: Sorghum kafirin was extracted from condensed tannin-free white cultivars PANNAR PEX 202 & 606. Milled sorghum was extracted using the fundamentals from Carter and Reck method (1970) with a solution of 70% (w/w) absolute ethanol in distilled water, 0.5% (w/w) sodium metabisulphite and 0.35% sodium hydroxide (w/w) (Taylor et al., 1984b; Hamaker et al., 1995) at 70 ^{0}C for 1 h. After centrifugation and solvent evaporation overnight in a fume cupboard, the protein suspension was acidified to approximately pH 5 with 1 M HCl to precipitate the protein. The wet protein concentrate was freeze-dried and then defatted three times each of 1 h with hexane at room temperature. The defatted protein powder was air-dried and had protein, fat, ash and total polyphenol (tannic acid equivalents) contents of 970 g kg^{-1}, 12.0 g kg^{-1} and 8.6 g kg^{-1} and 2.4 g kg^{-1} respectively on a dry mass (DM) basis. The molecular weight distribution by sodium dodecyl sulphate polyacrylamide gel electrophoresis showed the presence of α_1 and α_2 kafirin (23 and 25 k) and β kafirin (20 k) (see figure 2.10, track 1) as reported by Shull et al. (1991); and dimers and trimers without any other protein contaminants as reported by El Nour et al. (1998). The amino acid composition of the protein powder was nearly identical to the kafirin amino acid composition reported by Taylor and Schüssler (1986). Hence, the protein is referred as to kafirin.

Bovine serum albumin (BSA) (Sigma, St. Louis, USA, > 97% albumin) was used as a standard binding protein.

Phenolic compounds: Commercial phenolic compounds: ferulic acid (Sigma), catechin (Sigma), and tannic acid (Merck, Darmstadt, Germany) were used. Phenolic compounds were also extracted from a tannin (high tannin) red sorghum (Nola GH 91) and a condensed tannin-free red sorghum (PAN 8564). Milled sorghum (100 g) was first defatted with diethyl ether at room temperature (≈ 25 ^{0}C). The phenolics were then extracted with 500 ml of cold acetone/water (75/25; v/v) at 4 ^{0}C for 1 h (Kaluza et al., 1980). The crude extracts were stored at 8 ^{0}C and used within 48 h of extraction. The total solids, protein and total polyphenol contents (catechin equivalents) of the crude phenolics extract of condensed tannin-free red sorghum (PAN 8564) were 8.52 mgml^{-1}, 14 gkg^{-1} DM and 700 gkg^{-1} DM respectively. Total solids, protein, total polyphenol (catechin equivalents) and condensed tannin (catechin equivalents) contents for the crude phenolics extract high tannin sorghum (Nola GH 91) were 11 mgml^{-1}, 13 gkg^{-1} DM, 2545 gkg^{-1} DM and 2730 gkg^{-1} DM, respectively.

2.1.3.2 Analyses

2.1.3.2.1 Binding Assay

This was based on the ability of phenolic compounds to bind and precipitate protein. Phenolic compounds (1 ml): 0-750 μg tannic acid, catechin and ferulic acid, 0-440 μg (catechin equivalents) extracted phenolic compounds from condensed tannin-free red sorghum (sorghum flavonoids) and 0-2100 μg (catechin equivalents) extracted phenolic compounds from tannin sorghum (sorghum condensed tannins) were added to kafirin protein (1 ml at 10 mgml^{-1}) or BSA (1 ml at 10 mgml^{-1}) in capped test tubes. The kafirin and its added phenolic compounds were in a buffer solution of 75%(v/v) absolute ethanol in distilled water containing 0.02 M phosphate buffer at pH 4 (adjusted with lactic acid). BSA with the various phenolic compounds were in 20% (v/v) absolute ethanol in water containing 0.085 M sodium chloride and 0.1 M acetate buffer at pH 4 (adjusted with lactic acid) (Hagerman and Butler, 1978). The test tubes were incubated in a shaking water bath at 60 ^{0}C for kafirin and 30 ^{0}C for BSA for 1 h. The tubes were cooled to room temperature and chilled overnight at 4 ^{0}C to enhance haze formation. After haze formation, the tubes were incubated

at 30 ^{0}C for 30 min to distinguish between chill haze and permanent haze. The amount of phenolic compounds bound to the kafirin and BSA was quantified by measuring the amount of haze formation and total polyphenol bound.

2.1.3.2.2 Determination of Haze

This was determined according to the method of the American Society of Brewing Chemists (ASBC) (1976). The working standards were in the range of 0-10,000 ASBC Formazin Turbidity Units (FTU). The haze of the standards and incubated phenolic compounds/protein samples was measured at 580 nm (Morris, 1987). Haze was expressed as ASBC FTU.

2.1.3.2.3 Determination of total polyphenol bound to protein

Polyphenols were quantified by a modified International Organization for Standardization method (1988). The contents of the protein-phenolic compound tubes were vortexed and then centrifuged at 2000 x *g* for 5 min. The clear supernatant was discarded and 1 ml of buffer solution (as per binding assay) was carefully added to the side of the centrifuge tubes without disturbing the pellet and then centrifuged at 2000 x g for 5 min. The procedure was repeated twice to ensure minimal residual phenolic compounds which were not bound to the protein. The pellet was resuspended in either 5 ml 75% ethanol or 20% ethanol (v/v) for kafirin and BSA, respectively. One ml 1% carboxymethyl cellulose and 0.2% ethylene-diamine-tetra-acetic acid (w/v) in distilled water followed by the 0.2 ml ferric ammonium citrate reagent (1.75% w/w in water) and 0.2 ml ethanolamine alkali reagent (29% w/w in water) were added for colour formation. The absorbance was read at 525 nm. Catechin (Sigma) and tannic acid (Merck) were the standards. The amount of polyphenols bound was expressed as μg tannic acid for tannic acid and as μg catechin equivalent for the other phenolics, namely: catechin, the crude phenolic extracts from tannin sorghum (sorghum condensed tannins) and condensed tannin-free red sorghum (sorghum flavonoids).

2.1.3.2.4 Statistical analyses

Analysis of variance was performed on the data to establish significant ($P<0.05$) differences between the proteins and the various phenolic compounds. A paired t-test was also done to show significant ($P<0.05$) differences between the haze values between the two incubated temperatures (4 and 30 ^{0}C).

2.1.4 Results and discussion

Haze determination of kafirin with various phenolic compounds was determined to establish which types of phenolic compound bind to kafirin and the measurement of the polyphenol in the haze was performed to establish how much binds. BSA was used as standard binding protein for comparison as it is known to bind with condensed tannin from sorghum (Hagerman and Butler, 1978). The binding assay of various phenolic compounds with kafirin and BSA was done under different conditions because the two proteins have different properties. According to Sigma (2002), BSA is saline soluble. However, kafirin is soluble in aqueous ethanol at high temperature (Johns and Brewster, 1916).

Figure 2.2 shows the haze of kafirin and BSA as affected by phenolic compounds; and Figure 2.3 shows the amount and percentage polyphenols bound to kafirin and BSA (in relation to μg added). Ferulic acid, a phenolic acid, did not significantly increase haze for both kafirin and BSA (Fig. 2.2a). Thus, there was no haze formation between ferulic acid and kafirin, although it has been reported that ferulic acid might be covalently bound to hemicellulose in the cell wall of sorghum grain (Glennie, 1984). Similarly, gallic acid, another phenolic acid was not found to complex PRP (Siebert and Lynn, 1998). According to these authors, phenolic acids can be considered as 'single-ended' and cannot cross-link protein to form haze.

Catechin and the sorghum flavonoids (the phenolics extracted from the condensed tannin free sorghum) did not give any significant haze or bind to kafirin. However, a small increase in haze at high concentration of catechin (Fig. 2.2b) and the sorghum flavonoids (Fig. 2.2c) was observed for BSA. Similarly,

low binding by catechin (Fig. 2.3a) and sorghum flavonoids (Fig. 2.3b) with BSA was observed at high concentrations. According to Siebert and Lynn (1998), flavonoid type polyphenols can be considered as 'double-ended' compared to the tannins as 'multiple-ended'. These authors showed that these 'double-ended' polyphenols have low ability to complex PRP because they cannot cross-link proteins to form haze. These authors also suggested that low molecular weight polyphenols such as the flavonoids have low affinity to complex with protein.

With increase in tannic acid concentration, an initial increase in haze followed by a plateau was observed for both kafirin and BSA (Fig. 2.2d). Tannic acid gave more haze with BSA than kafirin. A maximum of about 20% of the added tannic acid was bound to kafirin initially, followed by a decrease to about 5% when the amount of added tannic acid was increased from about 100 to 750 μg (Fig. 2.3c). In contrast, about 80% of tannic acid was bound to BSA at all concentrations (Fig. 2.3c). This firstly indicates the low binding affinity of kafirin to tannic acid compared with BSA. Secondly, it seems that the proportion of tannic acid bound to kafirin stayed the same irrespective of the amount added.

For both proteins, an increase in haze was observed with increasing concentration of sorghum condensed tannins (crude phenolic extract from tannin sorghum) (Fig. 2.2e). With sorghum condensed tannins BSA gave more haze than kafirin. At high concentration (>900 μg) of sorghum condensed tannins, the haze did not increase suggesting some saturation. About 30-40% of sorghum condensed tannins were bound to kafirin, but about 70% was bound to BSA (Fig. 2.3d).

According to Bamforth (1999), haze can be reversible or irreversible. Chill haze is reversible and is soluble when the liquid is returned to room temperature. Irreversible haze is a permanent haze and is not soluble when the liquid is returned to room temperature. Table 2.1 shows the effect on haze of kafirin incubated with sorghum condensed tannins at 4 and 30 ^{0}C. As expected, when

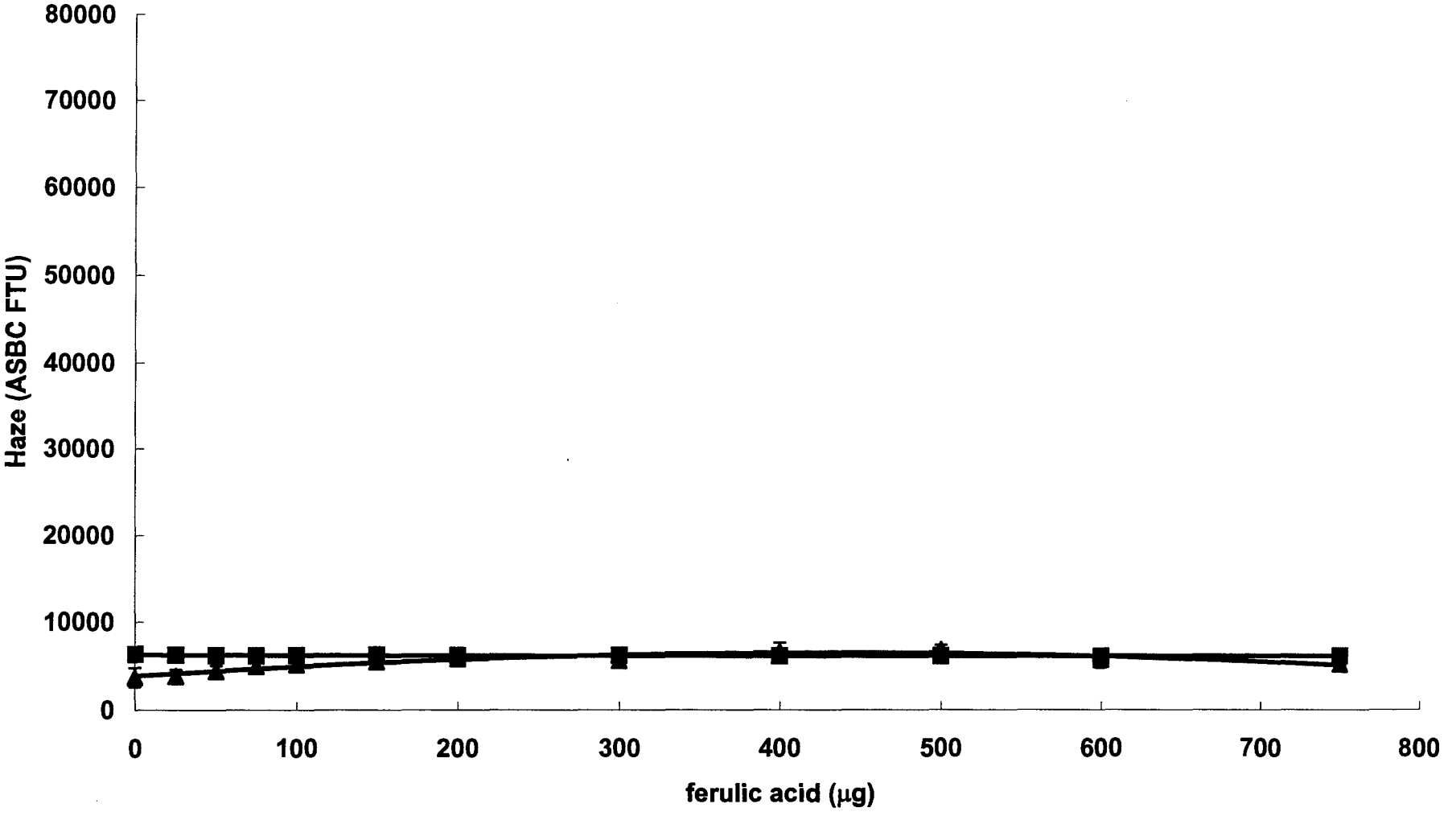
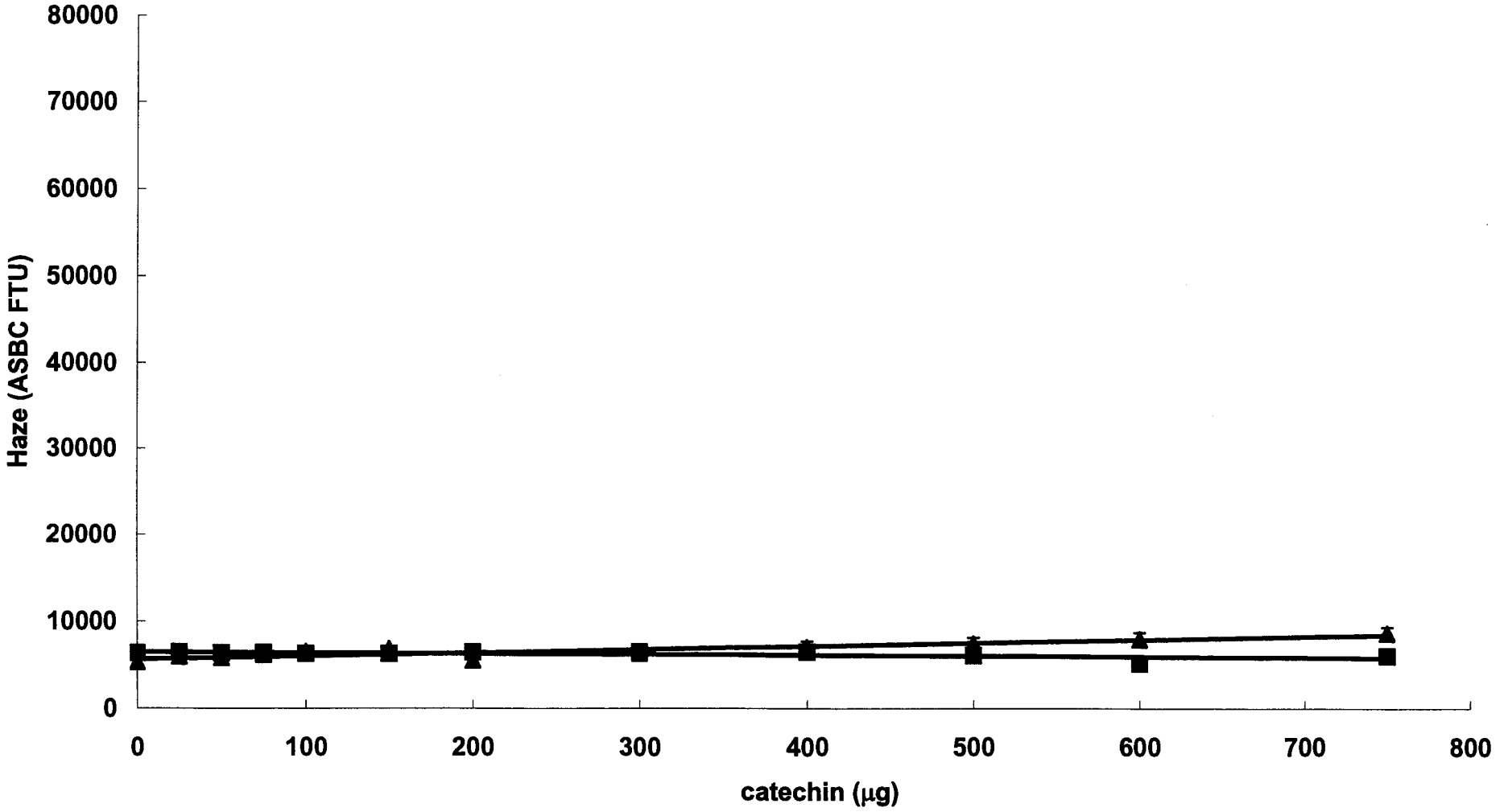


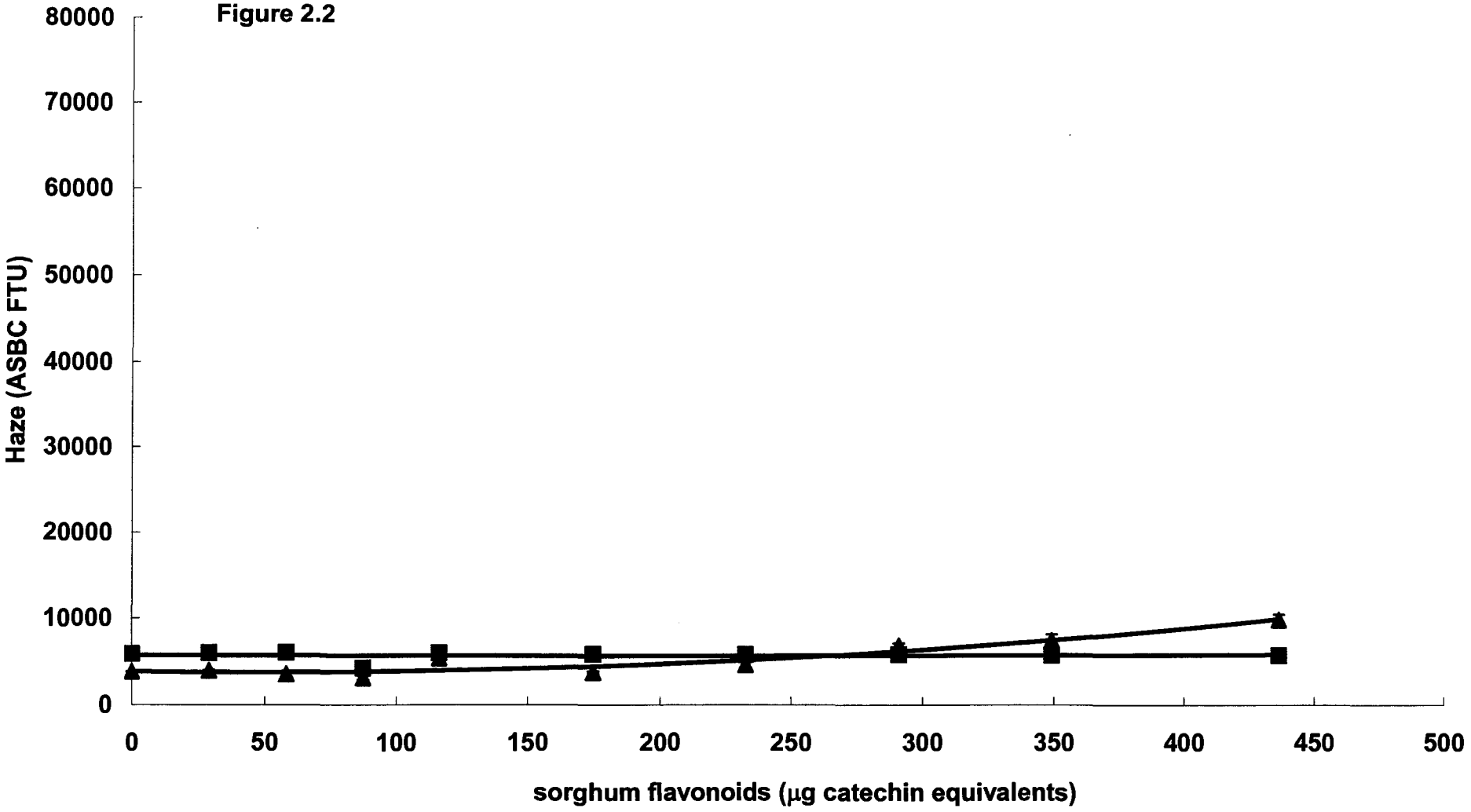
Figure 2.2 Haze formation of kafirin (■) and bovine serum albumin (▲) as affected by phenolic compounds
Error bars show ± one standard deviation of the corresponding mean
a) Ferulic acid

Figure 2.2



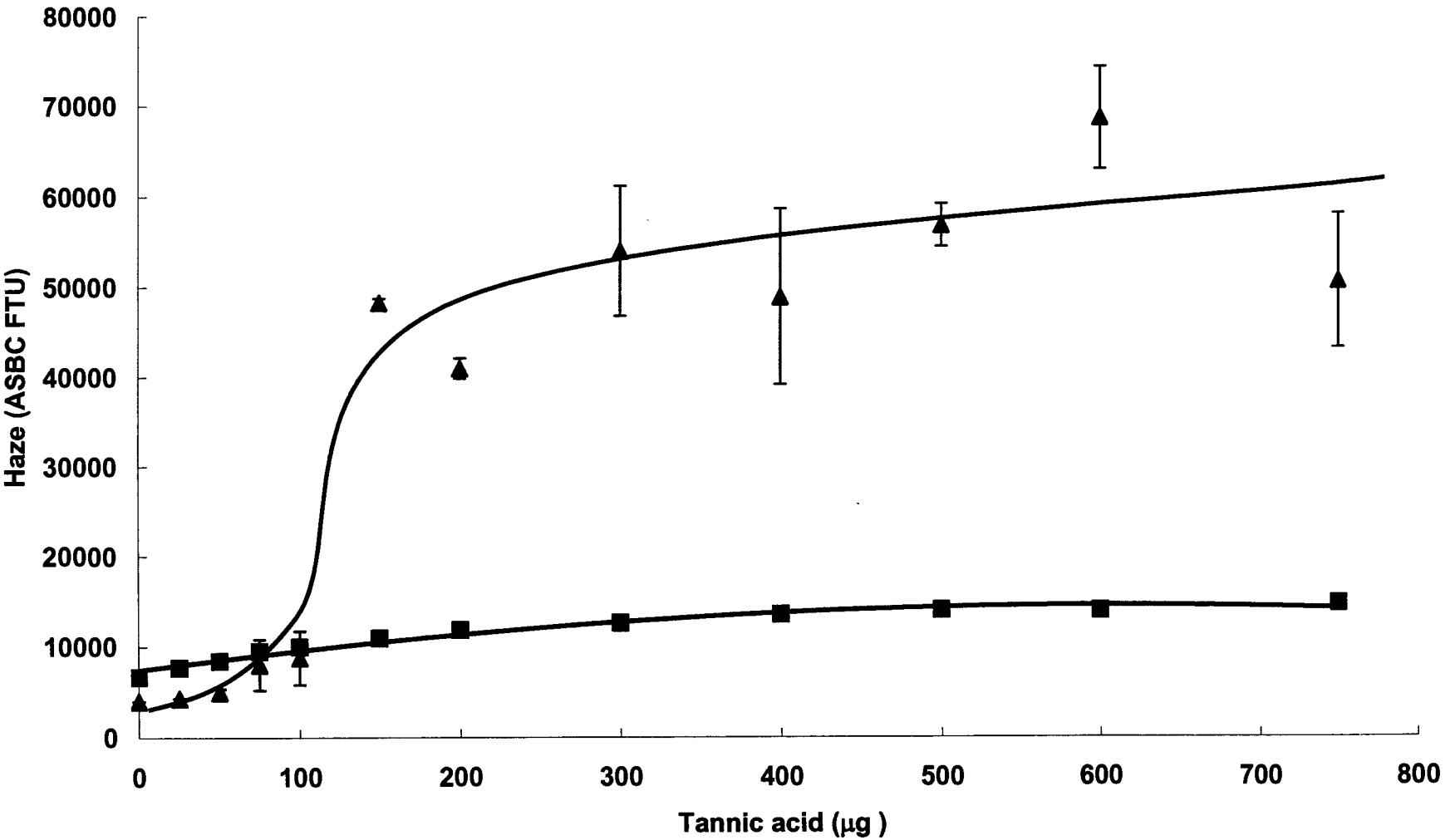
b) Catechin

Figure 2.2



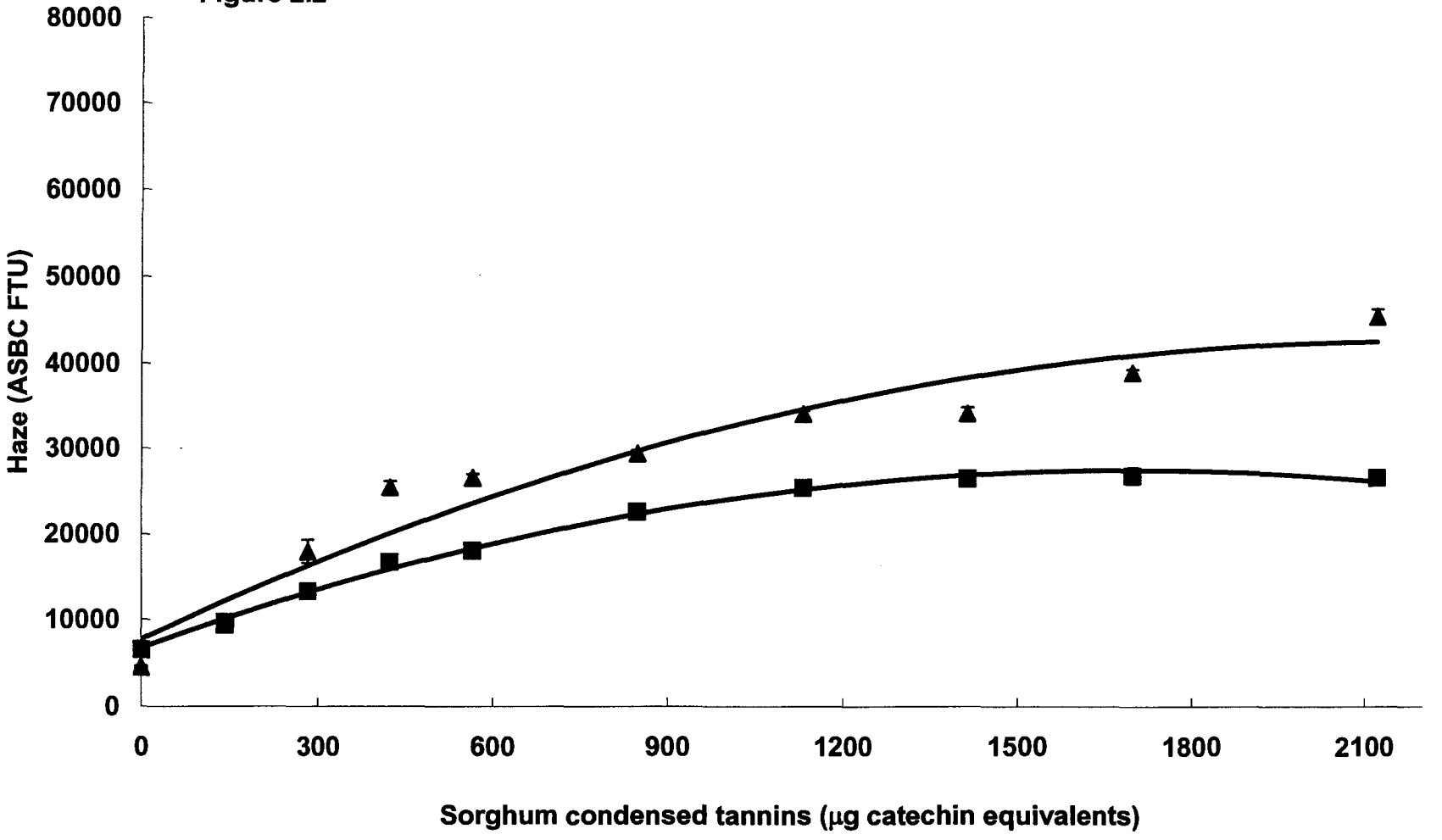
c) Sorghum flavonoids

Figure 2.2



d) Tannic acid

Figure 2.2



e) Sorghum condensed tannin

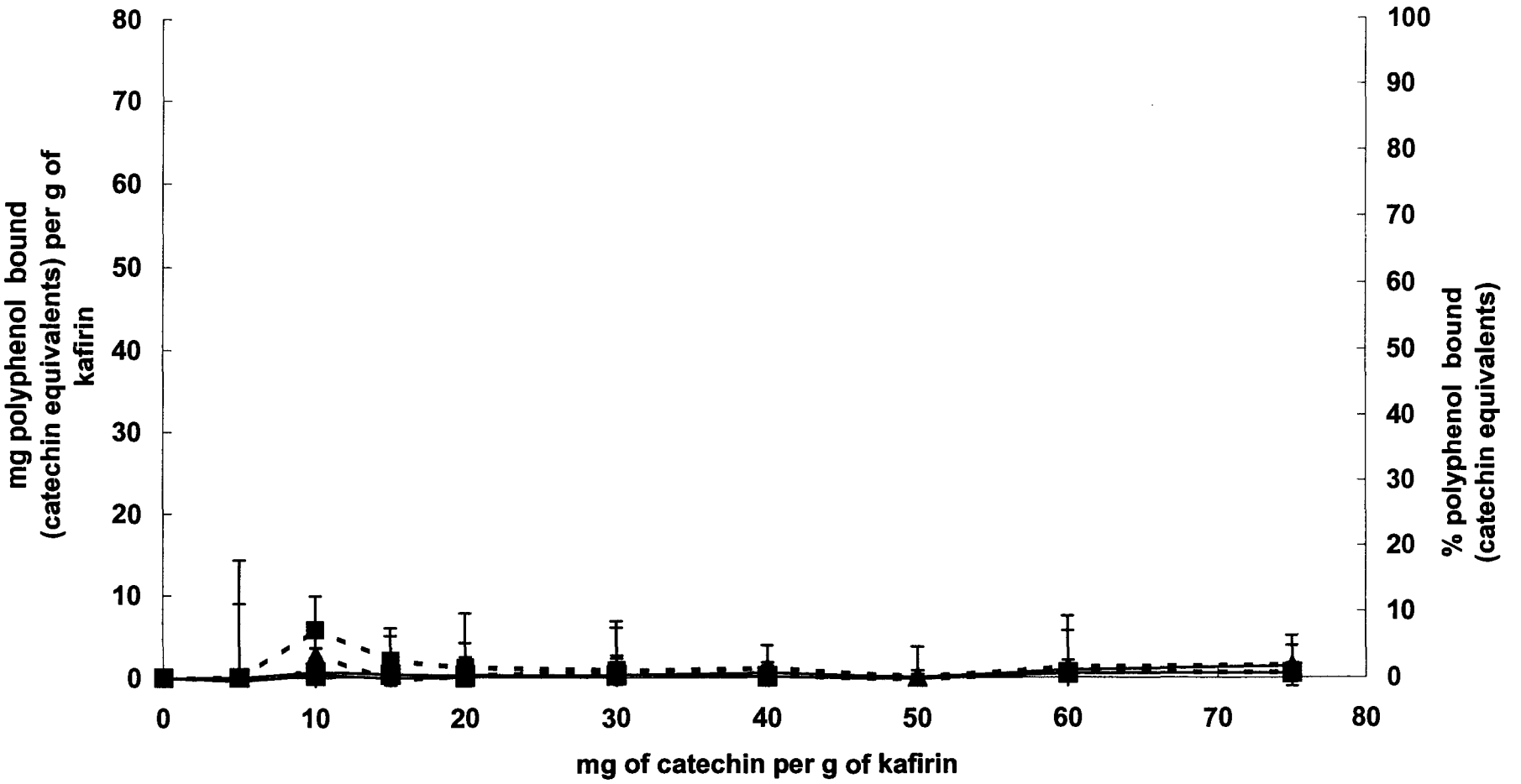
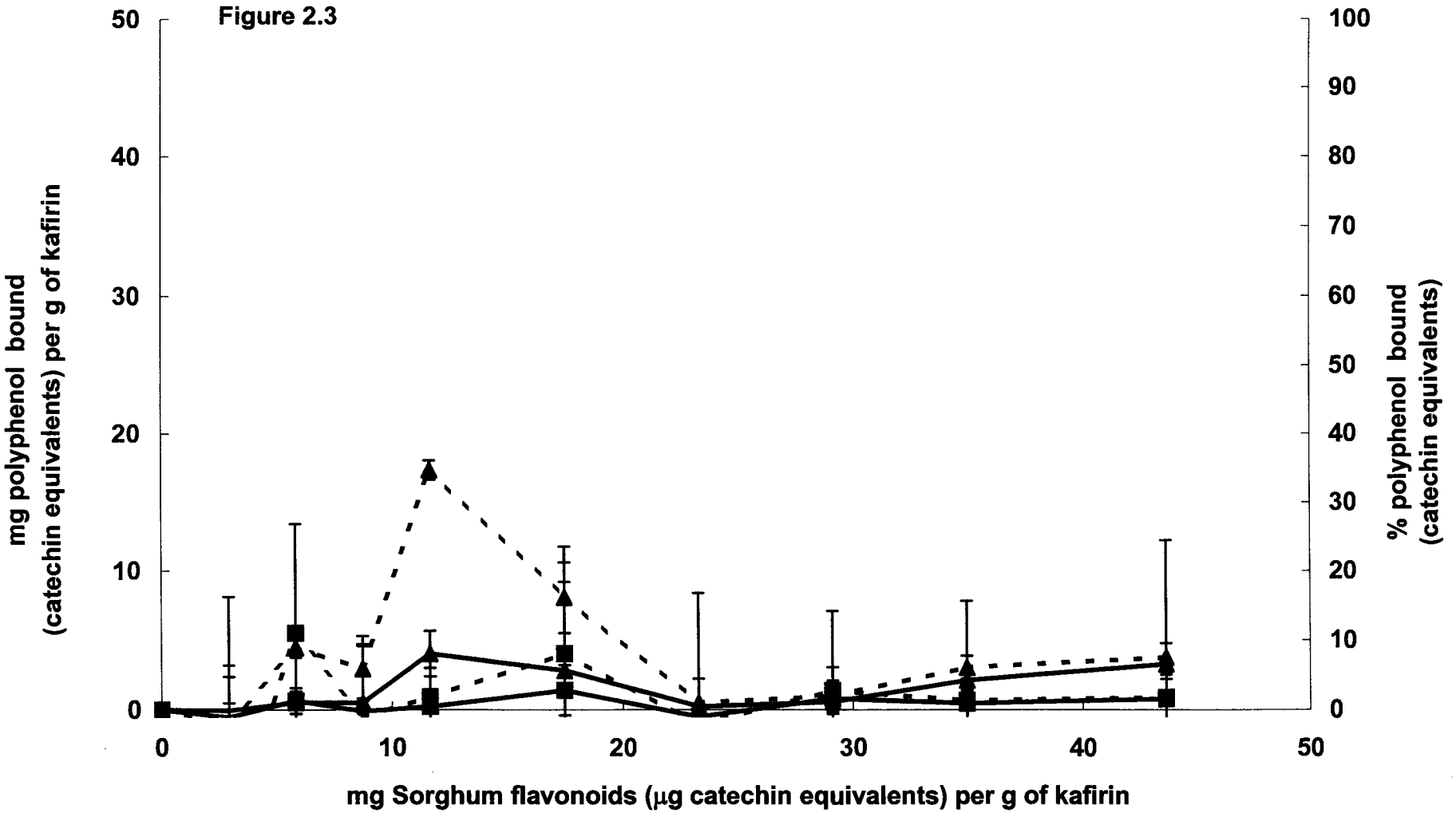


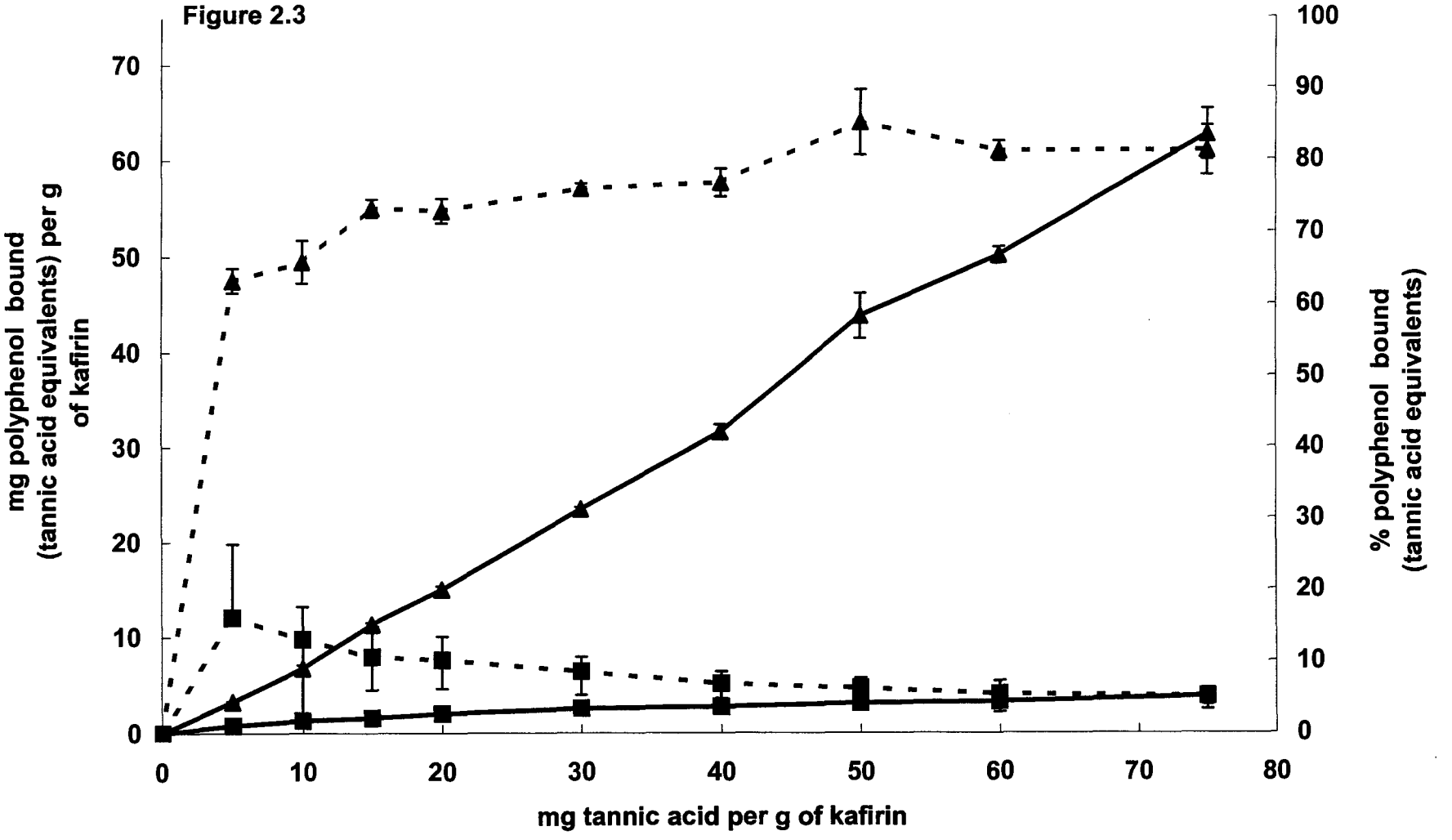
Figure 2.3 Quantity (——) and percentage (------) phenolic compounds bound to kafirin (■) and bovine serum albumin (▲)
Error bars show ± one standard deviation of the corresponding mean
a) Catechin

Figure 2.3

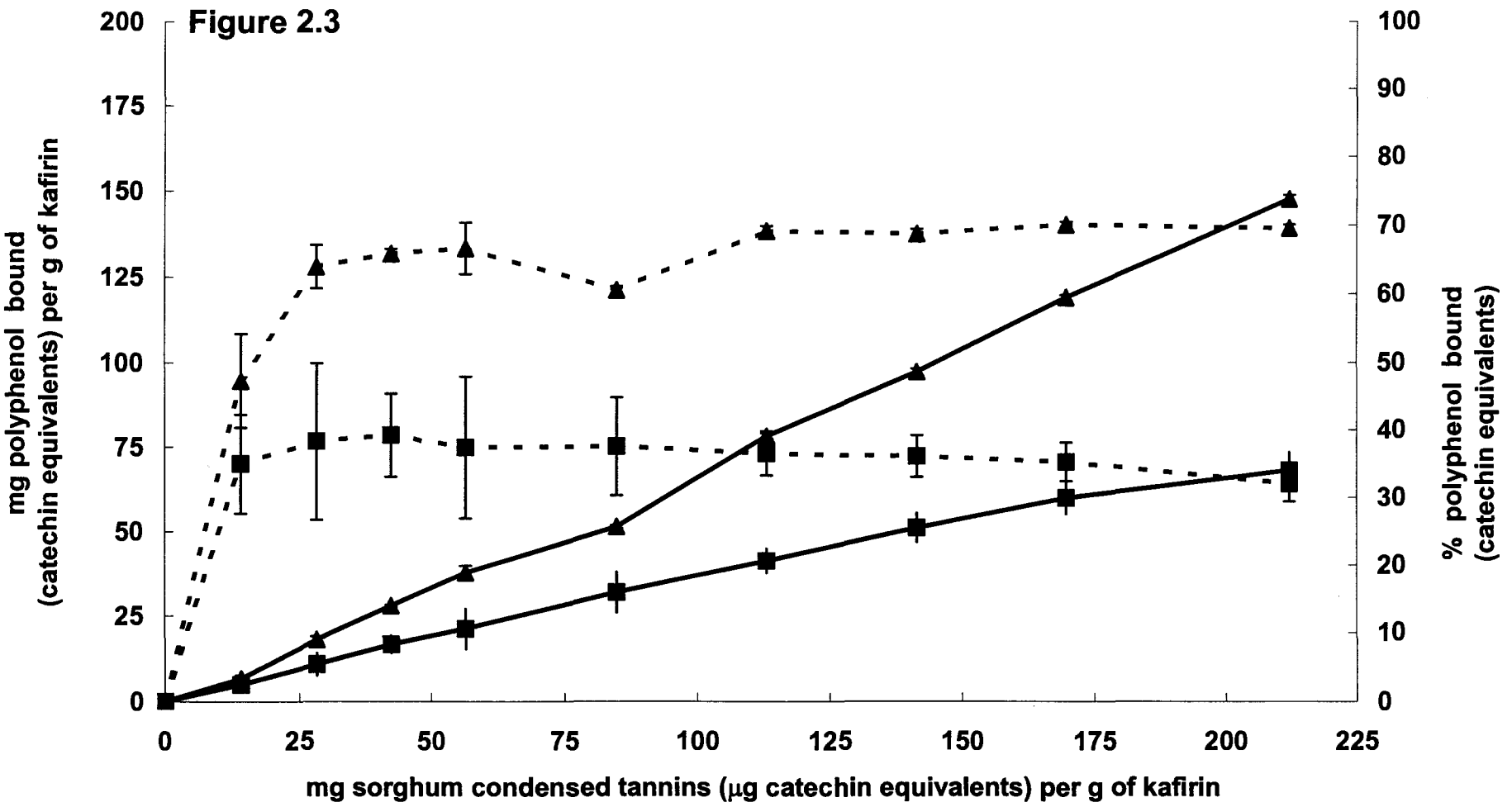


b) Sorghum flavonoids

Figure 2.3



c) Tannic acid



d) Sorghum condensed tannin

the polyphenol concentration increased from 707 to 1414 μg, haze formation increased. However, there was no significant decrease between the incubation temperatures from 4 ^{0}C to 30 ^{0}C. This shows that most of the light scattering was due to permanent haze and that the reaction between kafirin and sorghum condensed tannins was irreversible. It therefore indicates that the interaction between sorghum condensed tannins and kafirin is strong.

Table 2.1 Haze of kafirin protein as affected by sorghum condensed tannins at 4 ^{0}C and 30 ^{0}C

Sorghum condensed tannins	Haze[1] (ASBC FTU)	
(μg catechin equivalents)	4 °C	30 °C
707	19540 (±735) a	17813 (±456) a
1414	26370 (±1315) a	24920 (±1393) a

[1] Each value represents the mean of three replicates with standard deviation in brackets

Different letters show significant differences at $P<0.05$ in the same row

Besides kafirin, other prolamins such as barley hordein have been shown to interact with proanthocyanidins (Asano et al., 1982). Similarly, gliadin, the wheat prolamin can interact with commercial tannic acid to form haze (Siebert et al., 1996). Prolamins are rich in proline. PRP from saliva can be complexed by pentagalloyl glucose and by procyanidin dimer because they can form hydrophobic interactions between the aromatic ring and the pyrrolidine ring of proline (Baxter et al., 1997). In addition, hydrogen bonds between the hydroxyl groups of the tannins and the carbonyl groups of the protein peptide bonds are believed to stabilise the PRP-polyphenol complex (Murray et al., 1994).

It seems that sorghum condensed tannins had a higher affinity than tannic acid to complex kafirin. About 5 to 20% (μg bound to μg added) (Fig. 2.3c) of tannic acid was bound to kafirin in comparison to 30 to 40% (μg bound to μg added) of sorghum condensed tannins (Fig. 2.3d). This finding is in agreement with Baxter

et al. (1997) who showed that procyanidin dimer (condensed tannin) interacted more strongly with salivary PRP than pentagalloyl glucose (hydrolysable tannin). The mode of interaction was hydrophobic. Lu and Bennick (1998) also found that crude Quebracho condensed tannin had a higher binding affinity than commercial tannic acid to complex salivary PRP. The difference may be because of the higher molecular weight of sorghum condensed tannins compared with tannic acid. Tannic acid is a hydrolysable tannin, known as a gallotannin. According to Hagerman (2002), commercial tannic acid is comprised of mixtures of gallotannins from sumac (*Rhus semialata*) galls (Chinese gallotannin); Aleppo oak (*Quercus infectoria*) galls (Turkish gallotannin); or sumac (*R. coriaria*, *R. typhina*) leaves (sumac gallotannin). Chemically, commercial tannic acid is made up of heterogeneous and variable mixtures of galloyl esters ranging from 0.8 to 1.9 k (Pasch and Pizzi, 2002). The molecular weight of sorghum condensed tannins has been given as 4.9 k (Hagerman et al., 1998) and 10 to 50 k (McGrath and Smith, 1990).

The tannins (tannic acid and sorghum condensed tannins) had a higher affinity for BSA than kafirin. The difference may not lie in the proline content as kafirin has higher proline content than BSA, on average 9.6 g100 g^{-1} (Taylor and Schüssler, 1986) versus calculated 4.7 g100 g^{-1} for BSA (Anonymous, 2002b). However, molecular weight and structure may play a role. Hagerman and Butler (1993) showed that sorghum proanthocyanidins had a higher affinity for high molecular weight proteins. BSA is a higher molecular weight protein than kafirin. BSA has a molecular weight about 69 k (Anonymous, 2002b), whereas kafirin comprises several polypeptides in the range of 23 and 25 k, 20 k, and 28 k for α, β and γ- kafirin, respectively (Shull et al., 1991). The maize prolamin, zein, is thought to have a compact globular structure (Argos et al., 1982). It is probable that kafirin has the same structure because of its great homology with zein (DeRose et al., 1989). Hagerman and Butler (1993) also suggested that compactly structured globular proteins such as lysozyme and myoglobin have lower affinity for condensed tannin in comparison to an open globular protein like BSA. Thus, it can be inferred that BSA in the binding assay had a more

open globular structure than kafirin and the proline sites were more exposed for complexation by the tannins.

The haze values (Fig. 2.2d and 2.2e) for both kafirin and BSA as affected by the tannins (tannic acid and sorghum condensed tannins) suggested saturation, but the amount of the tannins bound (Fig. 2.3c and 2d) did not show saturation except in the case of kafirin and tannic acid complexes. An attempt was made to find the mole ratio of proline bound by the tannins. Proline is the major amino (imino in this case) acid reported to be bound by polyphenols such as procyanidin dimmer (Baxter et al., 1997; Murray et al, 1994) and pentagalloyl glucose (Murray, 1994). It was assumed that 1 mole of proline is bound by 1 mole of tannic acid (M_w 1701) and 1 mole of catechin (M_w 270.3) as an indication for sorghum condensed tannins. The data (Table 2.2) showed that in both proteins, kafirin and BSA, prolines were not saturated by the tannins, except possibly at concentration of greater than 1000 μg of sorghum condensed tannin for BSA. If it was assumed that 2 or 3 moles of proline are bound to 1 mole of the tannins, the ratios in Table 2 will double or triple, again suggesting no saturation of the proline binding sites. Thus, the difference in possible saturation of the haze values and the linear increase in the amount tannins bound with increase in added tannins suggests that not all the prolines are available to be bound by the polyphenols. This difference also suggests that there may be tannin-tannin interactions that will not lead to an increase in haze, but will increase the amount of tannin bound to the proteins. In fact Baxter et al. (1997) showed that procyanidin dimer and pentagalloyl glucose can self associate or stack when bound to PRP.

Table 2. 2 Mole ratio of proline[a] from kafirin and bovine serum albumin bound by tannic acid or sorghum condensed tannin as determined from the total polyphenol binding assay.

Tannic acid			Sorghum condensed tannins		
Quantity (μg tannic acid)	Mole ratio of proline to polyphenol bound		Quantity (μg catechin equivalent)	Mole ratio of proline to polyphenol bound	
	Sorghum	BSA		Sorghum	BSA
50	1669	211	67	46.3	17.6
100	1028	101	181	21.1	6.5
150	844	61	279	13.7	4.2
200	659	46	377	10.8	3.1
300	523	29	514	7.2	2.3
400	487	22	782	5.5	1.5
500	429	16	972	4.5	1.2
600	412	14	1188	3.8	1.0
750	350	11	1475	3.4	0.8

[a] the amounts of calculated proline in sorghum and BSA (Anonymous 2002b) were 7.9 and 4.0 μMole respectively in the binding assay and it was assumed that 1 mole of proline will be bound by 1 mole of tannic acid (M_w 1701) and by 1 mole of catechin (M_w 270.3) for sorghum condensed tannin.

2.1.5 Conclusions

Various phenolic compounds interact differently with sorghum kafirin. Phenolic acids and flavonoid type phenolics do not complex with kafirin to form haze, whereas sorghum condensed tannins and tannic acid have the ability to complex kafirin. As sorghum condensed tannins can complex kafirin, this suggests that this complexation may be involved in the decrease in protein digestibility of tannin sorghum and in the formation of haze in lager beer made from tannin sorghum. In contrast, it suggests that in condensed tannin-free sorghum, the endogenous phenolic compounds such as flavonoids and

phenolic acid may not play a significant role in the observed decrease in protein digestibility when such sorghums are wet cooked (Duodu et al., 2001).

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2.2 Sorghum kafirin film property modification with hydrolysable and condensed tannins

2.2.1 Abstract

Films made from kafirin, the prolamin protein of sorghum, could be an environmentally-friendly alternative to synthetic plastic packaging films. However, because protein-based films have inferior functional properties to synthetic plastic packaging. Tannic acid (TA) and sorghum condensed tannins (SCT) were added up to 20% (w/w to protein basis) as modifying agents during kafirin film casting. Both TA and SCT were bound to kafirin protein in the film. Scanning electron microscopy showed that TA modified films were less porous; and the SCT modified film appeared more globular in structure than unmodified films. Modification with both tannins at increasing levels resulted in an increase in tensile stress and Young's modulus by two fold and four fold, respectively but a decrease in% strain by three fold and water absorbed by 12 to 15%. Modification with TA and SCT did not change the apparent water vapour permeability. However, a significant quadratic decrease was observed for oxygen permeability. The glass transition temperature (T_g) of the films showed a quadratic increase with increase in TA and SCT level. These findings indicate that TA and SCT can modify the properties of kafirin films. This is probably by decreasing free volume in the film and possibly by decreasing chain mobility between kafirin polypeptides by cross-linking.

Key Words: sorghum, kafirin, film, tannin, plastic, tensile properties, oxygen permeability

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2.2.2 *Introduction*

Protein can be used as a biopolymer for food packaging and coating that can be both biodegradable and edible. Since synthetic plastic packaging is made from non-renewable resources and can be a hazard to the environment, protein biopolymers are thus an attractive alternative. Numerous articles have shown the potential of maize zein (Adyt et al., 1991; Park et al., 1994; Santosa and Padua, 2000; Yoshino et al., 2002), wheat gluten (Adyt et al., 1991; Gontard et al., 1992), soy protein (Bradenburg et al., 1993; Cunningham et al., 2000), sunflower protein (Orliac et al., 2002) and whey protein (Anker et al., 1998; Tien et al., 2000) as coatings and films. Zein, the prolamin protein of maize, has received considerable attention as it does not have religious concerns compared to animal proteins and is not considered to be allergenic (Skerritt, 1988). Kafirin, the prolamin protein of sorghum is extensively homologous to zein (DeRose et al., 1989). Films produced from kafirin have similar tensile and water vapour barrier properties to films from zein (Buffo et al., 1997). Kafirin is believed to be more hydrophobic than zein (Belton et al., 1997) and is less easily digestible than zein when it is wet heat processed (Duodu et al., 2002). Thus kafirin appears to have considerable potential as a biopolymer for food coatings and films.

Although research and development in edible and biodegradable coatings and films has intensified in recent years, only a few of them are used commercially as packaging systems because of their limitations in performance compared to synthetic plastic packaging. Chemical (Were et al., 1999), physical (Cunningham et al., 2000), enzymatic (Larre et al., 2000a) and plasticization (Santosa and Padua, 2000) modifications have been investigated to improve the mechanical performance and barrier properties of protein films. Orliac et al. (2002) found that plant tannins from tara and chestnut can be used as agents to improve the mechanical properties for sunflower protein isolate films. It is known that sorghum grain protein can be complexed by tannins. This complexation is responsible for the low protein digestibility of high tannin sorghum grain (Butler et al., 1984). Moreover, by *in vitro* binding assay it was found that tannic acid and sorghum condensed tannin have the ability to complex kafirin (section 2.1).

Thus tannins may have the potential to modify the functional properties of kafirin biofilms.

The present study describes the modification of kafirin films with hydrolysable and condensed tannins.

2.2.3 Experimental

2.2.3.1 Materials

Kafirin was extracted from a mixture of two condensed tannin-free white cultivars PANNAR PEX 202 & 606 using the fundamental (section 2.1) from the Carter and Reck patented method (Carter and Reck, 1970). Commercial tannic acid (TA) (Merck, Darmstadt, Germany), and extracted and air-dried sorghum condensed tannin (SCT) from tannin (high tannin) red sorghum (Nola GH 91) as described in section 2.1 were used as modification agents.

2.2.3.2 Film preparation

Films were produced using the casting method. Kafirin powder was weighed into a conical flask. Then a combination of plasticizers (1 glycerol: 1 polyethylene glycol: 1 lactic acid), followed by the tannins and ethanol (70% w/w) were added. The protein concentration was 16% (w/w) with respect to the aqueous ethanol. The plasticizers were at a concentration of 40% (w/w) with respect to protein, and the tannins (TA and SCT) were at 0 (control), 5, 10, 15 and 20% (w/w) with respect to protein. The flask was heated at 65-70 °C on a hot plate for 10 min with vigorous stirring. After protein and tannin solubilisation, the flask was placed in a shaking (100 rpm) water bath at 55 °C for 40 min to promote interaction between the kafirin and the tannins. Aliquots (4 g) of the solution were then poured into plastic petri dishes (9 cm diam) and gently swirled to evenly cover the bottom of the petri dishes. They were then placed on a level surface in a ventilated oven (not forced-draft) at 50 °C for 4 h to evaporate off the solvent and form the film. After careful removal of the films from the petri dishes, they were conditioned at 25 °C in a desiccator containing

saturated calcium nitrate solution to give a relative humidity (RH) of 50% for at least 48 h before analysis.

2.2.3.3 Analyses

2.2.3.3.1 Tannin bound by kafirin

Kafirin film (approximately 0.1 g accurately weighed) was suspended in a test tube with 2 ml 70% ethanol (w/w) in distilled water and shaken for 1 h at 25 °C to dissolve the unbound tannins. The tubes were then centrifuged at 2500 rpm (800 g) for 10 min. After which, the supernatant was decanted off to remove the unbound tannins. The modified International Organization for Standardization tannin method (International Organization for Standardization, 1988), as described in section 2.1, was used to quantify the tannin bound to the protein in the kafirin films. The results are expressed as tannic acid equivalents for the TA and catechin equivalents for the SCT.

2.2.3.3.2 Scanning electron microscopy (SEM)

Pieces (approx 10 mm^2) of the unmodified and modified kafirin films were freeze-fractured in liquid nitrogen at –80 °C. Films were mounted on aluminium stubs using double sided carbon tape, with the fracture surface exposed at the top. The freeze fracture surfaces of the films were then coated with chromium and viewed by SEM at 5 kV.

2.2.3.3.3 Tensile properties of films

These were determined essentially according to ASTM D 882-97 (American Society for Testing and Materials, 1988) using a Stable Micro Systems TA-TX2 Texture Analyser (Godalming, UK) fitted with a tensile rig grip. Strips of film (60 mm long and 6 mm wide) were cut with a scalpel, measured for thickness with a micrometer at 4 different points and then mounted between the tensile grips (40 mm apart). The films were subjected to tension with a crosshead speed of 0.4 mm sec^{-1}. The force (stress) and the elongation (strain) were recorded. The results were expressed as stress at maximum force (σ_y), stress at break (σ_b),

strain at break (ε_b), and Young's modulus (E) for the linear region of the stress-strain graph.

2.2.3.3.4 Water uptake by films

Kafirin film (approx. 0.5 g accurately weighed) was placed in a bottle containing 20 ml distilled water and incubated in a shaking (100 rpm) water bath at 25 °C for 24 h. The film was removed and the surfaces were gently blotted with filter paper to remove surface water. Water uptake by the film was then determined by weight difference of the sample after drying in a forced-draft oven at 103 °C (±0.5) for 3 h. The initial water content of the films was also measured.

2.2.3.3.5 Barrier properties of films

Oxygen transmission was measured by ASTM D 1307-90 method (American Society for Testing and Materials, 1990) using a Mocon Oxtran 2/90 (Modern Controls, Minneapolis, USA) at 50% RH. A square portion of film (25 cm^2) was masked between two aluminium sheets with an opening of 5 cm^2. The thickness of the film was measured at 5 points over the circular surface and placed in the instrument. Oxygen permeability was read from the instrument and expressed as $cm^3\ \mu m\ m^{-2}\ d^{-1}\ kPa^{-1}$.

The apparent water vapour permeability (WVP) of the films was assessed using a modified ASTM E 96-95 method (American Society for Testing and Materials, 1995). A circle of film (40 mm diam.) was cut, measured for thickness with a micrometer at four points around the edge and in the centre. The films were then mounted on top of Schott bottles (100 ml) with the plastic screw top drilled accurately to provide a circular opening (33 mm diam.). The bottle was filled with distilled water to leave a gap of 22 mm between the film and water level. Then a fibre tap washer (31 mm internal diam.) was placed on each film and the bottle was closed with the modified screw top to create a water-tight seal. The bottles were placed in an air-ventilated incubator at 25 °C. Weight change of the bottles was recorded daily for at least 10 days. The RH of the incubator was monitored daily and the mean RH was taken as the RH of the incubator for

calculation. The apparent WVP was then calculated according to the method of Gennadios et al., (1994a), which includes the correction of the stagnant air gap resistance between water and film.

2.2.3.3.6 Dynamic mechanical analysis (DMA)

DMA of the films was performed under tension at different temperatures and RHs using a Rheometrics RSA-II (Rheometric Scientific, Piscataway, USA) basically as described by Stading et al. (2001). The temperature scans were performed from -10°C to 60°C at 5°C min^{-1}. The potential loss of volatile plasticizers and water was controlled by coating the film with a layer of hydrophobic grease. The temperature at which the glassy modulus changed to transition modulus was used as the glass transition temperature (T_g) of the film. Linear regression was applied to fit a line to the glassy modulus and to the transition modulus. T_g was determined by the intersection of the two linear lines. The effect of humidity was investigated by changing the RH in steps of 10% from 40% to 60% RH at a constant temperature of 25°C. Once storage modulus (E′) had reached steady-state, E′ was recorded and the RH was changed.

2.2.3.3.7 Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data to determine if there were significant ($P<0.05$) differences between the equally spaced treatments: 0 (control), 5, 10, 15 and 20% (w/w protein basis) modification with TA and SCT. The treatments were repeated at least three times, unless stated otherwise. Following a significant difference ($P<0.05$) from ANOVA, least significant difference was calculated between the treatment means, and orthogonal polynomial contrasts were performed on the treatment means to determine if increasing modification with TA and SCT yielded linear, quadratic or cubic responses (Steel and Torrie, 1984).

2.2.4 Results and discussion

Both TA and SCT bound to kafirin in the film (Fig. 2.4a and 2.4b, respectively). The amount of TA and SCT bound per gram kafirin protein showed a linear increase with increase in the quantity of tannins added. Only about 15-25% of the added TA was bound to kafirin compared to 55-70% for SCT. It was also found by determining haze and total polyphenols bound to the kafirin in an *in vitro* binding assay (section 2.1), that kafirin interacted with TA and SCT. The amount of tannin bound to the kafirin was similar to that reported here for the kafirin film.

The mechanism of kafirin-tannin binding is not known but it is probably of significance that kafirin is rich in proline, on average 9.6 g100 g^{-1} (Taylor and Schüssler, 1986). Hydrogen bonds between the hydroxyl groups of tannins and the protein carbonyl groups are believed to be involved in the complexation of the salivary proline rich protein (PRP) (Murray et al., 1994). PRP can also interact with pentagalloyl glucose (a hydrolysable tannin) and procyanidin dimer (a condensed tannin) by hydrophobic interactions with the pyrrolidine ring of proline (Baxter et al., 1997). According to Charlton et al. (2002) when PRP analogues are complexed by hydrolysable and condensed tannins, the tannins can firstly coat the protein polypeptides and act as bridges to cross-link the polypeptides to form aggregates or agglomerates of proteins.

Scanning electron micrographs of unmodified and modified kafirin films are shown in Figure 2.5. The lower part of the fracture surface (B, side in contact with the petri dish) and the upper part of fracture surface (T, side in contact with the atmosphere) of unmodified kafirin film had different structures (Fig. 2.5a(i)). The upper part of the fracture surface had vertical striations with a uniform structure, whereas there were horizontal striations with a coarse structure in the lower part. During film formation in the oven, a skin was observed to form at the surface in contact with the atmosphere. Moreover at this stage, the clear film casting solution became milky, indicating the possibility of protein precipitation or phase separation of protein, solvent and plasticizers. The skin may correspond to the upper part of the films with the uniform structure. The

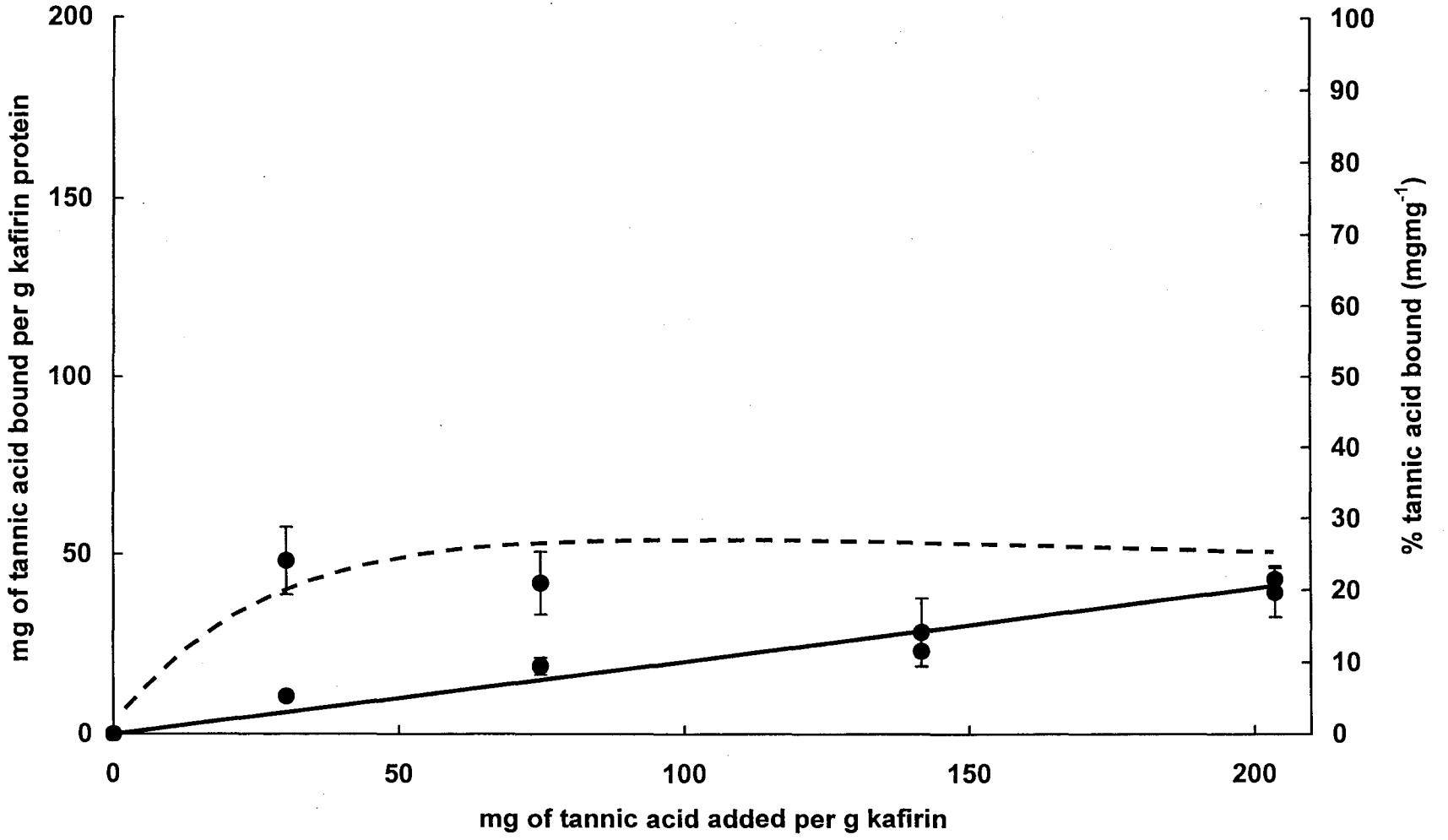
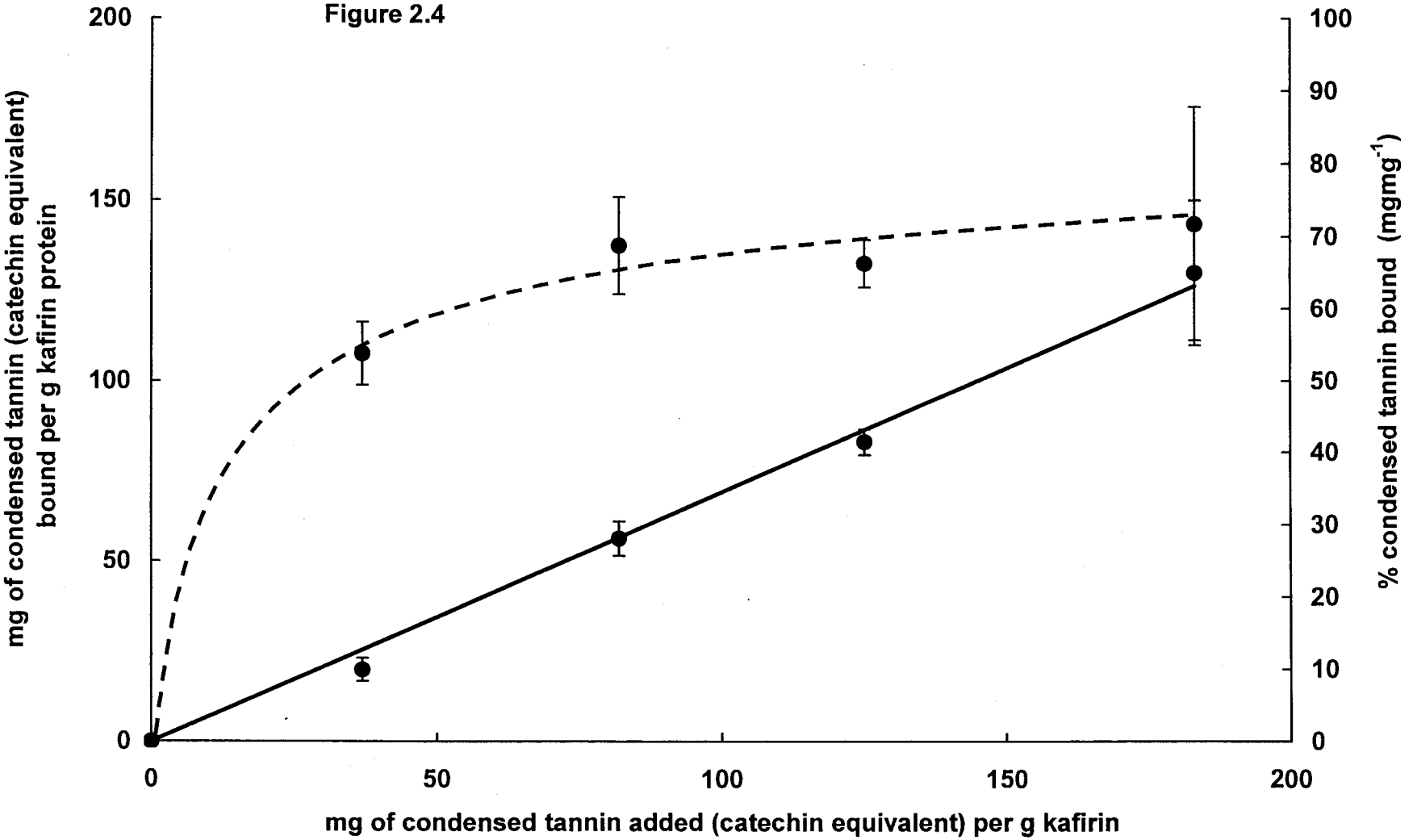


Figure 2.4 Quantity (——) and percentage (-----) of tannins bound to kafirin film
Error bars show ± one standard deviation of the corresponding mean.
a) Tannic acid

Figure 2.4



b) Sorghum condensed tannin

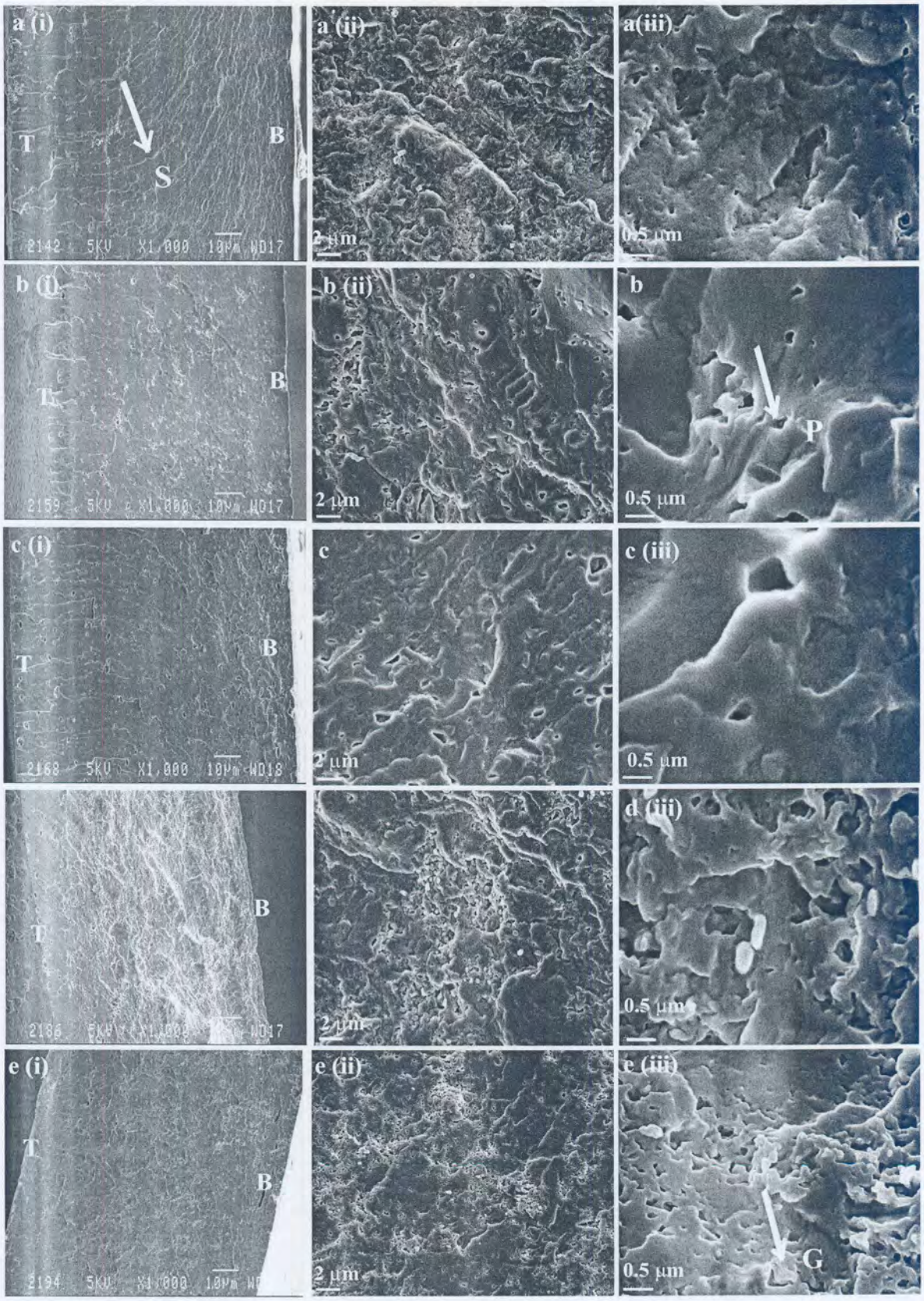


Figure 2.5 Scanning electron micrographs of freeze-fracture surfaces of unmodified and modified kafirin films.
(a) unmodified film, (b) modified film at 10 % tannic acid, (c) modified film at 20 % tannic acid, (d) modified film at 10 % sorghum condensed tannin, (e) modified film at 20 % sorghum condensed tannin. (I) whole fracture section ;(ii) and (iii) portion of the lower part (side in contact with the petri dish) of the fracture surfaces. T = upper part of the film fracture surface in contact with the atmosphere, B = lower part of the fracture surface in contact with the petri dish, S = striation, P = pores, G = globular like structures.

striations were more evident at high magnification (Fig. 2.5a(ii)). At highest magnification pores were observed (Fig. 2.5a(iii)). The vertical and horizontal striations as well as the pores are probably phase separated structures containing plasticizer and the remaining solvent. The striations and pores may be the result of plasticizer and solvent evaporation in the vacuum of the scanning electron microscope. This type of textural difference was also observed across a cross-section of soy-milk protein film (Okamoto, 1978). It was found that the upper part of the fracture surface was uniform, while the lower part had more pores. The formation of a skin at the upper part of the soy-milk protein during the initial stage of film formation was also observed. Film growth continued at the interface between the solid and liquid phases. Kafirin film modified with TA and SCT had different structures in the upper and lower layers (Fig. 2.5b(i), c(i), d(i) and e(i)) like the unmodified film. However, the lower part of the fracture surfaces (B) appeared different from the unmodified film. TA modification at 10 and 20% seemed to result in a less striated structure with larger and fewer pores (Fig. 2.5b (ii, iii) and 2.5c (ii, iii)). Modification with SCT resulted in a more striated structure, with globular type looking structures and larger pores (Fig. 2.5d (ii, iii) and 2.5e (ii, iii)). The globular type structures of the modified kafirin films with SCT at 20% suggest brittleness of the films. The above microstructural differences show that TA and SCT affect film formation.

The tensile properties of kafirin films, as affected by modification with TA and SCT, are shown in Figure 2.6. According to the American Society for Testing and Materials (1988) stress at maximum tensile force (σ_y) expresses the maximum force (the internal resistance to an external load) developed in a film during tensile test; strain at break (ε_b) is a representation of the film's ability to stretch and Young's modulus (E) is the ratio of stress to strain in the linear range of the stress-strain graph and measures the intrinsic stiffness of the film.

Mean σ_y, force at break (σ_b), ε_b and E of unmodified kafirin films were 2.4 MPa, 2.3 MPa, 109% and 40 MPa, respectively (Fig. 2.6a, b, c and d respectively). σ_y and ε_b of the films were similar to those reported by Buffo et al. (1997) for kafirin

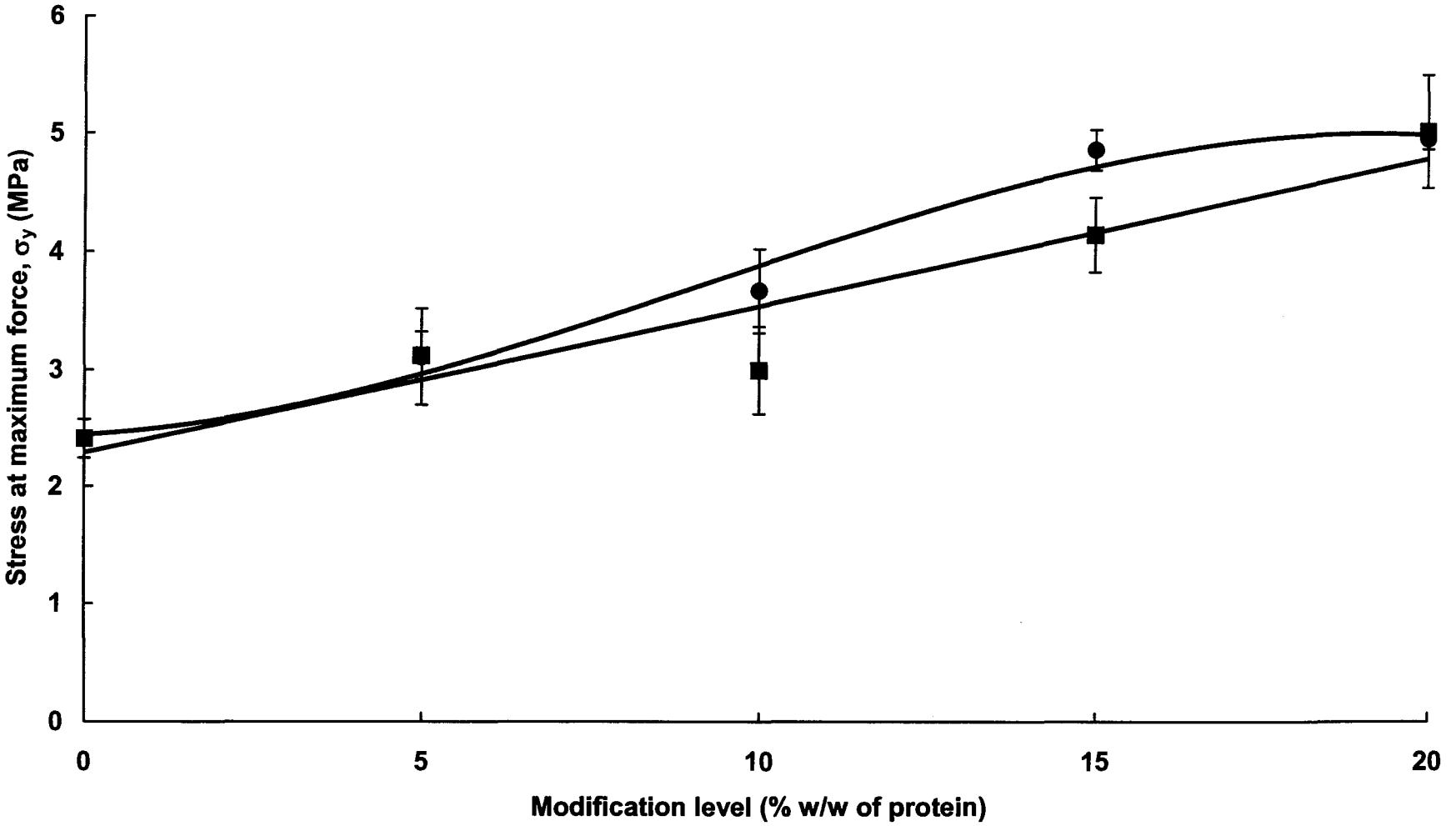
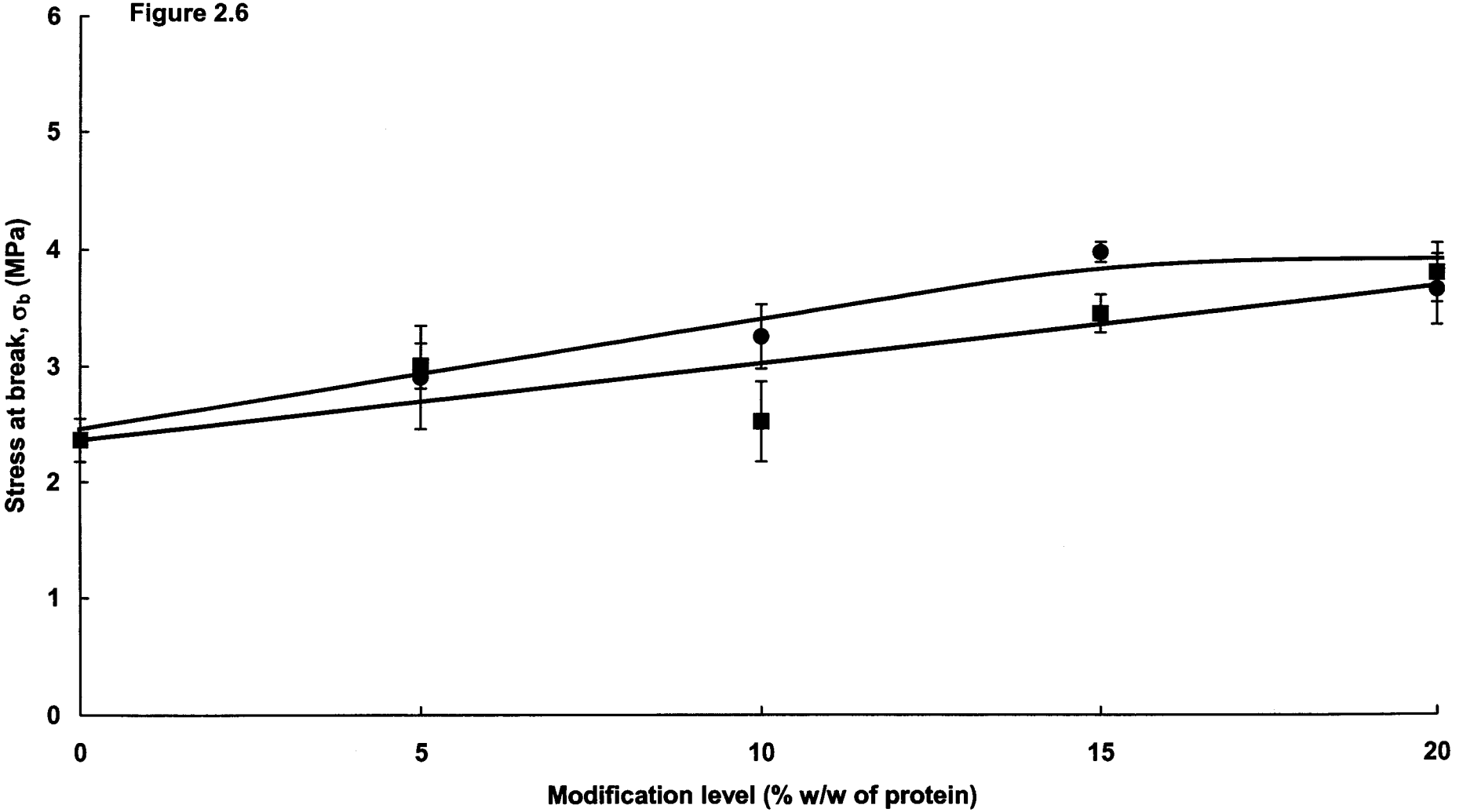


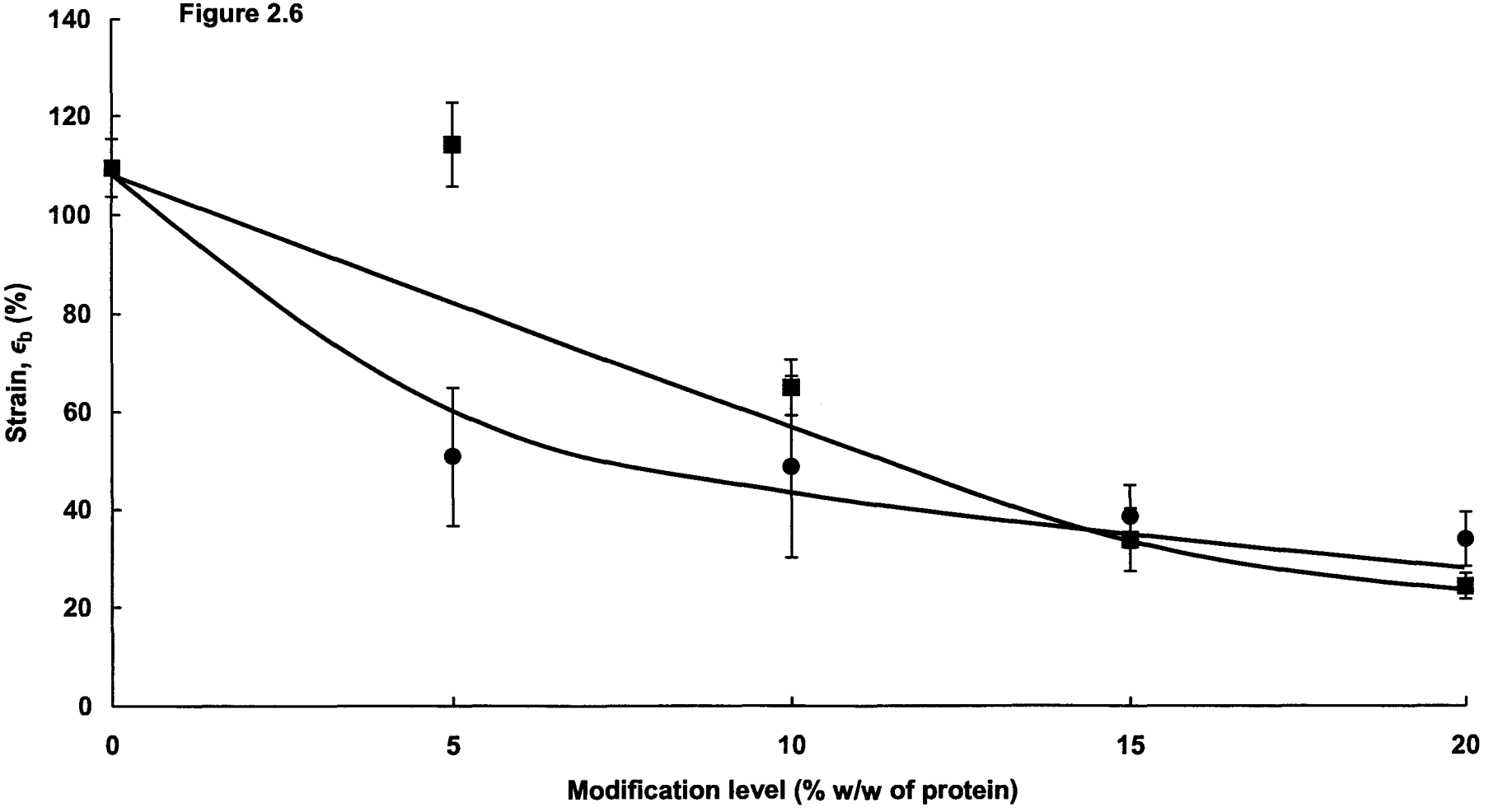
Figure 2.6 Effect of tannic acid (■) and sorghum condensed tannin (●) tensile properties of kafirin films Error bars show ± one standard deviation of the corresponding mean.
a) Stress at maximum force

Figure 2.6



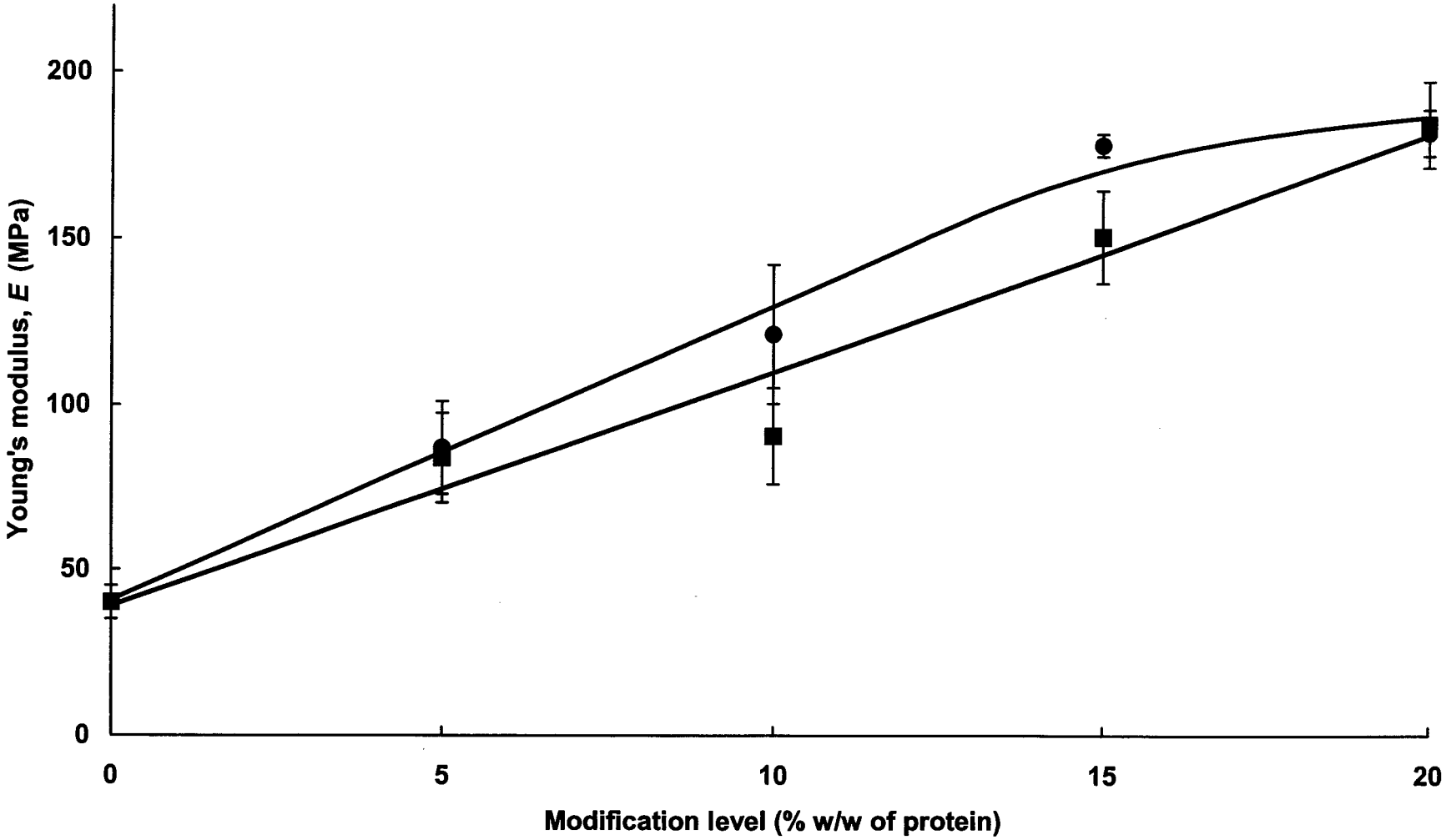
b) Stress at break

Figure 2.6



c) Strain at break

Figure 2.6



d) Young's Modulus

films. Modification of the kafirin films resulted in a significant linear increase in σ_y, σ_b and *E* with increasing concentration of added TA and SCT from 0 to 20% (Fig. 2.6a, b, and d, respectively). With a 20% level of addition, σ_y of kafirin films increased from about 2.4 MPa to 5 MPa for both TA and SCT, a 2-fold increase (Fig. 2.6a). Similarly the σ_b (Fig. 2.6b) increased from 2.3 MPa to about 3.8 MPa when modified with TA and SCT at 20%. ε_b of the kafirin films showed a significant decrease when the films were modified with increasing levels of TA and SCT (Fig. 2.6c). At a 20% level of addition of TA and SCT, ε_b of kafirin films decreased by about 4-fold and 3-fold, respectively. *E* of the film when modified at 20% with TA and SCT increased more than 4-fold (Fig. 2.6d). Thus, kafirin films become stiffer but less plastic when modified with TA and SCT.

An increase in σ_y was also observed by Orliac et al. (2002) when sunflower protein films were modified with gallic acid, chestnut tannins and tara tannins. These authors examined different ways to modify sunflower protein films. They found that the stress at maximum force of glutaraldehyde modified films was higher than phenolic compound (gallic acid, chesnut and tara tannins) modified films. They suggested that the phenolic compounds might act through 'weak interaction', as discussed above, rather than covalent bonding as is the case with glutaraldehyde. Similarly, when glutaraldehyde was used to modify collagen protein (Heijman et al., 1997), the tensile strength of the collagen increased from 7.2 MPa to 18 MPa, but modification with TA increased the tensile strength to only about 12 MPa. TA modification of collagen has been referred to as cross-linking because it resulted an increase in the hydrothermal shrinkage temperature (Heijman et al., 1997). Other types of modification such as covalent cross-linking of zein films with glutaraldehyde (Parris et al., 1998), cross-linking gluten films with dialdehyde (Lens et al., 1999), ultra violet radiation induced cross-linking of zein films (Rhim et al., 1999) and cysteine added soy protein-wheat gluten films (Were et al., 1999) have also resulted in an increase in tensile stress of protein-based films.

The decrease in ε_b of kafirin films modified by TA and SCT indicates that the tannins adversely affected the plasticity of the films (Fig. 2.6c). A decrease in ε_b has also been observed when gallic acid, chestnut tannins and tara tannins were used to modify sunflower protein films (Orliac et al., 2002) and when glutaraldehyde as a covalent cross-linking agent was used to modify gelatin films (Bigi et al., 2001). Plasticizers such as glycerol and polyethylene glycol, which are used to plasticize zein and kafirin films (Buffo et al., 1997), contain polar hydroxyl groups. They are believed to develop hydrogen bonds with polymers, and replace polymer–polymer interactions in the films (Gennadios et al., 1993), enhancing film plasticity. Consequently, direct interactions between protein molecular chains can be reduced, leading to an increase in polypeptide mobility, producing an extensible film. Thus, the decrease in the ε_b of kafirin films modified with TA and SCT suggests antiplasticisation effects, probably because of tannin and kafirin interaction.

Water uptake and solubility are important properties to be considered if films are to be used in a high moisture environment. After 24 h in distilled water, the control films and the films modified with 5, 10 and 15% TA and SCT could be removed without breaking, but those modified with 20% TA and SCT broke into pieces as they were brittle, suggesting over modification. Thus, the water absorbed by kafirin films modified at 20% TA and SCT could not be determined. The unmodified kafirin films absorbed about 70% moisture, but there was a significant ($P<0.01$) decrease in water uptake down to about 45% when kafirin films were modified with 10% TA and to about 56% with SCT at 5% (Table 2.3). There was no significant change in water uptake with further level of modification. When gelatin films were stabilised by cross-linking with genipin (Bigi et al., 2002) (a dark blue pigment from gardenia fruit and chemically is an iridoid glycoside with formula $C_{17}H_{14}O_5$ (Ozaki et al., 2002), and sunflower protein films were modified with chestnut and tara tannins (Orliac et al., 2002), a decrease in water uptake was also observed. Similarly, when gluten films were modified with dialdehyde by covalent cross-linking, the swelling in water decreased from 100% to about 15% (Lens et al., 1999).

The mean oxygen permeability of unmodified kafirin films was 135 cm^3 μm m^{-2} d^{-1} kPa^{-1} (Fig. 2.7). Modification of the kafirin films with TA and SCT resulted in a significant decrease in their oxygen permeability. Oxygen permeability decreased quadratically when the TA and SCT modification level was increased from 0 to 20%. At 20% level of modification, the mean oxygen permeability of kafirin films modified with TA and SCT was 48 and 57 cm^3 μm m^{-2} d^{-1} kPa^{-1}, respectively. Barrier properties of biodegradable and edible films depend on molecular mobility and T_g of the material (Cherian et al., 1995). The gas permeability of a polymeric material is high above its T_g and low below its T_g.

Table 2. 3 Effect of tannic acid (TA) and sorghum condensed tannin (SCT) modification on the water absorbed (%)[a] by kafirin films

Treatments	Water absorbed (%)
Unmodified film	70.7 (±3.2) c
5% TA	55.8 (±4.5) b
10% TA	45.4 (±12.4) a
15% TA	52.0 (±7.5) ab
5% SCT	55.7 (±6.5) b
10% SCT	56.8 (±2.3) b
15% SCT	57.3 (±2.5) b

[a] mean of 4 replicates with standard deviations in brackets
Different letters within the column show significant difference at $P<0.05$

T_g of the kafirin films as measured by DMA is shown in Figure 2.8. The T_g was taken as the transition change from the amorphous (glassy) to the rubbery phase, as calculated from the storage modulus, E′. T_g of the plasticized kafirin film was 34.6 °C. Modification with TA and SCT resulted in a quadratic increase in T_g. T_g of films can be influenced by plasticizer level (Yang and Paulson, 2000), moisture content (Jin et al., 1984) and degree of modification (Schacht et al., 1997). In the present study, the plasticizer level was kept constant. The moisture content of kafirin films modified with TA and SCT did not show any significant difference to the control (Fig. 2.8), except at high level (20%) of modification with TA where there was a decrease. Therefore, it can be inferred

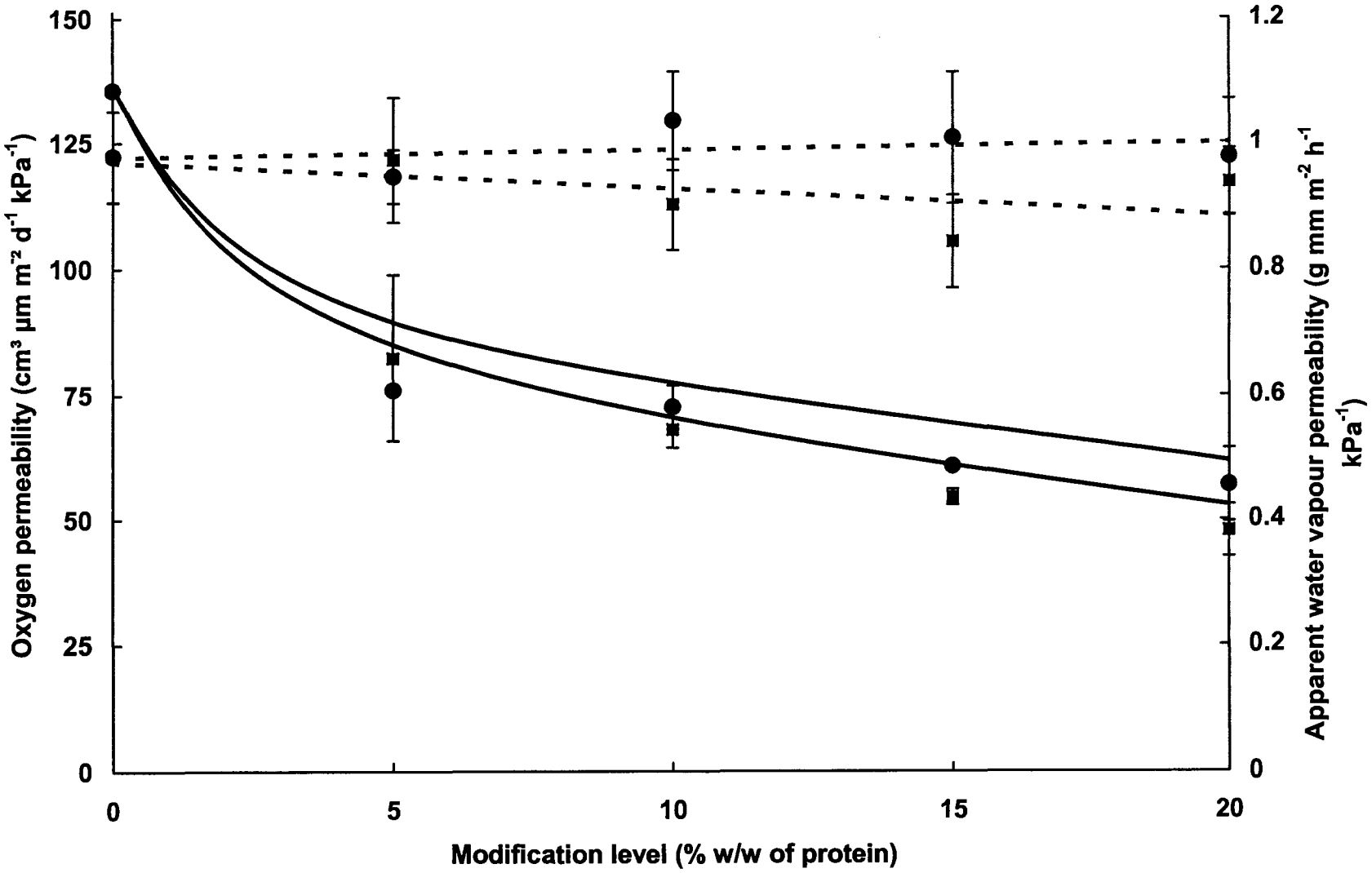


Figure 2.7 Effect of tannic acid (■) and sorghum-condensed tannin (●) on the oxygen permeability (——) and apparent water vapour permeability (-----) of kafirin films
Error bars show ± one standard deviation of the corresponding mean

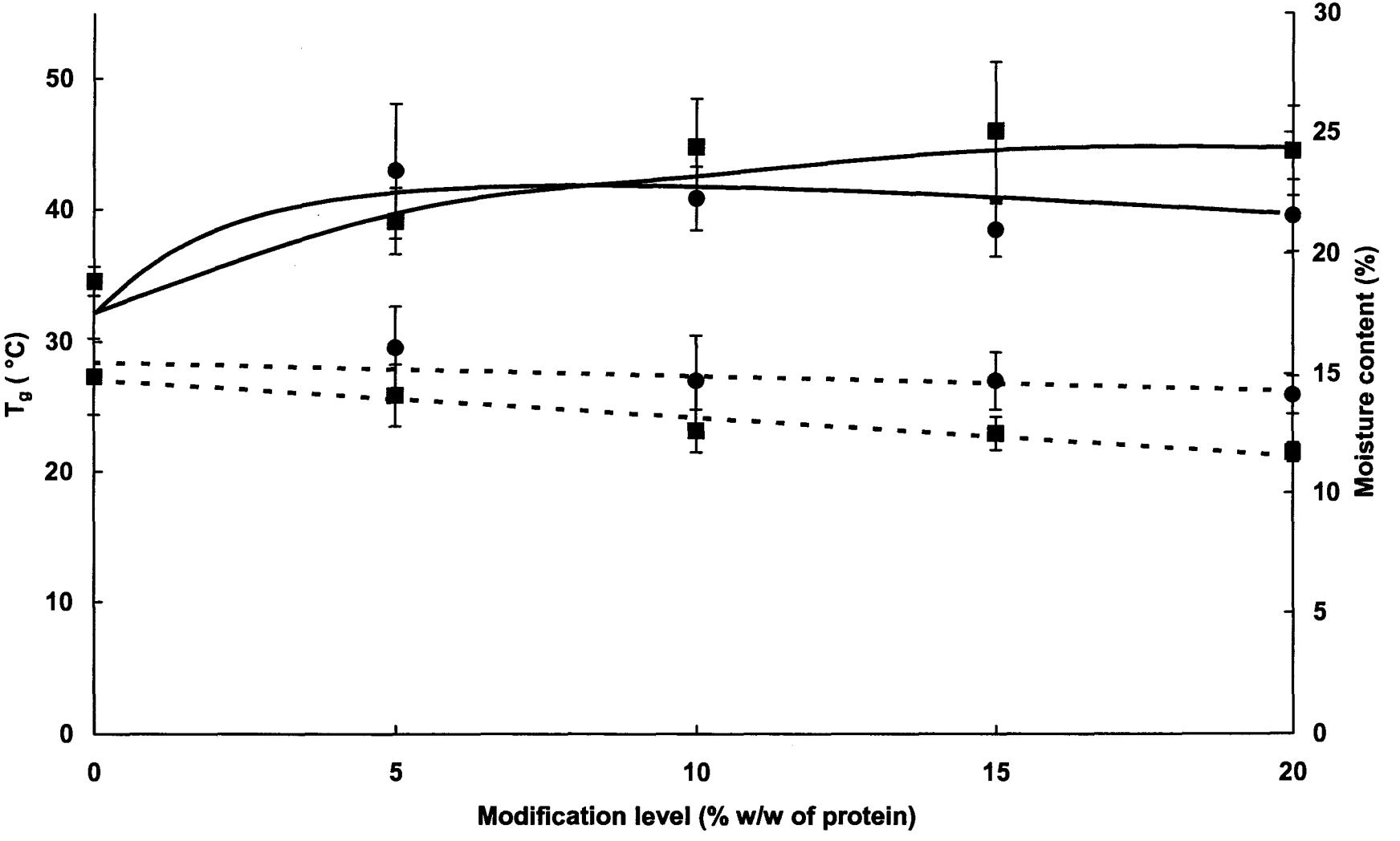


Figure 2.8 Effect of tannic acid (■) and sorghum-condensed tannin (●) 0n the glass transition temperature, T_g (——) and moisture content (-----) of kafirin films
Error bars show ± one standard deviation of the corresponding mean.

that the increase in T_g of kafirin films was a result of modification with TA and SCT. As T_g is a measure of molecular mobility, it can also be proposed that the increase in T_g of the kafirin films indicates a decrease in molecular mobility of polypeptide chains in the film. This is supported by the increase in stress and decrease in strain of the kafirin films when modified with TA and SCT (Fig. 2.6).

The oxygen permeability (Fig. 2.7) and the T_g (Fig. 2.8) of the kafirin films modified with TA and SCT showed opposite trends. The increase in T_g may have resulted in a decrease in free volume at the molecular level in the film allowing less space for gas diffusion (Pascat, 1986), hence the decrease in oxygen permeability.

The mean apparent WVP of unmodified kafirin films was 0.98 g mm m^{-2} h^{-1} kPa^{-1} (Fig. 2.7). Modification of kafirin films with TA and SCT did not result in any significant change in apparent WVP. This can be explained by the mechanism of moisture diffusion through the films. According to Debeaufort et al. (1994) water interacts by absorption, dissolution and desorption through the polymer matrix during its diffusion through a biodegradable and edible film. The apparent WVP determination was done under a RH gradient of about 85% (below the film) to 30% (atmosphere). Under such conditions (high RH gradient), extensive swelling of wheat gluten network was found to occur, caused by absorbed water (Gontard et al., 1993). The swelling increased water diffusion through gluten films.

The lack of an effect of modification with TA and SCT on apparent WVP of the kafirin films (Fig. 2.7) can be explained by the DMA data (Fig. 2.9). The increase in surrounding RH can increase the moisture content of the film. An increase in moisture content may cause plasticization of the amorphous part of the film, thereby leading to a drop in E' by decreasing T_g (Stading et al., 2001). As expected, a drop in E' was observed from 40 to 60% RH, indicating moisture absorption by the film at higher RH. In fact, overall there was a large decrease in E' for all the treatments from 40 to 60% RH. This may suggest that the films could change from a glassy to a more rubbery state at relative humidities higher

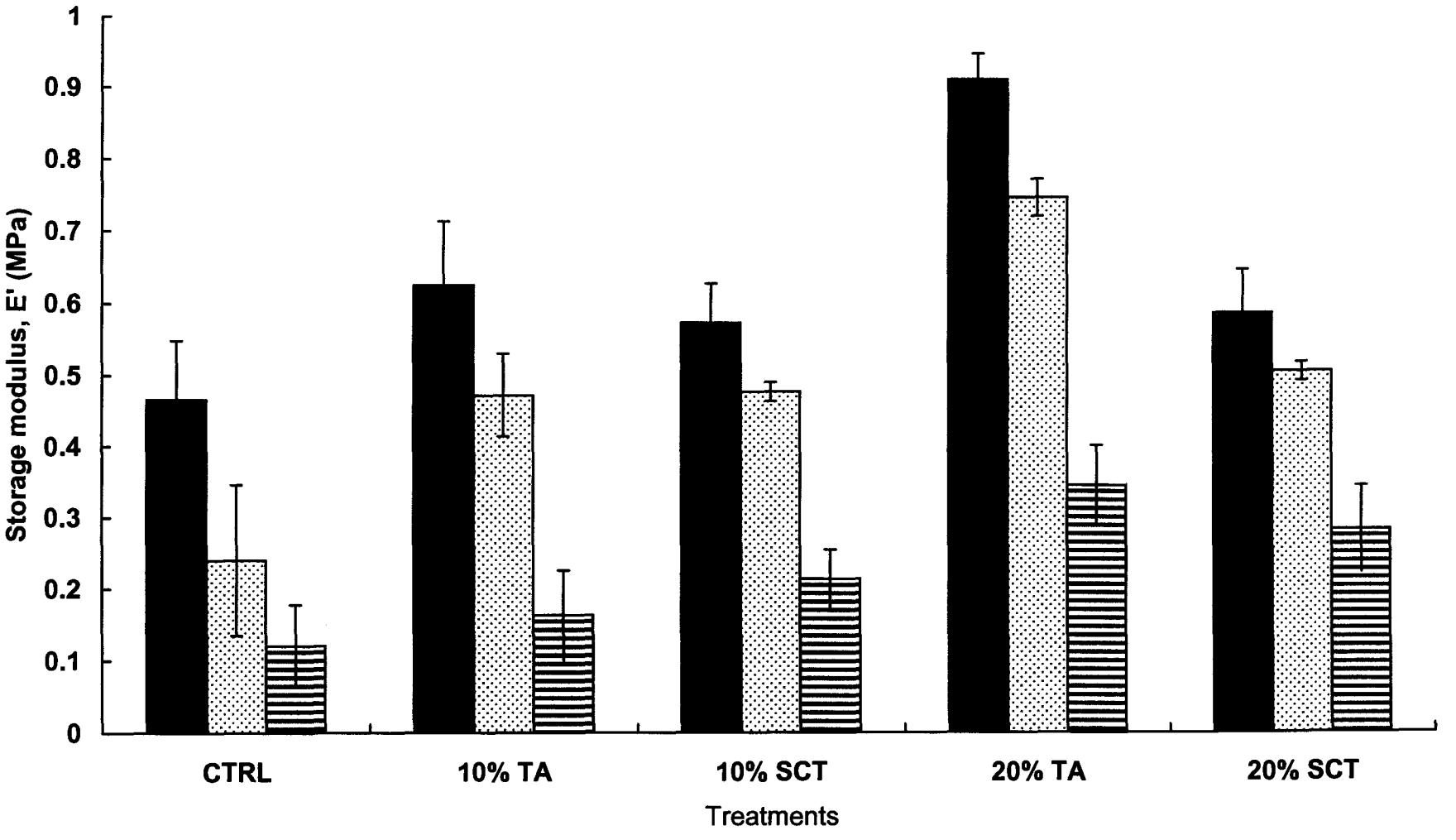


Figure 2.9 Storage modulus E' of unmodified (control) and modified kafirin films with tannic acid (TA) and sorghum condensed tannins (SCT) at 10% and 20% (w/w of protein) during DMA under changing RH
Solid bars 40% RH, dotted bars: 50% RH, horizontal bars: 60% RH. Error bars show ± one standard deviation of the corresponding mean.

than 60%. Thus, under the test conditions for the apparent WVP (RH gradient from 85-30%), the kafirin films could be changed to a more rubbery state by absorbed water, and this change could enhance water diffusion so that the tannin binding did not have any significant effect on the apparent WVP of kafirin films.

DMA of the films at different RH also showed that the tannin modified kafirin films had a higher *E'* compared to the control (Fig. 2.9), suggesting that the modified kafirin films can store more elastic energy. A bigger drop in *E'* with increased RH indicates more moisture absorption. The decrease in *E'* of the unmodified films (control) was significantly higher from 40 to 50% RH than from 50 to 60% RH. However, the decrease in *E'* was significantly higher from 50 to 60% RH than from 40 to 50% for the modified kafirin films at 10% and 20% with TA and SCT. This suggests that the modified kafirin films have a better mechanical stability at these higher RHs than unmodified kafirin films.

2.2.5 Conclusions

Tannins as additives can modify the properties of kafirin films. TA and SCT may be hypothesized to cross-link of kafirin protein polypeptide chains as shown by the decrease in T_g. Thus, TA and SCT modification could decrease protein chain. The decrease of kafirin polypeptide chain mobility and free volume caused by cross-linking with TA and SCT is probably responsible for the increase in tensile stress, Young's modulus; and decrease in tensile strain, water absorption and oxygen permeability of the films.

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2.3 Effects of tannins on the secondary structure of kafirin and kafirin films

2.3.1 Abstract

The interaction of sorghum kafirin protein with the tannins, tannin acid (TA) and sorghum condensed tannins (SCT) was studied by SDS-PAGE, Fourier Transform Infrared (FT-IR) and Raman spectroscopy. SDS-PAGE of kafirin-SCT complex under non-reducing conditions showed a large M_r band that did not enter the separating gel. Complexation of kafirin and modification of kafirin films with the tannins resulted in a change in the FT-IR spectra in the frequency range of 1700 to 1580 cm^{-1}. There was a decrease in absorbance at the frequency of about 1620 cm^{-1}, suggesting a decrease in β-sheet formation during kafirin-tannin interaction. The spectra of kafirin in solution showed a peak at 1650 cm^{-1}, but no peak at 1620 cm^{-1}. This suggests that during kafirin dissolution, the β-sheets are probably changed into random coils. Raman spectroscopy showed a shift in the TA peak at about 1710 cm^{-1} to about 1728 cm^{-1} for kafirin-TA complexes, suggesting participation of the carbonyl groups of TA in kafirin-TA interaction. The results indicate that the random coils, probably formed during kafirin dissolution, provide binding sites such as carbonyl groups for interaction with the tannins. The carbonyl groups of the random coils can probably form hydrogen bonds with the hydroxyl groups of the tannins. Thus, the carbonyl groups are not available to be reorganized into β-sheet structures during solvent cooling and evaporation. It further appears that the tannins cross-link several kafirin polypeptide chains to form large M_r kafirin-tannin complexes and thus probably prevent the formation of intermolecular β-sheets.

Key words: kafirin, tannin, protein secondary structure, FT-IR, Raman spectroscopy

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2.3.2 Introduction

Sorghum grain is an important dietary source of protein for many African and Asian people (Murty and Kumar, 1995). However, the presence of condensed tannins *syn*-proanthocyanidins or procyanidins in tannin (high tannin) sorghum cultivars can decrease protein digestibility (Butler et al., 1984), reducing their food value. The reduction of protein digestibility is probably due to formation of tannin-protein indigestible complexes (Butler et al., 1984) and possibly as a result of enzyme inhibition (Nguz et al., 1998). Tannins can also cause haze in beverages (Bamforth, 1999). These negative effects of tannins can be attributed at least in part to protein-tannin complexation (reviewed by Haslam et al., 1992). There is however limited knowledge about the interaction of tannins with the prolamin storage protein, kafirin, the major protein of sorghum grain (Taylor and Schüssler, 1986). In section 2.1, kafirin was shown to interact with tannic acid (TA) and sorghum condensed tannins (SCT) in an *in vitro* assay. The interaction resulted in haze, with the tannins binding with kafirin to form insoluble complexes. Section 2.2 showed that modification of kafirin films with TA and SCT increased the tensile stress, and decreased tensile strain and oxygen permeability of the films.

Fourier Transform Infra-Red (FT-IR) and Raman spectroscopy are non-destructive methods that can be used to study protein secondary structure (Thygesen et al., 2003). FT-IR has been used to show that the secondary structure of kafirin is changed when it is wet cooked (Duodu et al., 2001). The spectral changes in the amide I and II regions were interpreted as a conformational change of some α–helices into antiparallel intermolecular β–sheets with wet cooking. This conformational change was implicated in the well-known reduced protein digestibility of cooked sorghum (Duodu et al., 2003). FT-IR has also been used to study conformational changes in zein (the prolamin of maize) when lipid-like linolenic acid ethyl esters were added to the protein in the humid state (a_w 0.9) (Mizutani et al, 2003). Presence of the lipid decreased the content of α–helical structures of zein. Raman spectroscopy has been used to study the interaction of azodyes with wool fibre keratin during dyeing (Pielesz et

al., 2003). In this study, electrophoresis, FT-IR and Raman spectroscopy were used to investigate the kafirin-tannin interaction.

2.3.3 Materials and methods

2.3.3.1 Materials

Kafirin was extracted from a mixture of two condensed tannin-free white sorghum grain cultivars, PANNAR PEX 202 and 606, using the fundamental of Carter and Reck method (1970), as described in section 2.1. Commercial tannic acid (Merck, Darmstadt, Germany) and sorghum condensed tannin (SCT) powder extracted from tannin red sorghum (Nola GH 91), as described in section 2.1, were also used.

2.3.3.2 Preparation of kafirin complexed with tannins and kafirin films

TA and SCT at 0, 5, 10, 15 and 20% (w/w) basis tannin preparation to kafirin protein, were added during film casting and complex formation. For the tannin-kafirin complexation, the method is described in section 2.1. In brief, the tannins were added to kafirin solution, incubated at 55 °C, then chilled overnight at 4 °C to promote haze and precipitation. The next day, the mixture was centrifuged and the pellet freeze-dried and then ground into a fine powder. The pellet represented the insoluble kafirin-tannin complex. The freeze-dried pellets will be identified as uncomplexed kafirin (control), kafirin-TA complex or kafirin-SCT complex.

Kafirin films, unmodified and modified with TA and SCT, were produced using the casting method described in section 2.2. These films are identified as unmodified kafirin films (control) and TA or SCT modified kafirin films.

All samples were stored in a dessicator containing silica gel for at least 48 h to ensure minimal moisture before spectroscopic analysis.

2.3.3.3 Analyses

2.3.3.3.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE)

SDS-PAGE under non-reducing conditions was performed using 140 mm length and 1.5 mm thick gels on a Bio-Rad vertical electrophoresis system (Protean II xi Cell, Bio-Rad Laboratories, Hercules, USA). Separating and stacking gel were 12% and 3.9% (v/v) acrylamide, respectively. A ready to use solution of 40% acrylamide-bis was polymerized with 0.1% (w/v) ammonium persulphate and tetramethyl-ethylenediamanine (TEMED). Kafirin samples, 10 mg, was prepared with 3 ml of buffer solution. The buffer consisted of 25% 0.5 M Tris-HCl at pH 6.8, 20% (v/v) glycerol, 4% SDS (10% w/v) and 5% bromophenol blue (0.1% w/v) in distilled and deionised water. The samples were heated in boiling water bath for 10 min, vortexed and then boiled for a further 5 min. About 33 μg of protein was loaded per track. Premixed protein molecular markers (low range marker, Cat. No. 1 495 984, Roche Molecular Biochemicals, Indianapolis, USA) were also loaded. The molecular markers were phosphorylase B, M_r 97.4 k; bovine serum albumin, M_r 66.2 k; aldolase, M_r 39.2 k; triose phosphate isomerase, M_r 26.6 k; trypsin inhibitor, M_r 21.5 k; and lysozyme, M_r 14.4 k. Electrophoresis was carried out at 13 mA per gel and 120 V (constant) for 4.5 h. The gel was stained with 1% (w/v) Coomassie blue R-250, destained and then scanned.

2.3.3.3.2 FT-IR spectroscopy

Spectra of uncomplexed and complexed kafirin in dry form; and the unmodified and TA and SCT modified kafirin films were measured using a FTS 175 spectrometer (Bio-Rad, Hemel Hempstead, UK) by a Golden Gate diamond Horizontal Attenuated Total Reflectance (ATR) (Specac, Hemel Hempstead, UK). Kafirin, TA and SCT samples (2 mg) were spread and kafirin film sample (10 mm x 10 mm) was placed on the ATR crystal to cover the crystal surface area. The sample was gently squeezed by a screw to promote contact with the crystal. The spectra (128 scans at 2 cm^{-1} resolution) were collected with the frequency range 4000 to 800 cm^{-1}. The angle of incidence for the ATR crystal

was 45°. The empty crystal was used as background. The FT-IR spectra were Fourier deconvoluted to enhance resolution.

2.3.3.3.3 Raman spectroscopy

The Raman spectra of kafirin in dry form, SCT and TA samples were measured with a FT-Raman spectrometer (Bio-Rad, Hempstead, UK). About 2 mg was placed in the cavity in a circular aluminium stub. The sample was aligned to the incoming laser. A Near Infra-Red NdYAG laser (1065 nm, 300-500 mW power) was used for excitation. The number of recorded spectra was 256 at a resolution of 8 cm^{-1} and light scattering was performed at a reflection of 180°.

2.3.4 Results and discussion

Kafirin in dry form, uncomplexed kafirin in dry form and kafirin-SCT complex at 10% showed electrophoretic bands with M_r of approximately 24 k, 22 k, and 18 k (Fig. 2.10). These bands can be identified as α_1-, α_2-, β-kafirin polypeptides (El Nour et al., 1998). Some dimers and trimers of kafirin polypeptides could also be seen. The major difference in pattern band between the samples was the occurrence of high M_r band that did not enter the separating gel in the kafirin-SCT complex sample. Sarni-Manchado et al. (1999) applied SDS-PAGE under reducing conditions to salivary protein complexed with grape seed tannins, and also found a band that did not enter the separating gel. The band that did not enter the separating gel can thus be identified as a very high M_r kafirin-SCT complex.

Figure 2.11 shows the FT-IR deconvoluted spectra of kafirin in dry form and solution, TA and SCT in the frequency range of 1725 to 1475 cm^{-1}. In this frequency range the absorbance of kafirin started to increase at about 1700 cm^{-1} to give a first peak at about 1650 cm^{-1} and a second peak at about 1620 cm^{-1}. After these two peaks, the absorbance decreased and then there was an increase at about 1575 cm^{-1} to give another two peaks at about 1540 and 1517 cm^{-1}. The frequency range of 1700 to 1590 cm^{-1} is regarded as the amide I vibration for protein and the region between the frequency range 1575 to 1480

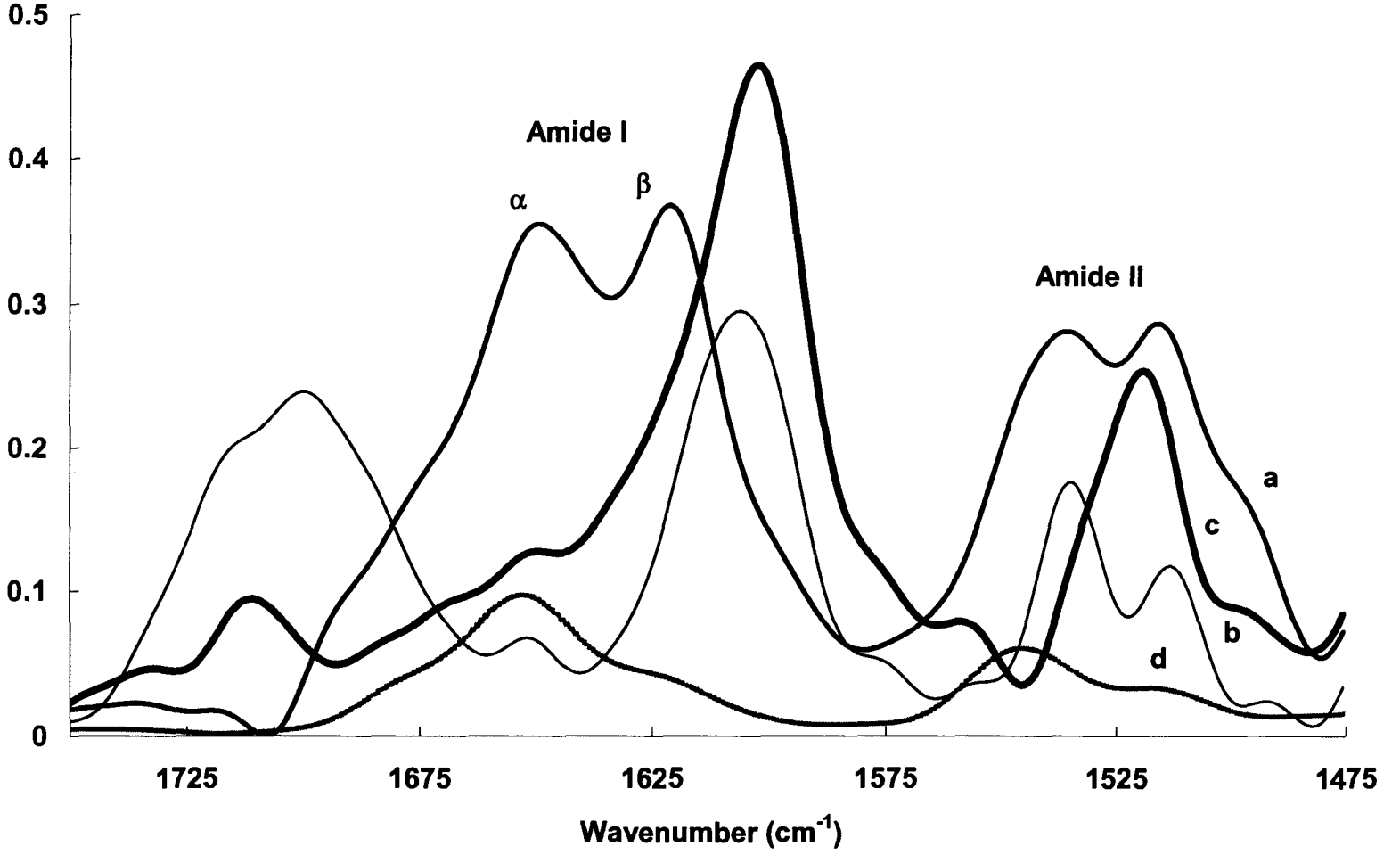


Figure 2.11 FT-IR spectra of kafirin in dry form (a), tannic acid (b), sorghum condensed tannins (c) and kafirin solution (d)

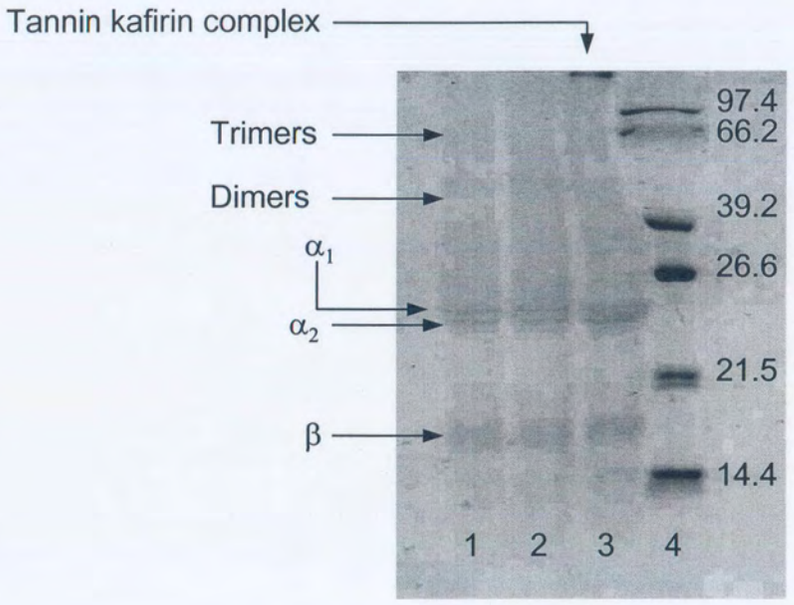


Figure 2.10 SDS-PAGE under non-reducing condition of kafirin and complexed kafirin

Track 1 = kafirin powder, track 2 = uncomplexed kafirin powder, Track 3 = kafirin-SCT complex at 10%, track 4 = molecular weight standards with their size in k on the right

cm^{-1} is regarded as amide II vibration (Bandekar, 1992). The amide I vibration corresponds mostly to C=O stretching, coupled with some contribution from CN stretch, CCN deformation and in plane NH bending modes of groups in the polypeptide chain (Bandekar, 1992). The amide II vibration is believed to be an out of phase combination of CN stretch and in plane NH deformation mode of groups in the polypeptide chain (Bandekar, 1992). In the amide I region, the frequency range of 1650 to 1658 cm^{-1} has been assigned to α-helices and the frequency range 1620 to 1640 cm^{-1} has been assigned to the β-sheet structures of protein (Kretschmer, 1957). In the amide II region, α-helices can be assigned to 1545 to 1547 cm^{-1} and β-sheets to about 1524 cm^{-1} (Kretschmer, 1957). Thus the peaks in Figure 2.11 at 1650 and 1540 cm^{-1} can be assigned to α-helical secondary structure. The peaks at 1620 and 1517 cm^{-1} can be considered as indicative of β-sheet secondary structure.

The spectra of TA and SCT in the frequency range of 1725 to 1475 cm^{-1} showed several peaks with a major one at about 1605 cm^{-1} (Fig. 2.11). The other peaks for TA were about 1700, 1535 and 1515 cm^{-1}. SCT gave a peak at 1520 cm^{-1}. Chemically, TA has a glucose core surrounded by several galloyl groups and SCT is a polymer of flavonoids like flavanols (Hagerman, 2002). Thus, the main peak at about 1605 cm^{-1} for TA and SCT is considered to be due to the presence of the aromatic rings with OH groups. (Kemp, 1987; Rao and Rao, 2002). The peak for TA at about 1700 cm^{-1} most probably corresponds to the carbonyl (C=O) (Omoike and Vanloon, 1999) from the galloyl group of TA. The peaks at 1605 and 1700 cm^{-1} for TA, and 1605 cm^{-1} for SCT, respectively did not overlap with the peaks at 1650 and 1620 cm^{-1} corresponding to the amide I region of the kafirin. However, some absorbance peaks from TA (1535 and 1515 cm^{-1}) and SCT (1520 cm^{-1}) overlapped with the peaks (1540 and 1517 cm^{-1}) corresponding to the amide II. Therefore, only the amide I was taken into consideration when the effect of TA and SCT on kafirin secondary structure was assessed.

It seems that when kafirin in dry form was complexed with TA and SCT (Fig. 2.12a and b, respectively), there was a decrease in the absorbance of the peak

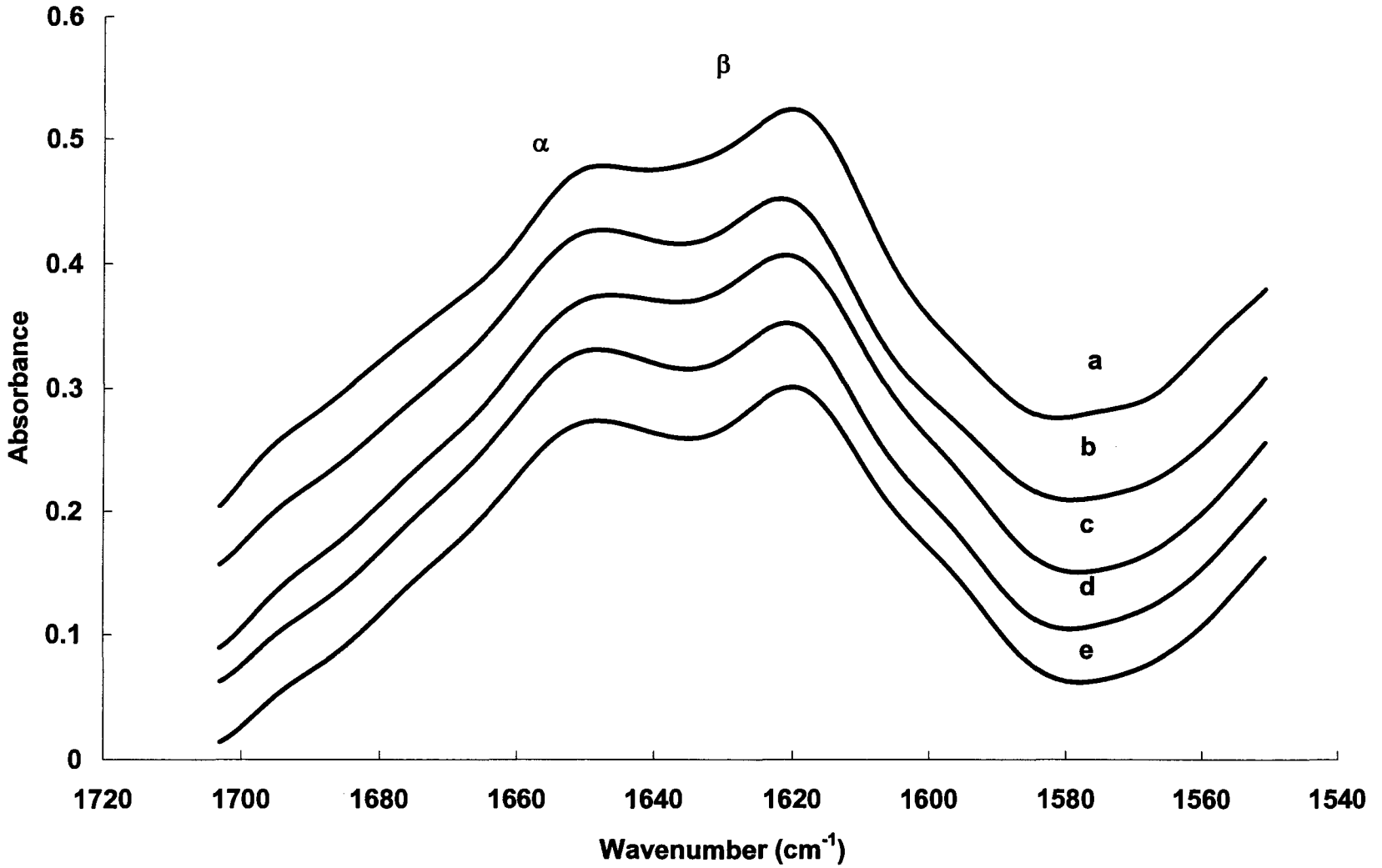
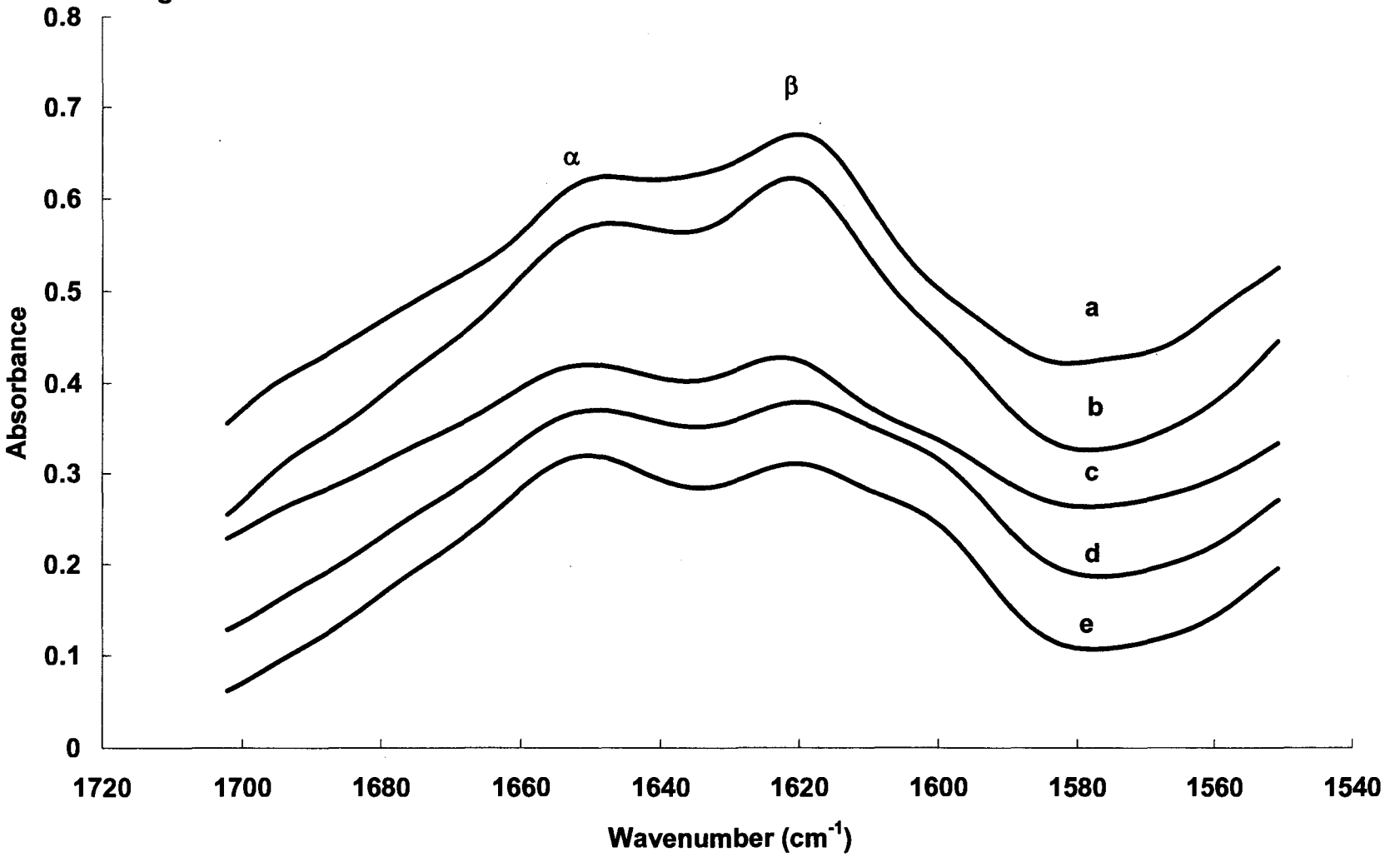


Figure 2.12 FT-IR spectra of kafirin-tannins complexes
a = uncomplexed kafirin, b = 5 %, c =, 10 %, d = 15 %, e = 20 % kafirin tannin complexes.
a) Tannic acid

Figure 2.12



b) Sorghum condensed tannin

at about 1620 cm^{-1}. The peaks of all the spectra at about 1650 cm^{-1} were normalized (maximized) to the same absorbance level, and the decrease in the absorbance of the peak at about 1620 cm^{-1} for the kafirin-TA and kafirin-SCT complex was calculated relative to the uncomplexed kafirin. At the 5% level of kafirin-TA complexation, the 1620 cm^{-1} peak decreased by about 6% compared to the uncomplexed kafirin. There was no further decrease in the peak at higher level of complexation. There was no change in the relative absorbance of the peak at about 1620 cm^{-1} when kafirin was complexed at 5% with SCT. In contrast, SCT addition in the complex at 10, 15 and 20% resulted in a decrease in the absorbance of the peak by 10, 10 and 15%, respectively.

Similarly, there was a change in the absorbance peak at 1620 cm^{-1} when kafirin films were modified with TA and SCT (Fig. 2.13a and b, respectively). At 5, 10, 15 and 20% levels of modification with TA in the films, the absorbance peak at about 1620 cm^{-1} of kafirin decreased by about 7, 25, 27 and 40%, respectively, in comparison to unmodified kafirin films. Modification with SCT at 5, 10, 15, 20% in the films resulted in a decrease in absorbance of the peak at about 1620 cm^{-1} by 12, 19, 24 and 31%, respectively, when compared to unmodified kafirin film.

As the peak at 1620 cm^{-1} is associated with β-sheet conformation (Kretschner, 1957), it seems that the interaction of TA and SCT with kafirin decreased β-sheet structure formation in the kafirin molecules. Kafirin was dissolved at high temperature (about 65 °C with vigourous stirring) and incubated at 55 °C with the tannins for complexation. This treatment was the same when kafirin is prepared for film production. It is suggested that because of heat application during kafirin dissolution and incubation, the hydrogen bonds between the intermolecular β-sheets are broken leading to random coils. Duodu et al. (2001) suggested that heat can also unravel some α-helical chain structures, then these unravelled chains can aligned and reorganize into β-sheet conformation when sorghum is wet cooked. Presumably as an intermediate, the unravelled α-helical chains are random coils, when they re-organise into β-sheet structure. The random coils could provide binding sites for tannins to interact. The binding

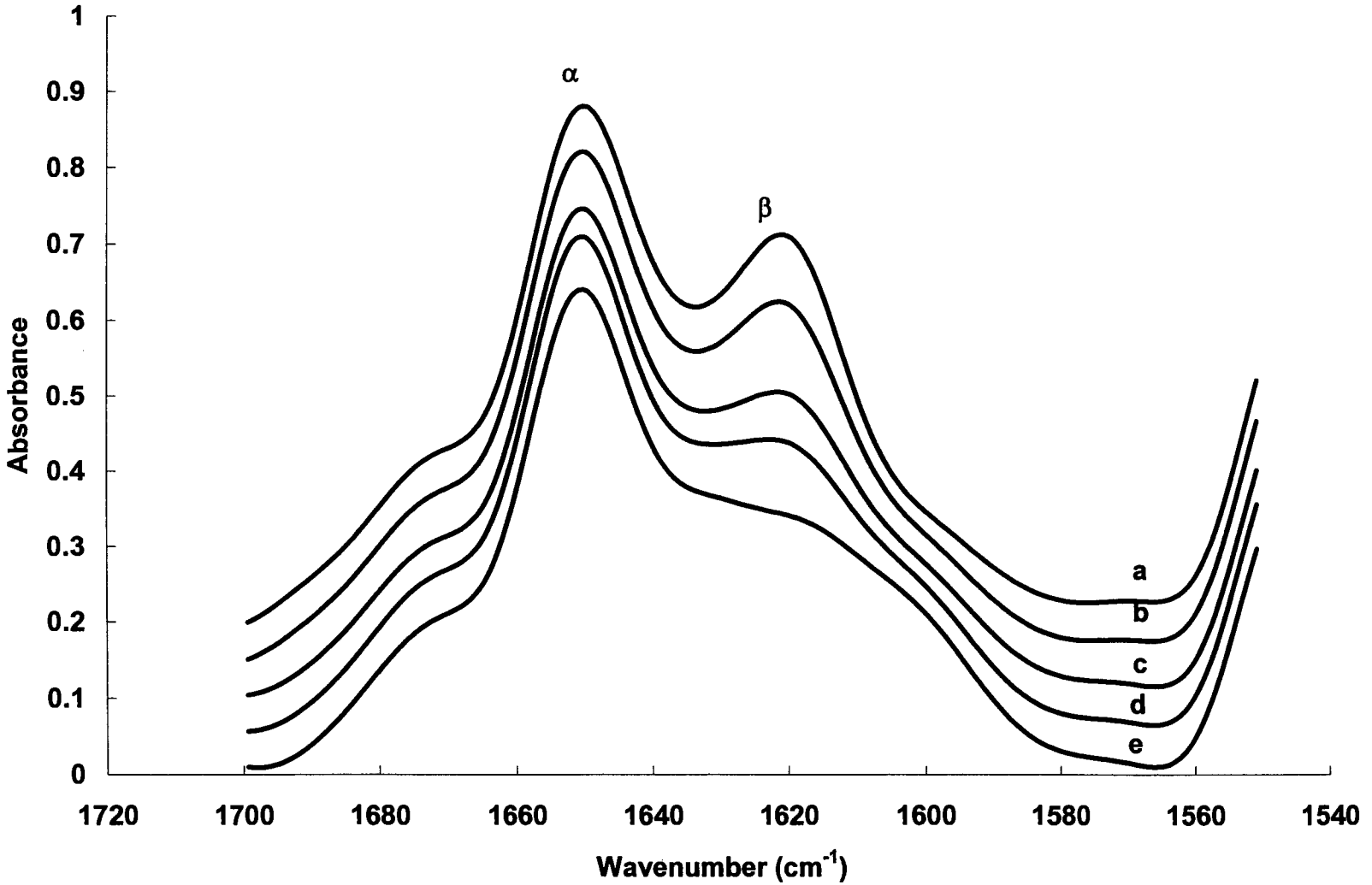
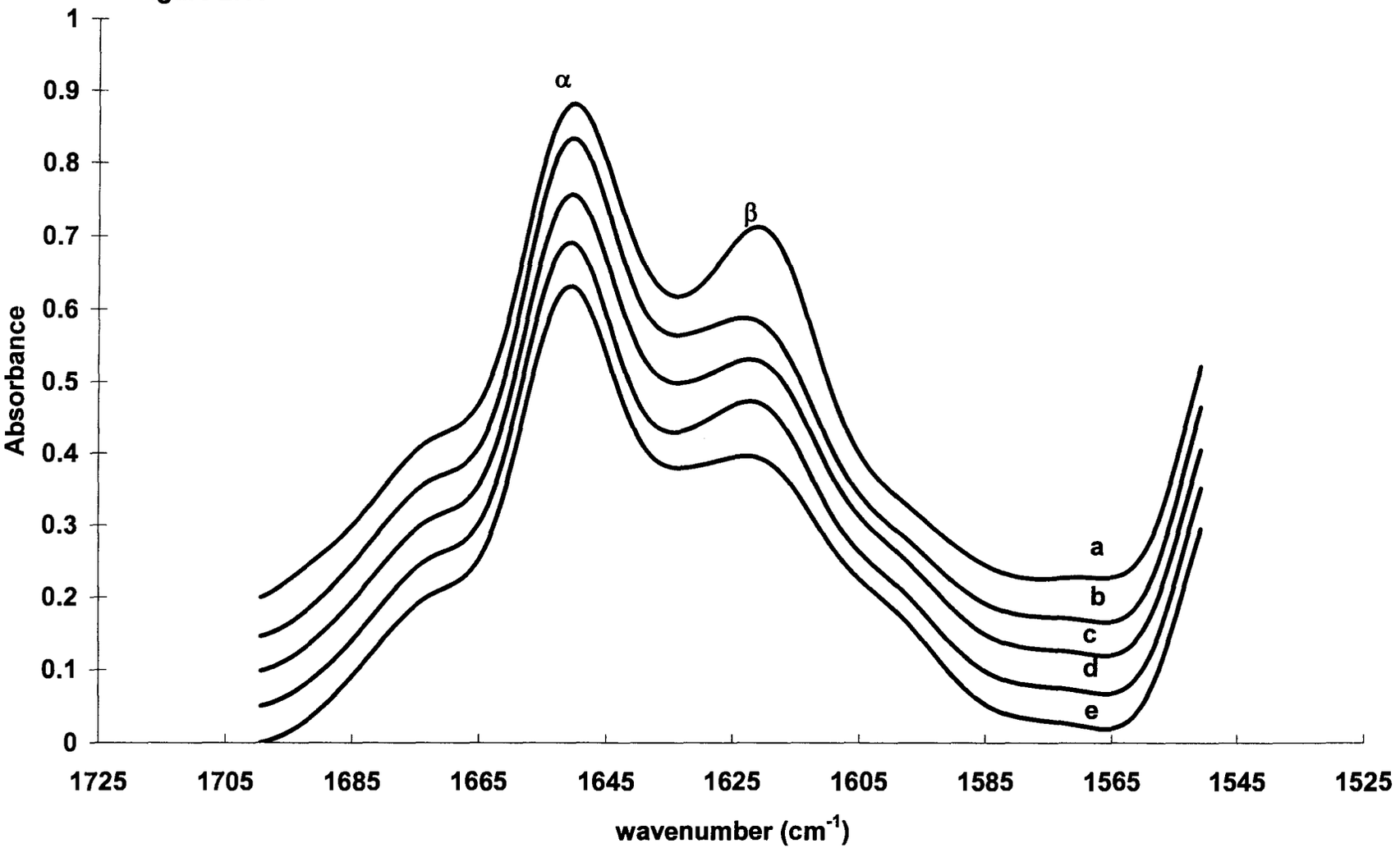


Figure 2.13 FT-IR spectra of kafirin films modified with tannins
a = unmodified film, b = 5 %, c =, 10 %, d = 15 %, e = 20 % level of modification.
a) Tannic acid

Figure 2.13



b) Sorghum condensed tannin

sites could be the carbonyl groups of the polypeptide to form hydrogen bonds with the hydroxyl groups of the tannins. Then during cooling of kafirin solution (4 °C) to promote complexation and solvent evaporation during film formation, the tannins can probably occupy the carbonyl groups in the protein to prevent interaction of the random coils with other polypeptides to reorganize into β-sheet structures. To test the possible occurrence of random coils of kafirin, the spectra of kafirin in solution before modification with the tannins was measured.

Kafirin in aqueous ethanol solution showed a peak at 1650 cm^{-1} only and no peak at 1620 cm^{-1} (Fig. 2.11). It is difficult to distinguish between assigned α-helices and random coils in solution due to their overlapping nature (reviewed by Singh, 2000). Thus the peak at 1650 cm^{-1} could be α-helices or random coils. There was no peak at about 1620 cm^{-1}. The absence of the β-sheet peak suggests that the hydrogen bonds between the β-sheets in the kafirin in dry form were broken down to probably form random coil structures in solution.

The Raman spectra of kafirin films modified with tannins were not determined, as the films did not scatter light when scanned. The Raman spectra of TA within the frequency range of 1550-1800 cm^{-1} shows peaks at 1613 and 1710 cm^{-1} (Fig. 2.14a). The peak at 1613 cm^{-1} has been assigned to the aromatic ring with hydroxyl groups (Rao and Rao, 2002). The peak at about 1715 cm^{-1} can be assigned to carbonyl groups (Omoike and Vanloon, 1999), probably from the galloyl rings. The Raman spectra of the kafirin-TA complex showed an increase in intensity of the peak at 1613 cm^{-1} with increased level of TA complexation. This indicates the presence of an increasing concentration of tannic acid in the kafirin-TA complex. Similarly, an increase in the intensity of the Raman spectra was observed at about 1613 cm^{-1} for the kafirin-SCT complex compared to uncomplexed kafirin (Fig. 2.15). The complexed kafirin in dry form also showed a peak at about 1710-1728 cm^{-1} compared to no peak for the uncomplexed kafirin in dry form. In addition to the increased intensity, there was a Raman shift of the peak from 1710 to about 1728 cm^{-1} when kafirin was complexed with TA. The Raman shift increased with increase in level of TA addition. This shift

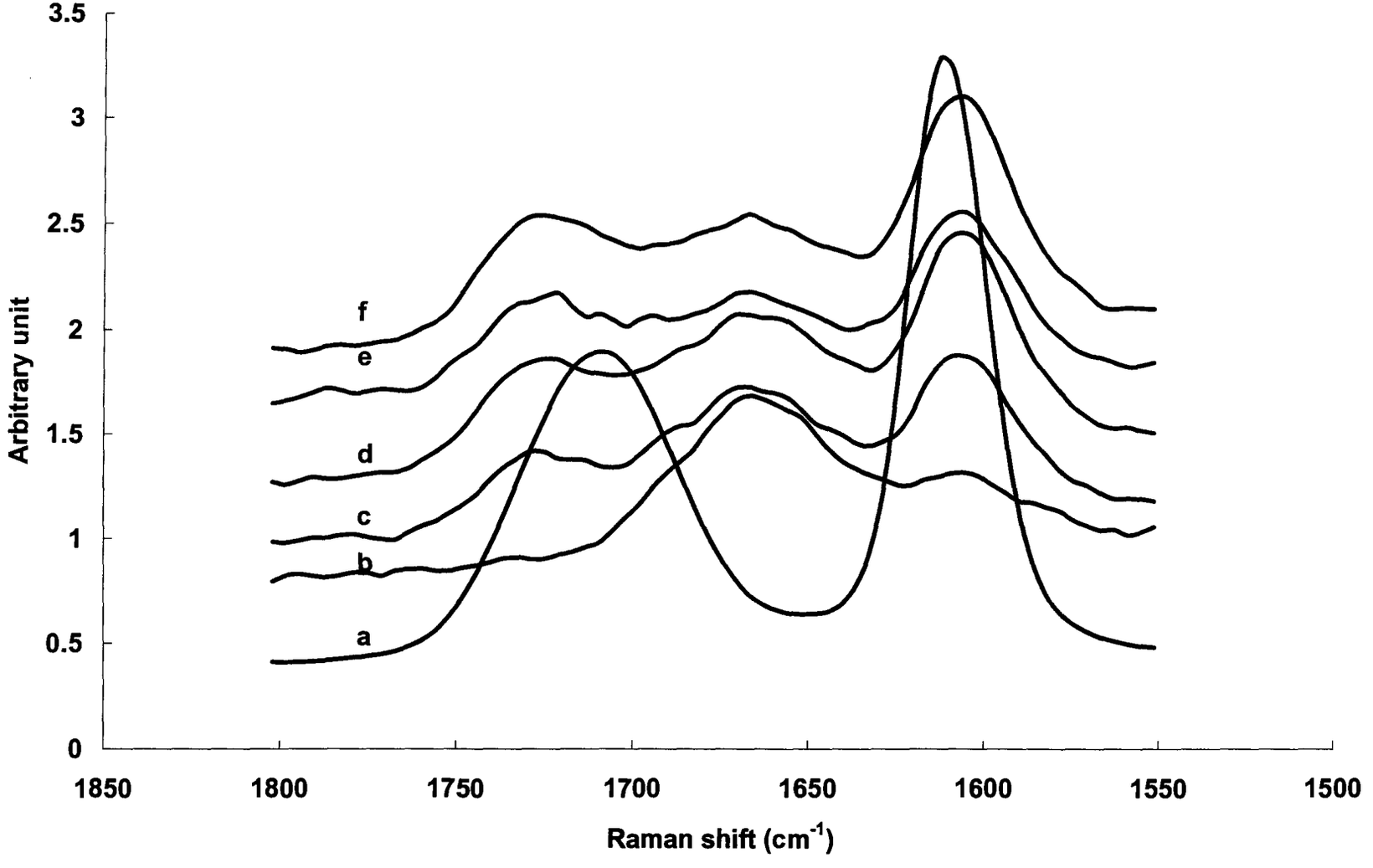


Figure 2.14 Raman spectra of tannic acid and kafirin-tannic acid complexes
a = tannic acid, b = uncomplexed kafirin, c = 5 %, d =, 10 %, e = 15 %, f = 20 % kafirin-TA complexes.

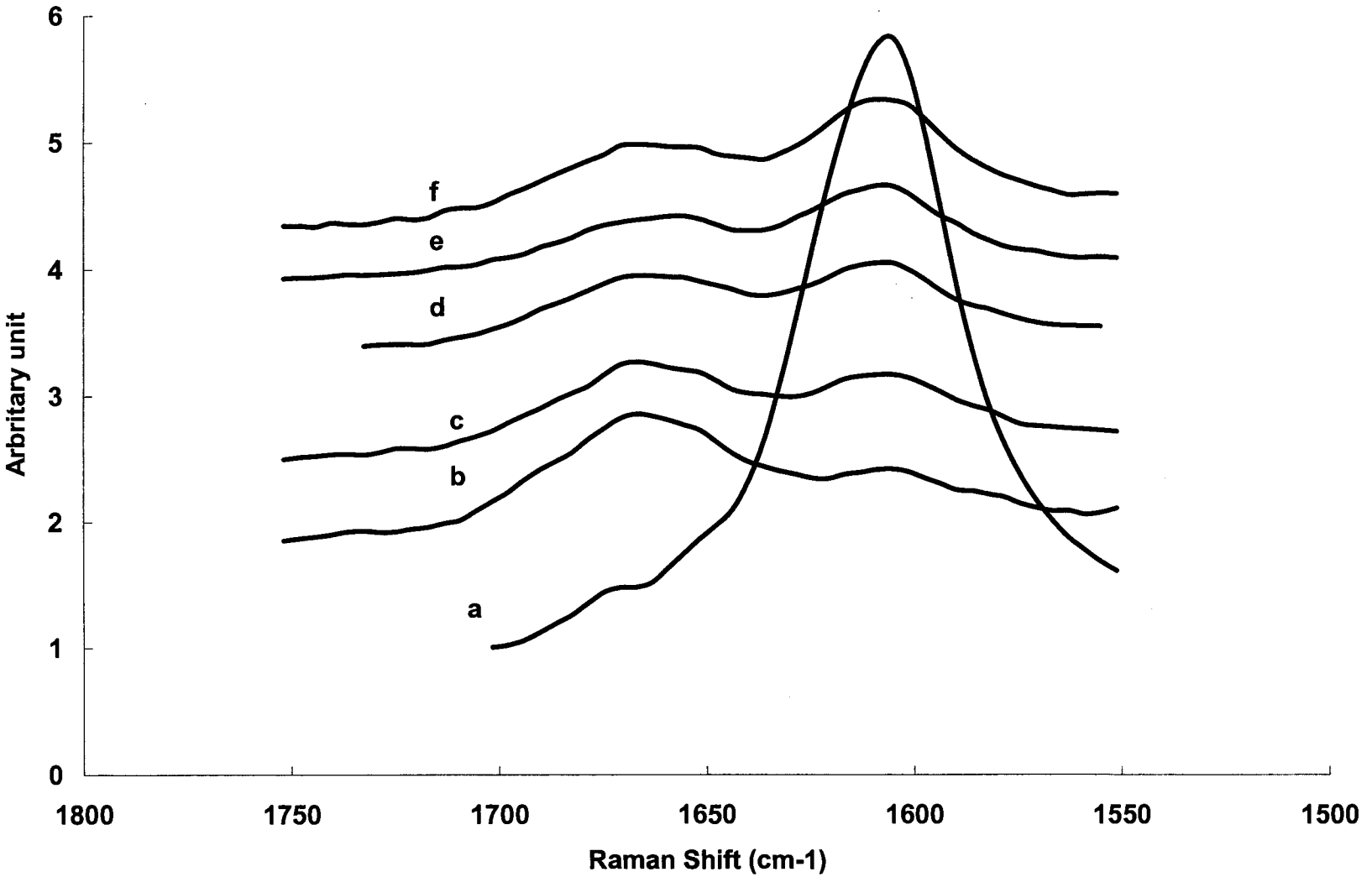


Figure 2.15 Raman spectra of sorghum condensed tannin and kafirin-sorghum condensed tannin complexes a = sorghum condensed tannin, b = uncomplexed kafirin; c = 5 %, d =, 10 %, e = 15 %, f = 20 % kafirin-SCT complexes.

suggests that the kafirin can sterically interfere with the C=O vibration of TA. This interference can probably happen by hydrogen bonding between the carbonyl groups of tannic acid and hydrogen from the amide groups of the kafirin polypeptide chains.

The above results indicate that the probable mode of interaction of tannin with kafirin can be through hydrogen bonds. Hydrogen bonds are also believed to occur between salivary proline rich protein (PRP) and pentagalloyl glucose tannin (Murray et al., 1994). It has also proposed that tannins bind with several polypeptide chains of salivary PRP (Baxter et al., 1997). This multiple binding can cross-link the salivary PRP polypeptide chains to form large M_r complexes that precipitate out (Charlton et al., 2002). Because of the haze formation when kafirin interacts with TA and SCT (section 2.1) and the occurrence of the very high M_r kafirin tannin-complex, it is proposed that the tannins can cross-link kafirin molecules. Cross-linking would suggest that the tannins can bind with several kafirin molecules and prevent the formation of β-sheets between kafirin molecules. The cross-linking probably caused a decrease in molecular mobility and free volume of modified kafirin. This in turn is probably the cause of the increased tensile stress, decreased tensile strain, increased T_g, decreases oxygen permeability of modified kafirin films (section 2.2).

2.3.5 Conclusions

TA, a hydrolysable tannin and SCT, a condensed tannin, can interact with kafirin to change its secondary structure. Complexation of kafirin and kafirin lead to a decrease in β-sheets formation of the protein. Hydroxyl groups of the tannins probably form hydrogen bonds with the carbonyl groups from the random coils of kafirin polypeptide chains in solution. Thus the carbonyl groups from kafirin random coils may be unavailable to interact to form β-sheets. As the tannins can have several hydroxyl groups as binding sites, it is suggested that the tannins can simultaneously bind with more than one polypeptide to cross-link kafirin molecules. This cross-linking could result in the observed very high M_r tannin-kafirin complexes.

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3. GENERAL DISCUSSION

The general discussion chapter firstly deals with the methodological considerations of the research project, then the interactions of kafirin with phenolic compounds specifically the tannins. Next the modifications in kafirin films brought about tannin interaction are discussed. Lastly, some potential uses of tannin modified kafirin films in food systems are explored.

3.1 Methodological considerations

Various methods were used in this research project. The major methods will be discussed in terms of their scientific principles and their advantages, limitations and any deviations to the methods will be examined.

The method to study the interaction of various phenolic compounds with kafirin was an *in vitro* binding assay. Haze formation as a result of binding was determined. The haze was also precipitated out to determine the amount of the phenolic compounds in the precipitate. The amount of haze and phenolic compounds bound are indication of the strength of interaction of the different phenolic compounds with kafirin. The assay thus assumes that the interaction will lead to the formation of insoluble complexes. Hagerman and Butler (1978) developed the binding assay for quantitative determination of tannin in sorghum grain. They found out that the amount of sorghum condensed tannin precipitated by BSA (bovine serum albumin) was linearly related to the amount of tannin present in sorghum grain. Hence they suggested this method for tannin determination.

A potential problem with the binding assay as applied in this study is that phenolic compounds can interact with protein to form soluble complexes in addition to insoluble complexes (Hagerman and Robbins, 1987). Spencer et al. (1988) suggested that soluble complexes could be formed when the phenolic compounds can form a monolayer around the protein molecule, but precipitation only occurs when the monolayer is more hydrophobic than the protein. Thus, if the monolayer is less hydrophobic than the protein, like in the case of phenolic

acids, precipitation is unlikely to occur. Time, temperature and concentration of reactants can affect insoluble and soluble complex formation (Hagerman and Robbins, 1987). Incubation of protein and phenolic mixtures for 24 h at 4 °C can form more insoluble complex than 15 minutes at room temperature. The latter conclusions were therefore used in this study. Soluble complexes also occur when a tannin protein mixture contains excess protein (Hagerman and Robbins, 1987). Therefore different concentrations of phenolic compounds were used in the binding assay for this study.

To the best of my knowledge, this is the first published work using kafirin to interact with phenolic compounds in a binding assay. Thus, BSA was used as reference protein. Hagerman and Butler (1981); and Hagerman et al. (1998) used BSA to study tannin-protein interactions. They found that sorghum condensed tannins have a high affinity for BSA. Hence, BSA can be a good reference protein in this particular approach. However, it is difficult to compare kafirin and BSA directly. This is because they are different types of proteins. According to Sigma (2002), BSA is saline soluble and kafirin is aqueous alcohol soluble (Johns and Brewster, 1916). Thus, there was no common solvent that could be used for both BSA and kafirin. In the binding assay as used in this study, the phenolic compounds were dissolved in the same solvents as the BSA and kafirin. The phenolic compounds were completely soluble in the 20% (w/w) and 65% (w/w) ethanol solution used to dissolve BSA and kafirin, respectively. After chilling overnight at 4 °C, the phenolic compounds were still in solution and no visible precipitate was observed.

Hazes can be measured by the degree by which they scatter light. Haze was determined by the standard American Society of Brewing Chemists method (American Society of Brewing Chemists, 1976), where the degree of light scattering is related to a standard. The standard is formazin. Formazin is an insoluble complex formed between hydrazine sulphate and hexamethylenetetramine compounds in solution (American Society of Brewing Chemists, 1976). The method recommends the use of a turbidimeter or nephlometer. These are instruments that measure scattered light (Morris,

1987). However in this project, a spectrophotometer was used. Spectrophotometer measures the transmitted or absorbed light, but does not measure scattered light (Morris, 1987). A formazin standard curve read from the spectrophotometer gave a linear fit of R^2>0.99 (section 2.1). Morris (1987) found similar results when haze values were read with both a nephelometer and a spectrophotometer. He suggested that a spectrophotometer can be used if a nephlometer is not available.

Various methods can be used to quantitatively determine phenolic compounds. The choice of method depends on the type of phenolic to be determined. Complexation of phenols with ferric ions for example from ferric ammonium citrate measures all types of phenols and this method is not selective to specific phenolic compounds under alkaline conditions (Mole and Waterman, 1987). The main advantage of the method is an instantaneous reaction producing a stable colour lasting for several hours (Mole and Waterman, 1987). However, these authors found that the ferric reagent could form different colours with different types of phenolic compounds. Condensed tannin from sorghum can yield green complexes, but hydrolysable tannin such as tannic acid can yield blue complexes (Mole and Waterman, 1987). This suggests that different types of standard should be used. In this study, tannic acid was used as standard for the binding assay of kafirin with TA. Catechin was used as standard for assaying the polyphenol bound to kafirin for catechin and extracted flavonoids due to their structural similarity. Catechin was also used as standard to quantify the amount of sorghum condensed tannin (SCT) bound to kafirin. This is because when SCT is hydrolysed, it can yield flavanols, a flavonoid type phenolic compound with structural similarity to catechin (Hagerman, 2002). To alleviate the problem of different standards, the amount of phenolic bound was also expressed as a percentage of the amount added.

The methodology used to determine the mechanical and barrier properties of protein-based films were adapted from standard methods of the American Society for Testing and Materials (ASTM) for plastic sheets. The film mechanical properties were determined essentially using the ASTM method D 882-97 tensile test for thin plastic sheeting (American Society for Testing and

Materials, 1988). A tensile test expresses the ability of the film to deform when an external load is applied to extend the film (American Society for Testing and Materials, 1988). The results are then expressed as stress and strain of the material. The oxygen permeability was determined by an adaptation of the ASTM method F 1307-90 (American Society for Testing and Materials, 1990), which measures oxygen transmission rate through dry packages using a coulometric sensor. The water permeability was determined by the modified method E 96-95 (American Society for Testing and Materials, 1995), which measures water vapour transmission of material like plastic films and paper. All the methods as used were described in section 2.2.

The determination of the functional properties of kafirin films, like those other films, are affected by temperature (Ali et al., 1997) and relative humidity (Debeaufort et al., 1994). For example, thermally treated gluten films at different temperatures gave different tensile and WVP (Ali et al., 1997). Gluten films had different WVP when determined at different RH (Debeaufort et al., 1994). The ASTM methods recommend the conditioning of the plastic films or sheets before property determination. Ideally all the films should be conditioned and the test should be carried out in a climate controlled room at a specific temperature and relative humidity (RH). However such equipment was not available for this project. To minimize experimental errors due to ambient temperature and RH, the films were kept (conditioned) in a dessicator containing saturated calcium nitrate solution to create a RH of 50% at 25 °C (Lai and Padua, 1998). Tensile properties of the films were measured at ambient temperature and RH within two minutes removal from the dessicator.

The modified ASTM method for the WVP determination used adapted Schott bottles. The main drawback is that the bottles were not uniform in diameter throughout their height. The diameter of the neck of the bottle was smaller than the body of the bottle. This non-uniformity affected the rate of water evaporation. To minimize this experimental error, water was filled up to the neck that has similar diameter to the opening. However, as a result of water loss during the test, the water level fell below the neck where the diameter

increases. The stagnant air layer between the water surface and the films could also have affected the RH at the surface of the film facing the water surface (Gennadios et al., 1994b). This underestimated the WVP due to the difference in the RH gradient (Gennadios et al., 1994b). The stagnant air layer was taken into consideration using the calculation according to Gennadios et al. (1994b). However, due to these factors the measured WVP was not an absolute value.

WVP was determined over a 10-day period instead of 24 h as reported by other workers working with edible films (Gontard et al., 1993; Debeaufort et al., 1994; Gennadios et al., 1994b) The ASTM method does not specify the time period. In this respect the values obtained can be compared between the treatments in this project, but not specifically to other published data. The use of a long period as opposed to short period can be advantageous as the films are intended to be applied as coating of fruits such as litchi, and other products like nuts (Enviropak, 2002). Fresh fruits such as litchi (lychee) can a have shelf life of about 5-10 days. Shelled and roasted nuts can have a shelf life for months depending on the storage conditions. Thus data over a longer test period will be more relevant.

Dynamic mechanical analysis (DMA) of the modified and unmodified kafirin films was performed at different temperatures to determine the T_g of the films. At low temperatures, polymers mostly exist in a glassy state (Gupta 2000). This has been shown by the high storage modulus (E') compared to a low viscous modulus (E'') of the polymers at low temperatures from the DMA data (Gupta, 2000). A high storage modulus was also found for the kafirin film at low temperatures (section 2.2). When temperature is increased, the storage modulus falls rapidly and exhibits a maximum. Thus, these data can be used to identify the thermal or glass transition temperature (T_g). T_g is the indication of the molecular mobility of material. Although T_g measured from DMA is slightly higher than that measured with different scanning calorimetry DSC, the values correspond very well between both methods (Rieger, 2001). Gearing (1999) suggested that DMA has better sensitivity than DSC to measure T_g of polymeric

materials like amorphous homopolymers, copolymers, adhesives and coatings. Thus DMA is a better choice to measure T_g of kafirin films.

Methods used to determine the functional properties of protein-based films have been adapted from the relevant ASTM methods. As stated, the ASTM methods have been developed mostly for plastic sheets. Synthetic plastics are quite different polymers from biopolymers (Van de Velde and Kiekens, 2002). Plastic film such as polyethylene is made up of linear polymers, quite homogenous in structure, and the repeated units are the same. In contrast, kafirin like other proteins are branched, and are made up of different polypeptide units. For example kafirin is made up of α-, β-, γ-kafirin. SEM of kafirin films in section 2.2 showed that the film is not homogenous. Kafirin films can be considered as a multiphase system comprising of kafirin polypeptides, a mixture of plasticizers and residual solvent. Thus because of the differences in the polymer nature between biopolymers and the synthetic polymers, the ASTM methods commonly used for property determination of protein-based films need to be applied with caution.

Scanning electron microscopy (SEM) uses electrons instead of light for image creation. The image resolution of electron microscopy is many times greater compared to light microscopy (Bozzola and Russell, 1992). Image formation in electron microscopy is by electrons focused with magnetic lenses rather than photons focused on glass lenses as in light microscopy. In SEM, there is minimal sample preparation that can interfere with the structure of kafirin films. However, the identification of the structures from the SEM image was the major problem. It was difficult to distinguish between the protein and the plasticizers in the film. Across the cross section, the films showed different structures, indicating non-homogeneity of film. A major limitation of SEM is the vacuum that evaporates any solvent from the sample. The pores seen from the micrographs (section 2.2) could be due to evaporation of phase separated plasticizer, water, ethanol or other components in the films under vacuum during SEM.

Infrared is the wavelength (λ) range of 2.5 to 25 μm (wavenumbers ranging from 400-4000 cm^{-1}) of the electromagnetic spectrum (reviewed by Kemp,

1987). Atoms in a molecule are in constant vibration motion, which may be by stretching or bending vibration. When infrared light is passed through a molecule, some frequencies are absorbed and some are transmitted. A plot of frequency (as x axis) versus the percent frequency absorbed or transmitted gives an infrared spectrum (reviewed by Kemp, 1987). Spectroscopic methods like infrared can produce interferograms (interference patterns) as they are in the time domain. They are difficult to explain, but the mathematical conversion from the time domain to frequency domain can reduce the interferograms. This conversion is known as Fourier transformation, hence the name Fourier Transform Infrared (reviewed by Kemp, 1987).

Proteins and polypeptides are long chains of amino acids connected by peptide bonds. Three major spectral regions for proteins: amide I, amide II and amide III have been identified based on theoretical and experimental studies (reviewed by Singh, 2000). The frequencies at which amide bond vibration occur can be attributed to different secondary structures (reviewed by Singh, 2000). The differences in vibration of amide bonds are probably due to differential pattern of hydrogen bonding. Each amide region can be made up of α-helices, β-sheets, turns and non-ordered polypeptide fragments (Surewicz and Mantsch, 1988). The spectra corresponding to these different structures can overlap extensively and can be narrowed to enhance the resolution by a technique known as Fourier self deconvolution (Byler and Susi, 1986).

The spectra of the samples needed to be carefully interpreted (as discussed in section 2.3). Results in section 2.3 showed that some peaks of TA and SCT overlap in the amide II region of the kafirin. Thus amide I was used to study the effects of tannin on kafirin secondary structure, as the tannins did not have peaks in the amide I region. Plasticizers such as lactic acid due to the C=O group can probably affect the amide I region of the kafirin films. This is because amide I is mostly due to the C=O vibration of protein polypeptides (Bandekar, 1992). However, preliminary results with and without lactic acid at the concentration used in this work suggested that it had minimal or no interference.

Thus spectra obtained by FT-IR should be carefully interpreted. Hence, only the trends from the tannin-kafirin complex were discussed.

Raman spectroscopy is also a branch of vibrational spectroscopy. It is based on shifts in the wavelength or frequency of an exciting incident beam of radiation that result from inelastic scattering on interaction between the photons and the sample molecules (Li-Chan, 1996). Raman spectroscopy is still a new technique in the area of biomolecules and food science. The kafirin film did not produce a good Raman signal as compared with the kafirin-TA complex in dry form (section 2.3). This may be because of the plasticizers in the films that could have interfered with the Raman signal. The Raman signal could not distinguish between the protein structures in the amide regions, but the tannins showed an intense signal in the spectrum. This is probably because of the numerous aromatic rings in the tannins that can be produce good signals in Raman (Merlin et al., 1993).

3.2 Kafirin interaction with phenolic compounds

As reported, the various types of phenolic compounds showed different abilities to bind and form haze with kafirin in solution (section 2.1). Among the phenolic compounds examined, ferulic acid, a phenolic acid, did not form haze with kafirin. Extracted flavonoids (mosltly anthocyanins) from sorghum condensed tannin-free red cultivar, and catechin as another flavonoid did not form haze or bound with kafirin. Tannic acid (TA), a hydrolysable tannin, and extracted sorghum condensed tannins (SCT) formed haze and bound with kafirin. Thus TA and SCT were used for further studies to determine if they could modify kafirin film to change its functional properties and to understand the mechanism of tannin-kafirin interaction. TA and SCT were found to modify kafirin film as shown by significant changes in the functional properties, like increase in tensile stress, decrease in tensile strain, increase in T_g and decrease in the oxygen permeability (section 2.2). The modification of kafirin films by TA and SCT was suggested to be probably as a result of cross-linking of kafirin polypeptide chains by the tannins. Analysis of the complexed kafirin in dry form and modified kafirin films by the TA and SCT using spectroscopy indicated that

these tannins affected the kafirin secondary structure (section 2.3). The FT-IR spectra suggested that tannins like TA and SCT can interact with kafirin by cross-linking between kafirin molecules probably through hydrogen bonding.

Kafirin molecules in solution contained mainly α-helices and some random coils, as interpreted from the FT-IR spectra of kafirin solution (section 2.3). During kafirin dissolution under high temperature in aqueous ethanol, heat could break the hydrogen bonds to unravel some α-helices and the β-sheets to form random coils. It has been proposed that heat can unravel the α-helices of kafirin protein during wet cooking of sorghum (Duodu et al., 2001).

Kafirin molecules in solution may have binding sites to allow interaction with the tannins. The most probable binding sites of the tannin with kafirin molecules would be the random coils. Random coils can have exposed carbonyl groups (Darby and Creighton, 1993). Thus carbonyl groups of the random coils can potentially form hydrogen bonds with the hydroxyl groups of the tannins. Hydrogen bonds between the carbonyl groups of protein polypeptide chain and hydroxyl groups of tannin are believed to be involved in the interaction between salivary PRP and pentagalloyl glucose (tannic acid) (Murray et al., 1994). The results from section 2.3 in fact suggest this. Spectroscopic analysis of complexed kafirin in dry form and modified kafirin films with TA and SCT showed a decrease in the absorbance intensity of the peak in the amide I region assigned to β-sheet structure, relative to the absorbance intensity of the α-helices. This effect can be attributed to binding of tannins at the carbonyl groups on the random coils. Thus, the carbonyl groups were probably not available to form hydrogen bonds with the hydrogens in the amide groups from other polypeptide chains to reorganize into β-sheet structures.

Other potential binding sites can be with proline residues in kafirin molecules. Kafirin is rich in proline residues, on average 9.6 g100 g^{-1} (Taylor and Schüssler 1986). Proline residues have been emphasized in many research papers to be the important binding sites for phenolic compounds (reviewed by Spencer et al., 1988). NMR studies has shown that among the amino acids, proline residues in salivary PRP polypeptides have the maximum chemical shift when complexed

with pentagalloyl glucose (Luck et al., 1994). The pyrrolidine rings of proline residues probably form hydrophobic interactions with the aromatic rings of the tannins (Murray et al., 1994). Thus the hydrophobic region of a protein such as kafirin, which in part can be contributed by proline residues, can also be a potential binding site for the tannins.

Luck et al. (1994) also suggest that glutamine is also involved in the salivary PRP complexation with pentagalloyl glucose (hydrolysable tannin). This is because there was a chemical shift for the glutamine residues in the salivary PRP as measured by NMR. Kafirin is also rich in glutamine residues, on average 22 g100 g^{-1} (Taylor and Schüssler, 1986). Glutamine can form hydrogen bonds because of the presence of the carbonyl group in its side chain (Damodaran, 1996). Thus, the glutamine residues of kafirin could also be potential binding sites to form hydrogen bonds with tannin molecules.

The potential molecular interactions between the tannins and kafirin polypeptide chains are illustrated in Figure 3.1. The interaction of the tannins with probable binding sites such as carbonyl groups in the random coils, the proline residues in the random coils and the glutamine are shown.

Because precipitation, as observed by haze formation, occurred when kafirin interacted with TA and SCT (section 2.1), it can be inferred that these tannins bind with more than one polypeptide chain at a time. The changes in the mechanical and barrier properties of modified kafirin films found in this work also suggest the possibility of the kafirin polypeptide chains being cross-linked by the tannins (section 2.2). Tannins like pentagalloyl glucose and procyanidin dimer have been reported to simultaneously bind with more than one polypeptide chain to cross-link proteins such as salivary PRP (Baxter et al., 1997). These workers proposed that complex polyphenols such as hydrolysable and condensed tannins can interact with salivary PRP in a 'multidentate fashion' to cross-link the protein to form aggregate that precipitates out.

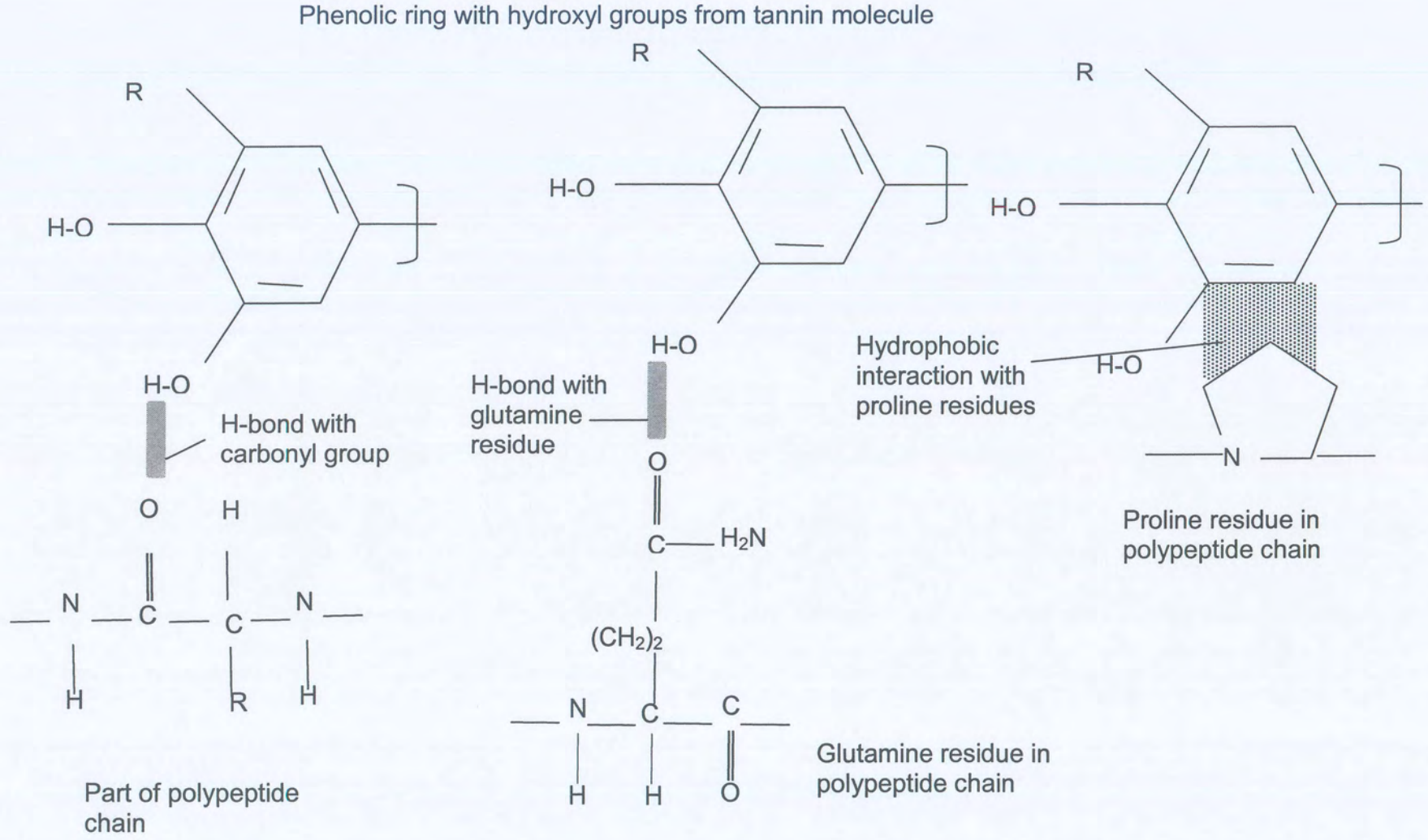

Figure 3.1 Potential molecular interactions between the tannins and kafirin polypeptide chains to show the possible binding sites where R=H or OH

FT-IR spectroscopic analysis of the kafirin complexes and kafirin films modified with TA and SCT showed a decrease in absorbance intensity of the peak assigned to β-sheets relative to the α-helices. Beta-sheet structures can be intramolecular or intermolecular. Whether the β-sheets were inter- or intramolecular could not be distinguished from the FT-IR spectra. However, this decrease in β-sheets can be interpreted as the tannins interacting with two polypeptide chains of the random coils to prevent the reorganization into intermolecular β-sheets. Tannins like TA and SCT contain numerous aromatic rings and therefore numerous hydroxyl groups can be involved in hydrogen bonding. The aromatic rings can also form hydrophobic interactions with several pyrrolidine rings from proline residues (Baxter et al., 1997). Thus, it is proposed that the tannins can interact by cross-linking of kafirin molecules through hydrogen bonds and hydrophobic interaction. Murray et al. (1994) suggested that hydrogen and hydrophobic interaction can both occur when tannins such as pentagalloyl glucose interact with protein such as salivary PRP.

A model for the cross-linking of kafirin molecules is presented in Figures 3.2 and 3.3 to show the formation of aggregates that can potentially precipitate out. Figure 3.2 shows the multiple binding sites of TA that can cross-link two polypeptide chains of kafirin through hydrogen bonds between the carbonyl groups of the polypeptide chain and the hydroxyl groups of the tannic acid. Figure 3.3 shows the multiple interaction of tannins with kafirin polypeptides through hydrophobic interaction.

In the literature, the role of proline residues as binding sites for tannins has been emphasized (reviewed by Spencer et al., 1988). Although experiments were not done to determine the involvement of proline residues in kafirin-TA and kafirin-SCT interaction in this study, the probable role of proline should not be ignored and thus it will be discussed. Kafirin is rich in proline residues and the different kafirin polypeptides contain different amounts of proline residues. Alpha and β-kafirin contain 8.9 and 9.7 mole% of proline residues, respectively (Shull et al., 1992). Gamma-kafirin is very rich in proline, 22.6 mole% (Watterson et al., 1990). The amino acid sequence of γ-kafirin has repeats of

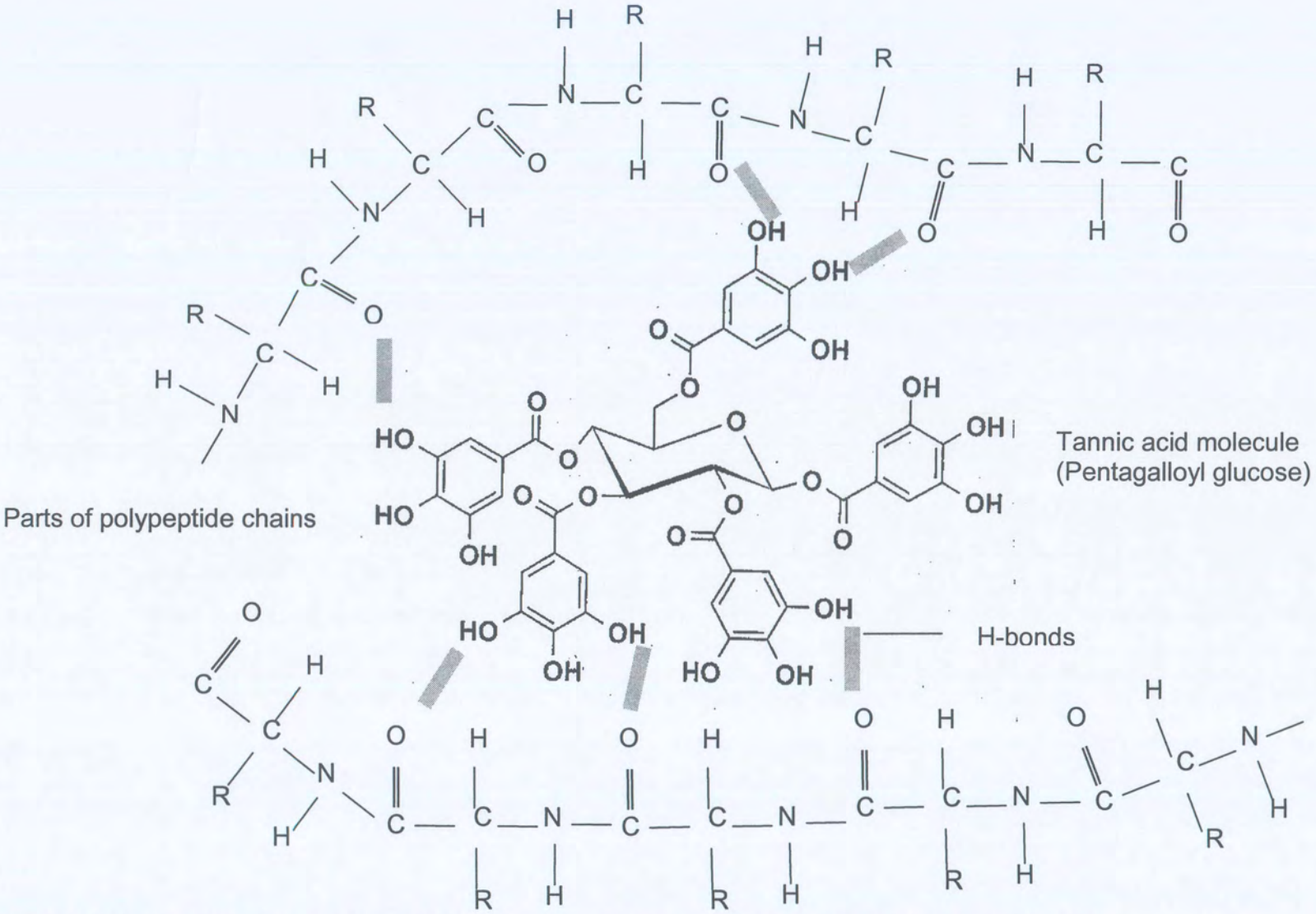

Figure 3.2 Cross-linking of two polypeptide chains of kafirin by tannic acid through hydrogen bonds.

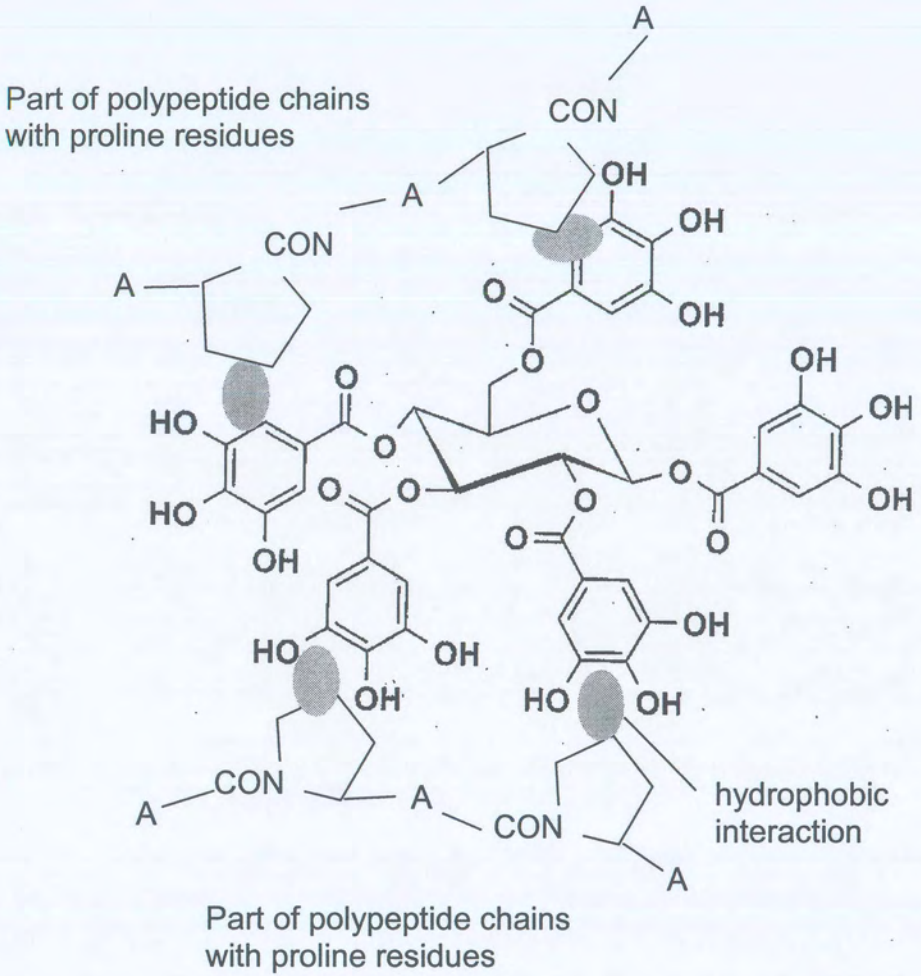

Figure 3.3 Hydrophobic interactions between galloyl rings of tannic acid and proline residues of different polypeptide chains of kafirin (A represent other amino acids in polypeptide chain)

proline residues (Anonymous, 2002c). The number of two, three, four and five repeats of proline residues in gamma kafirin is three, three, one and one, respectively. Baxter et al. (1997) found that tannins have high affinity for salivary PRP, not only because it is rich in proline, but also because it has repeat proline residues. NMR of salivary PRP has shown it has an extended and unstructured conformation (Murray and Williamson, 1994). This conformation was promoted by the repeat proline residues in salivary PRP. The high binding affinity of salivary PRP to tannins in solution was also attributed to its conformation (Murray and Williamson, 1994).

Kafirin is considered to be hydrophobic (Belton et al., 1997). The different kafirin polypeptides have different degrees of hydrophobicity. Relative hydrophobicities of kafirin can be determined from the free energy of hydration calculated from the summation of the amino acids. The more negative the free energy of hydration, the less hydrophobic is the protein. The hydration energies of α- and γ-kafirin are –140.36 and –113.63 k calmol^{-1}, respectively, as calculated by Duodu et al. (2003). The hydrophobicity of β-kafirin was not calculated, as its amino acid sequence has not yet been reported. Thus γ-kafirin is more hydrophobic than α-kafirin. Hydrophobic interaction arises from non-polar groups when they come close together. Phenolic compounds contain several non-polar groups such as the aromatic ring consisting of methylene groups, and the carbon-hydrogen skeleton of the sugars in hydrolysable tannins. Hydrophobic interaction as a mode of interaction between the pyrrolidine rings of the proline residues in salivary PRP and the galloyl rings of pentagalloyl glucose is believed to occur as indicated by NMR studies (Murray et al., 1994). Thus, it is highly that hydrophobic interaction between the hydrophobic γ-kafirin and tannins can occur.

When comparing the different kafirin polypeptides, γ-kafirin has a high concentration of proline residues, a repeat proline residues in the amino acid sequence, and high relative hydrophobicity. This suggests that γ-kafirin preferentially interacts with the tannins. However, further research like binding

assays and spectroscopic analysis of the different kafirin polypeptides with different types of tannins such as TA and SCT is required to confirm this theory.

The cross-linking mechanism of the interaction between the kafirin and tannins can also be shown schematically in a model. Kafirin protein is believed to be structurally similar to zein, the maize prolamin (DeRose et al., 1989). Argos et al. (1982) proposed a model for zein structure. Because of the similarity between zein and kafirin, the model proposed by Argos et al. (1982) can probably be used to schematically represent kafirin molecules. The model was proposed based on the amino acid sequence of the α-zein molecules with M_r 19 and 20 k. However, zein and kafirin can also exist as β- and γ- forms with different M_r. This is not taken into consideration in the model. The model assumes that zein exists as oligomers and polymers linked by hydrogen bonds between the glutamine rich ends of the molecules. The occurrence of disulphide bonds that can occur between cystine residues of β- and γ- species of the protein that lead to formation of oligomers and polymers are not shown in this model. Thus, the model can be regarded as α-kafirin. So far, there is no conclusive evidence for the Argos et al.(1982) model and it is only speculative. Not withstanding these criticisms, the model proposed by Argos et al. (1982) can be used to show schematically the cross-linking mechanism of kafirin by tannins.

Kafirin molecules exist as oligomers or polymers (El Nour et al., 1998). Kafirin oligomers or polymers are probably broken down to form monomers during dissolution or solubilisation in aqueous ethanol at high temperature. During kafirin dissolution, heat application can probably break the hydrogen bonds between the intra and intermolecular β-sheets of kafirin in dry form used to probably form random coils (as discussed in section 2.3). In addition, heat can probably break the hydrogen bonds between the glutamine residues. The net effect of kafirin dissolution as schematically illustrated in Figure 3.4 would be to produce individual kafirin molecules containing α-helices and some random coils.

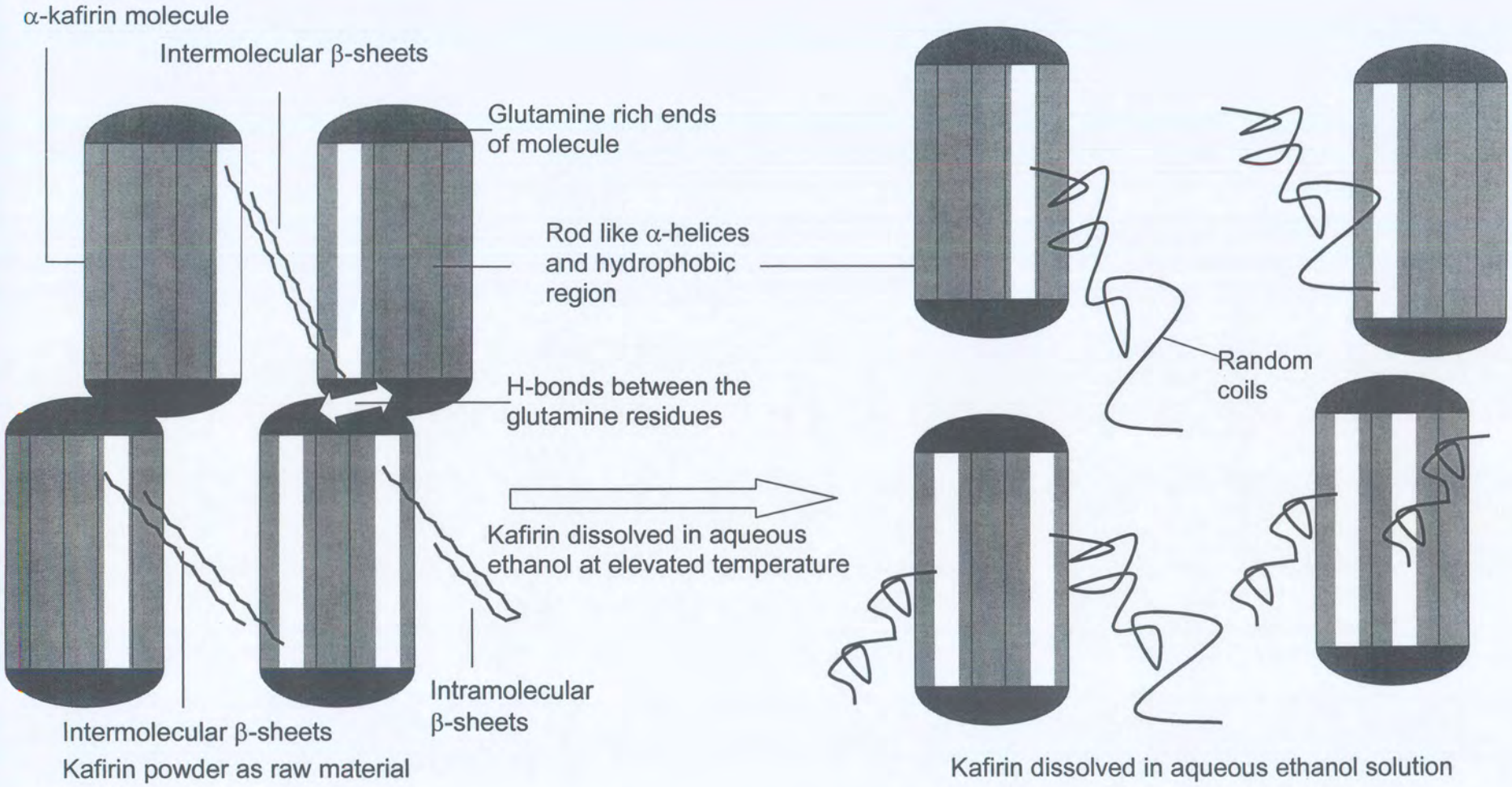

Figure 3.4 Schematic model for kafirin monomers and oligomers from the Argos et al. (1982) model showing the proposed form of kafirin in dry state and kafirin in solution.

Tannins can be illustrated as circular shape with spikes to represent their binding sites. The binding of tannins at the hydrophobic region of the kafirin molecule, and with glutamine residues by hydrogen bonds as earlier discussed, is schematically shown in Figure 3.5. In addition, the figure also shows that the tannins can bind with two polypeptide chains at the same time. These polypeptides would have formed β-sheet structures in the absence of tannins when the hot ethanol solvent is cooled down or when evaporated during film formation. The tannin binding can thus cross-link two polypeptide chains. The cross-linking would result in high molecular weight protein that would probably precipitate out. The model also supports the occurrence of restricted molecular mobility that would cause the increase in T_g ,and decrease in free volume to decrease oxygen permeability of modified kafirin films. It can also support the observed increased strength of modified kafirin films (section 2.2).

As shown in section 2.1, the various types of phenolic compounds had different binding affinity for kafirin. The hydrolysable tannin, TA, gave lower haze and less TA was bound to kafirin than SCT. There were also differences in effects on kafirin film ultrastructure, as shown by SEM with modification by TA as compared to SCT (section 2.2). FT-IR analysis of the precipitate formed when kafirin was complexed with TA indicated a smaller change in kafirin secondary structure when compared to SCT (section 2.3). These data indicate that SCT can interact more with kafirin than TA. The differences also suggest that the mode of interaction of hydrolysable tannins with kafirin may be different from that of condensed tannin-kafirin interaction. Hagerman et al. (1998) found that the hydrolysable tannin pentagalloyl glucose had lower binding ability with BSA than extracted and purified SCTs. These authors also suggested different mechanisms for the interaction between BSA with hydrolysable tannin compared with condensed tannins to account for the differences in binding activity. Pentagalloyl glucose probably interacts with BSA by forming a hydrophobic coat around the protein to precipitate it out, whereas condensed tannin from sorghum probably forms hydrogen-bonded cross-links between

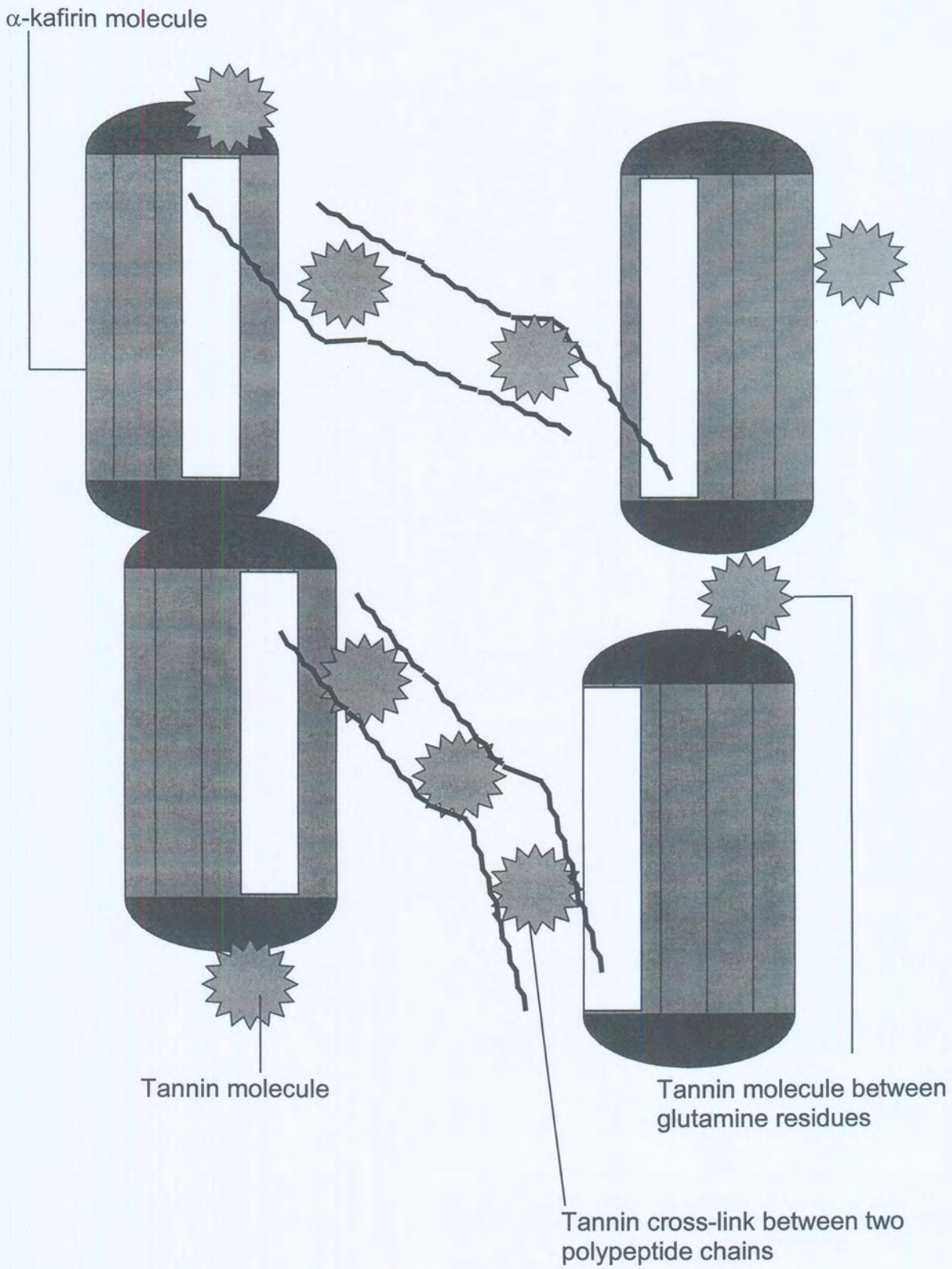

Figure 3.5 Cross-linking of the kafirin molecules by tannins

protein molecules. In this study, based on the lower binding of TA, it is possible that TA may form a hydrophobic coat around kafirin molecules. Spencer et al. (1988) suggested that phenolic compounds can form a coat around protein during interaction, but precipitation only occurs when the coat is more hydrophobic than protein. However, as kafirin is so hydrophobic (Duodu et al., 2003), the hydrophobic coat formed by TA molecules could possibly be less hydrophobic than the kafirin, and could thus not cause precipitation. This may be the reason for the low binding and haze forming of TA compared with SCT when bound to kafirin. It can also be inferred that TA can form soluble complexes with kafirin that do not precipitate. In fact, soluble complexes have been suggested to occur during the interaction of tannic acid with BSA (Hagerman and Robbins, 1987).

The mechanism of kafirin cross-linked by TA and SCT as explained above shows that the numerous hydroxyl groups of the tannins can interact with different polypeptide chains of kafirin to cross-link the protein. This suggests that kafirin molecules can dimerize. These dimers could further interact with the tannins by the same mechanism to form kafirin oligomers and polymers. The net effect of dimerization, oligomerization and polymerization of kafirin by the tannins would be to produce large molecular mass proteins or more specifically protein aggregates that form haze, which can precipitate out of solution. Charlton et al. (2002) proposed that dimerization, oligomerization and polymerization occurs in the reaction between salivary PRP and pentagalloyl glucose to form large molecular mass tannin-protein aggregates. Sarni-Manchado et al. (1999) using SDS-PAGE under reducing conditions of complexed salivary PRP protein with grape seed tannins, showed the occurrence of an electrophoresis band that did not penetrate the separating gel as compared to non-complexed protein, which entered the separating gel. Results in section 2.3 also showed the occurrence of this type of very high M_r protein-tannin complex that did not penetrate the separating gel.

3.3 Modification of kafirin films with tannins

This work has shown that kafirin films can be chemically modified with tannins such as TA and SCT to change their functional properties. In brief, the modification of kafirin films with TA and SCT decreased the tensile strain, increased the tensile stress, increased the Young's modulus and decreased the oxygen permeability of the films (section 2.2). Here the properties of the tannin modified kafirin films are compared with modification of other protein-based films modified by other methods, and with synthetic films in Table 3.1.

As can be seen, modification by various means can bring about an increase in the tensile stress and decrease in the tensile strain of protein-based films. Modification with aldehydes, cysteine, UV radiation and transglutaminase results in cross-linking of the protein molecules as reviewed in section 1.2. The cross-linking is most likely through covalent bonds. This can increase the molecular forces between the protein molecules to provide more resistance against extension. Thus, there is a general increase in the tensile stress during modification of protein-based films. The increase in tensile stress and decrease in tensile strain of modified kafirin films with TA and SCT can also be attributed to cross-linking of kafirin molecules by the tannins. However as discussed above, cross-linking is probably through intermolecular hydrogen bonds and hydrophobic interaction between kafirin molecules and the tannins.

As reported (section 2.2), oxygen permeability of kafirin films decreased when modified with TA and SCT. A similar effect was obtained when soy films were modified with cysteine (Table 3.1). The probable reason as explained in section 2.2 could be a decrease in free volume as a result of reduced molecular mobility as indicated by an increase in T_g of the modified kafirin films.

Modification of the kafirin films with TA and SCT did not significantly change the WVP. Similarly, modification of soy protein films with cysteine did not decrease the WVP (Were et al., 1999). Although the reason why there is no decrease in the WVP is not known, it is suggested that the similarity may be explained by the mechanism of water permeation through a film during the WVP

Table 3.1 Mechanical and barrier properties of kafirin films modified with tannins and other protein-based films modified by various methods, and synthetic film

Film type with plasticizer	Tensile stress (MPa)	Tensile strain (%)	Oxygen permeability (cm^3 μm m^{-2} d^{-1} kPa^{-1})	Water vapour permeability (cm^3 μm m^{-2} d^{-1} kPa^{-1})
Kafirin films (plasticizer similar relative to Buffo et al., 1997) [a]				
Unmodified	2.4	109	135	0.98
Modified with TA at 10% (w/w)	3.0	65	68	0.90
Modified with TA at 20% (w/w)	5.0	24	47	0.94
Modified with SCT at 10% (w/w)	3.6	49	72	1.03
Modified with SCT at 20% (w/w)	4.9	24	57	0.98
Sunflower protein films (33% of glycerol relative to protein) [b]				
Unmodified	2.8	37	ND	ND
Modified with formaldehyde at 4.5%	4.1	19	ND	ND
Modified with gluteralmdehyde at 4.5%	3.9	35	ND	ND
Modified with chestnut tannin at 6%	3.5	22	ND	ND
Modified with tara tannin at 6%	4.5	22	ND	ND
Modified with gallic acid at 6%	2.0	33	ND	ND
Zein films (15% of glycerol relative to protein) [c]				
Unmodified	5.2	4.4	ND	ND
Modified with 50% glutaraldehyde solution	19.8	3.0	ND	ND

Table 3.1 (continued)

Soy films (25% of glycerol relative to protein)[d]				
Unmodified	3.70	28.4	578	A decrease of 5.5% with
Modified with 1% cysteine (w/w)	6.72	7.46	494	modification
Soy films (50% of glycerol relative to protein)[e]				
Unmodified	3.7	124	ND	No change
Modified with ultra violet radiation at 103.7 Jm^{-2}	6.1	85.8	ND	
Deamidated gluten films (35% of glycerol relative to protein)[f]				
Unmodified	1.14	376	ND	ND
Modified by transglutaminase action	2.34	455	ND	ND
Gelatin films (no plasticizer) [g]				
Unmodified	1	211	ND	ND
Modified with genipin at 2%	6.8	13	ND	ND
Synthetic films [h, i, j, k]				
Low density polyethylene	8.6-17	500	1870	0.08
High density polyethylene	17-35	300	427	0.02
Ethylene-vinyl alcohol	14	650-800	0.1	0.25

ND is not determined, [a] own data, [b] Orliac et al. (2002), [c] Parris et al. (1998), [d] Were et al. (1999),[e] Gennadios et al. (1998), [f] Larre et al. (2000a), [g] Bigi et al. (2002), [h] Biddle (1986), [i] Smith (1986), [j] Foster (1986), [k] Salame (1986)

determination using the cup method, as explained in section 2.2. Water can be in the liquid phase in films during permeation (Debeaufort et al., 1994). Water can act as a plasticizer and can increase WVP of starch-based films (Chang et al., 2000). It has also been shown to promote phase separation and an inhomogeneous microstructure in amylose films, to increase gas permeation (Stading et al., 2001). Thus these factors may have been responsible for the lack of effect of tannin modification on kafirin film water permeability.

Modification of kafirin with TA and SCT changed the functional properties of kafirin films, but the functional properties were still not similar to synthetic plastic films (Table 3.1). This indicates that further modification could be required. As stated, tannin modification probably affected the mechanical and barrier properties of kafirin films by cross-linking of the protein polypeptide chains through hydrogen bonding and hydrophobic interaction. Hydrogen bonds and hydrophobic interactions are much weaker than covalent bonds. Thus, creation of covalent type of cross-links as well as hydrogen bond cross-links may be a potential way to further modify the functional properties of kafirin films. When sunflower protein films were modified with aldehydes to promote covalent cross-links, the tensile stress of the modified films were higher to those modified with tannic acid (Orliac et al., 2002). Similarly, Heijman et al. (1997) found that the tensile stress of collagen modified with glutaraldehyde was higher than that modified with tannic acid. Phenolic compounds can be oxidized to form ortho-quinones. The oxidation reaction can occur by the enzyme polyphenol oxidase, metal ions or autocatalysis in mildly basic media (reviewed by Haslam et al., 1992). These ortho-quinones can react with the amides groups of proteins to form polyphenol-protein complexes.(reviewed by Haslam et al., 1992). These complexes are covalently linked. A similar approach could be used for kafirin modification by the tannins to promote covalent cross-links.

Another way to promote covalent cross-links is through enzymatic modification. Enzymes such as transglutaminase can introduce covalent cross-links between added glutamine residues of proteins (Larre et al., 2000a). The action of transglutaminase has been found to increase tensile stress and decrease the

tensile strain of gluten films (Larre et al., 2000a). As kafirin is rich in glutamine residues (Taylor and Schüssler, 1986), transglutaminase could be an alternative to promote covalent cross-links. The Argos et al. (1982) model for zein and kafirin suggests that glutamine residues may be linked at the two ends of kafirin molecules.

Although modification with TA and SCT did not produce kafirin films with mechanical and barrier properties similar to common synthetic plastic films, they could be used in applications such as coating of fruits and vegetable or other foods, flavour encapsulation, and probably in non food applications like in the pharmaceutical industry for drug encapsulation. Two examples will be taken to explore the potential of modified kafirin films. Kafirin will be used as a thin film to coat litchi and nuts to evaluate its potential to extend the shelf life of these products in the Enviropak project (Enviropak, 2002). These two food products are potential export products for Southern Africa to Europe.

Litchi is a seasonal fruit cultivated in tropical areas. Litchi pericarp browning is a postharvest aesthetic defect that involves the loss of the red leathery pericarp colour (Ray, 1998). Enzymatic oxidation of the red anthocyanin phenolic pigment to brown pigments (Underhill, 1992), and decolorisation of the red pigments when the acidity of the pericarp decreases (Underhill and Critchley, 1994) mainly cause this colour defect. Pericarp desiccation and microcracking enhance enzymatic activity (Underhill and Simons, 1993). Sulphur dioxide treatment with an acid dip can inhibit the enzymatic activity (Zauberman et al., 1991) and can maintain the red anthocyanin colour (Underhill et al., 1994). However, sulphur dioxide treatment may not be accepted in certain countries for example in Europe due to the allergy to sulphur dioxide (Paull et al., 1998). A cellulose-based coating at low pH with citric acid was found to control pericarp browning by controlling moisture loss to reduce pericarp desiccation and microcracking, and by inhibition of polyphenol oxidase activity, and maintaining anthocyanin stability (McGuire and Baldwin, 1996). Another alternative could be coating of the litchi fruit with tannin-modified kafirin. The higher tensile stress of modified kafirin films can form a strong film around the fruits to probably prevent microcracking of the fruits. The modified kafirin coatings would provide extra

phenolic compounds that can enhance the colour of the fruits. The water barrier properties of the kafirin coating could to a certain extent prevent water loss reducing pericarp desiccation. In addition, the kafirin film casting solution contains lactic acid. This can promote to lower the pericarp pH and thus to probably prevent the oxidation of the anthocyanins and maintain the pericarp colour.

Metabolic activity of fresh litchi fruit can be decreased through reduction in respiration rate by modified atmosphere packaging to extend the shelf life of the fruit. However, It is important to prevent anaerobic respiration of fruits and vegetables during modified atmosphere packaging as this can cause off-flavours (Schlimme and Rooney, 1994). A low oxygen concentration of 3-4% under modified atmosphere was found to maintain a shelf life of litchi up to 22 days under refrigeration (Thekallat et al., 1997). Increased level of modification with tannins gave a decreased oxygen permeability of kafirin films. This suggests that kafirin film could be designed to fit the oxygen requirement without causing anaerobic respiration, but at the same time reduce respiration rate to decrease metabolic processes. Thus, modified kafirin films could provide combination effects that can potentially maintain the postharvest quality of litchi.

Another example of the application of tannin modified kafirin to maintain food quality is coating of nuts. Nuts are generally low moisture and high fat foods. The common mode of spoilage of nuts is lipid oxidation (reviewed by Trezza and Krochta, 2002). This causes rancidity and staleness (reviewed by Trezza and Krochta, 2002). Several methods such as cold storage, use of antioxidants and use of high oxygen barrier plastic films can maintain the quality and extend the shelf life of nuts (reviewed by Trezza and Krochta, 2002). The modification of kafirin films with TA and SCT decreased the oxygen permeability. This suggests that the modified kafirin films could be used for coating nuts to maintain a low oxygen concentration to decrease lipid oxidation.

Nut coated with tannin modified kafirin films could have an additional benefit in extending the shelf life quality of nuts. Tannins in tannin–protein complexes can also be antioxidants because they can act as radical scavengers and radical

sinks (Riedl and Hagerman, 2001). This could help in the prevention of fat oxidation in the nuts by quenching free radicals that lead to peroxide development and subsequent generation of off-flavour compounds (Coultate, 1996). Synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been incorporated as additives in zein used for coating of nuts to prevent rancidity (Andres, 1984). These synthetic antioxidants are not popular with consumers due to safety concerns (reviewed by Gennadios et al., 1994a). Thus, the presence of tannins as additive can be a natural alternative for the synthetic antioxidants. Furthermore, tannins as additives in kafirin films can provide an additional advantage as it could be marketed as a potential health benefit. Tannins are known as 'bioactive compounds' as they may have some health benefits in terms of the prevention cancer and cardiovascular diseases (Scalbert et al., 2002). The phenolic compounds can have antioxidant activity to scavenge some radical species to potentially prevent cancer (McAnlis et al., 1999). They have also been associated with inhibiton of the oxidation of low density lipoprotein to help prevent cardiovascular diseases (Kerry and Abbey, 1997).

4. CONCLUSIONS AND RECOMMENDATIONS

Different types of phenolic compounds have different abilities to bind with kafirin to form haze. Ferulic acid, a phenolic acid does not form haze with kafirin. Crude extracts from condensed tannin-free sorghum representing flavonoid type phenolics and catechin flavonoid do not bind with kafirin to produce haze. Tannic acid (TA), a hydrolysable tannin, and extracted sorghum condensed tannins (SCT) bind with kafirin and form haze. The high binding capacity of tannins is probably due to their high number of the aromatic rings. SCT has a higher affinity for kafirin than TA, probably because it is of higher molecular weight.

TA and SCT can bind and complex with kafirin to modify kafirin films. Modification of kafirin films with TA and SCT increases the tensile stress, decreases the tensile strain, increases the Young's modulus, and decreases the oxygen permeability. However, modification does not have an effect on the film apparent water vapour permeability. The T_g of kafirin films increases when they are modified with TA and SCT, suggesting a decrease in the molecular mobility of the modified films. The higher T_g of the modified kafirin films also suggests a decrease in free volume of the molecules in the films. This can probably reduce the oxygen permeability.

From the FT-IR and Raman data it appears that TA and SCT interact with kafirin to affect secondary structure of the protein when they bind with kafirin and modify kafirin film. TA and SCT probably prevent the formation of β-sheet structures in kafirin. The hydroxyl groups of the tannin aromatic rings probably form hydrogen bonds with the carbonyl groups on the random coils of kafirin polypeptide chains. Thus the carbonyl groups from kafirin may be unavailable to reorganise into β-sheet structures. It can be inferred that proline residues, which are rich in kafirin, can be potential binding sites for the tannins through hydrogen bonds and hydrophobic interaction. Tannic acid and kafirin binding can also be hydrogen bonded between carbonyl groups of the galloyl groups of tannic acid and hydrogens from kafirin. Because the tannins have numerous

hydroxyl groups, it is possible that tannins can occupy several carbonyl groups simultaneously. The binding of several kafirin molecules by tannins may suggest that the tannins will decrease intermolecular β-sheets rather than intramolecular β-sheets. Thus, the tannins can probably cross-link kafirin polypeptide chains through numerous hydrogen bonds to form high M_r kafirin-tannin complexes that cause haze. The cross-linking is probably the cause to decrease molecular mobility of the kafirin polypeptides which increases the tensile stress, decreases tensile strain, and decreases oxygen permeability of modified kafirin films.

It can be theorized that γ-kafirin can preferentially interact with tannins in comparison to α-, β- kafirin. This is probably because of the high content of proline residues, the repeat sequence of proline residues and relatively high hydrophobicity of γ-kafirin. Further research like binding assays and spectroscopic analysis of the kafirin polypeptides is recommended to confirm this theory.

The increase in tensile strength of modified kafirin films suggests that it can form a stronger coating to reduce litchi pericarp desiccation. Modified kafirin films can have potential application in coating nuts to reduce oxidative rancidity as these films have low oxygen permeability. In addition, the presence of TA and SCT could act as antioxidants to prevent oxidative rancidity of fats in the nuts.