Protein-Phenolic Interactions in Food

By

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ABSTRACT

Interest in protein-phenol interactions in biological systems in general has increased substantially during the past two decades. More recently, there has been particular interest in protein-phenol interactions in food system, as a result of widespread reports on the role of phenolic compounds in nutrition and health. Many phenolic compounds are now recognized for their nutraceutical properties. In the case of some of these biological active phenolic compounds, the proposed nutritional and health effects can be associated with their relationship with certain proteins. A good example is the recent recognition of soybean proteins containing isoflavones, for their health benefits and for prevention of certain diseases. In order to understand the exact roles of proteins and phenols in these protein-phenol relationship, it is essential to determine the nature of the chemical/physicochemical interactions between the proteins and the biologically active phenols. Our objective was to investigate the mode of interaction between selected food proteins and phenolic compounds. Bovine serum albumin (BSA), bovine β-lactoglobulin, and soybean glycinin were used with the following phenolic compounds; 3,4,5-trihydroxybenzoic acid (gallic acid), 3,4-dihydroxy cinnamic acid (caffeic acid), *p*-hydroxycinnamic acid (courmaric acid), and 5,7-dihydroxy 4-methoxy isoflavone (biochanin A). The interaction was investigated using incubation temperatures of 35°, 45° and 55°C at pH 5, 7 and 9. Native and SDS-polyacrylamide gel electrophoresis (PAGE), differential scanning calorimetry (DSC), and Fourier transform infrared (FTIR) spectroscopy were used to identify protein-phenol interactions. Certain phenolic compounds combined with BSA and prevented protein aggregation. In general, the thermal stability of the proteins increased as a result of interaction with phenolic compounds; the most pronounced effect was observed with β-lactoglobulin in the presence of gallic acid at pH 7. The interaction of the phenols with the proteins resulted in changes in protein secondary structure. Of the phenols investigated, the most pronounced effects were observed with gallic acid, while the least effects were observed with the isoflavone biochanin A.

RÉSUMÉ

L' intérêt pour les interactions protéines-phénols dans les systèmes biologiques en général a augmenté considérablement durant les deux dernières décennies. Plus récemment, un intérêt tout particulier a été porté sur les interactions protéines- phénols dans les systèmes alimentaires, résultant d'une augmentation de l'information sur les rôles des composés phénoliques dans les domaines de la nutrition et de la santé. Plusieurs composés phénoliques sont aujourd'hui reconnus pour leurs propriétés nutraceutiques. Dans le cas de certains composés phénoliques biologiquement actifs, les effets présentés dans cette étude sur la nutrition et la santé peuvent être reliés à leur relations avec certaines protéines. Par exemple, il a été reconnu récemment que les protéines de soya contenant des isoflavones présentaient des bénéfices pour la santé, comme la prévention de certaines maladies. Il est essentiel de déterminer la nature des interactions entre les protéines et les phénols biologiquement actifs afin d'estimer les rôles exacts des protéines et des phénols dans ces relations. Notre objectif a été d'étudier le mode d'interaction entre certaines protéines alimentaires et les composés phénoliques. L'albumine de serum de bovin (ASB), la -lactoglobuline bovine, et la glycinine de soya furent utilisées avec les composés phénoliques suivant; l'acide 3,4,5-trihydroxybenzoique (acide gallique), l'acide 3,4-dihydroxycinnamique (acide caffeique), l'acide *p*-hydroxycinnamique (acide coumarique), et la 5,7-dihydroxy 4-methoxy isoflavone (biochanine A). Les interactions furent étudiées sous des températures d'incubation de 35°, 45° et 55C° et à pH 5, 7 et 9. Gel sur polyacrylamide electrophoretic (natal et SDS) (PAGE), calorimétrie à scanner différentiel (DSC), et la transformée infrarouge de Fourier (FTIR), ont été utilisés pour identifier les interactions protéines-phénols. Certains composés phénoliques se sont liés à l'ASB et ainsi empêché l'aggregation protéique. En général, la stabilité thermique des protéines a augmenté résultant de l'interaction avec les composés phénoliques; l'effet le plus marqué fut observé avec la -l actoglobuline en présence de l'acide gallique à pH 7. Les interactions des phénols avec les protéines ont induit des changements dans la structure protéique secondaire. Parmi les phénols étudiés, les effets les plus prononcés furent observés avec l'acide gallique, en revanche, les plus moindres effets furent constatés avec l'isoflavone biochanine A.

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CHAPTER NO. 1

INTRODUCTION

Protein-phenolic interactions have received considerable attention in recent years. The importance of food proteins as a part of staple diet is well documented. Phenolic compounds are constituents of plants, including many that are consumed widely by humans. Data from human, and animal studies suggests that dietary phenolic compounds play important roles in protection against a wide range of human diseases including, cardiovascular diseases, certain types of cancers, and prevention of osteoporosis.

Protein-phenolic interactions can be categorized as either, reversible or irreversible. Reversible protein-phenolic interactions lead to the formation of insoluble complexes in solution; however, entirely new product can result from irreversible reactions. Tightly coiled, globular proteins show less affinity for phenolic compounds as compared to proteins that have random coil or lose conformation. Both the aromatic nuclei and the hydroxyl groups of the aromatic ring of phenolic compounds provide the principle binding sites for protein-phenol complexation. Hydrogen bonding and hydrophobic interaction have been found the primary attractive forces between protein molecules and phenolic groups.

Soybean is a recognized source of certain complex phenolic compounds in Asian diet. Soybean, besides being excellent source of protein (35-40%) and fat/oil (15-20%), also contains several isoflavones as a group of phenolic compounds (Messina, 1999). Soybean isoflavones have been reported to confer significant health benefits. The interest in soybean isoflavones has become intense to an extent that recently two international conferences have been convened to report the state of knowledge in the field.

Soybeans and soy products contain three isoflavones (genistein, daidzein, and glycitein) which occur in four possible forms, the aglycon, the glucoside, the malonylglucoside and the acetylglucoside (Wang and Murphy, 1994). Isoflavones are present in soy food as β-glucosides (Barnes et al, 1994) which, are hydrolyzed by the gut bacteria to release the highly absorbed aglucones. In October 1999 Food and Drug Administration (FDA) of United States responded to a petition by Protein Technologies International, by authorizing claims that soybean protein is good for heart function. According to FDA, in take of 25g soy protein a day can reduce the risk of heart diseases when used with low fat diet, the 25g soy protein recommended by FDA contains approximately 25mg of isoflavones. The isoflavones in soybean can be strongly attached with the proteins in the seed (Palevitz, 2000).

Though isoflavones show a strong interaction with soybean proteins, this protein-isoflavone relationship has not yet been fully investigated. The overall objective of our study is to use model system to investigate the interaction between proteins and phenolic compounds. Whereas, specific objectives are: 1) use PAGE to detect protein-phenolic interactions and 2) use DSC and FTIR to study effect of phenolic compounds on thermal characteristics of proteins.

CHAPTER NO. 2

LITERATURE REVIEW

2.1A Protein-phenolic interactions

The role of food proteins in nutrition and health has long been well established. More recently, the roles of phenolic compounds in human nutrition and health have become known. Consequently, investigations on protein-phenolic interactions have become the study of interest in many areas of food, nutrition and health. Polyphenols have been known to confer beneficial effects by acting as antioxidants, however they can also be harmful (Haslam, 1998). The nutritional effects of polyphenols are considered to be related to their capacity to bind and precipitate proteins (Jansman, 1993). The ability of polyphenols to form insoluble complexes with proteins, interferes with utilization of dietary proteins; these effects can be manifested in decrease in growth rate and/or feed conversion, as well as lower egg production (Myer and Gorbet, 1985). Polyphenols are known to bind proteins in beer, wine and fruit juices, resulting in undesirable turbidity and colloidal haze (Seibert, 1999). The complexation of polyphenols, as well as their enzymatic and non enzymatic oxidation products, with protein in seed, meals or flours has been reputed to reduce nutritional value of proteins from these sources (Haslam, 1998). Oxidized phenols can react with amino acids and proteins and inhibit the activity of proteolytic enzymes such as trypsin and lipases (Milic et al, 1968).

Tannins, which are polymers of phenolic compounds, have been investigated for their interactions with proteins; numerous studies on factors affecting tannin-protein interactions, such as pH, temperature, phenolic structure, protein size, and amino acid

composition and solvent characteristics in the medium have been reported (Serafini et al, 1997).

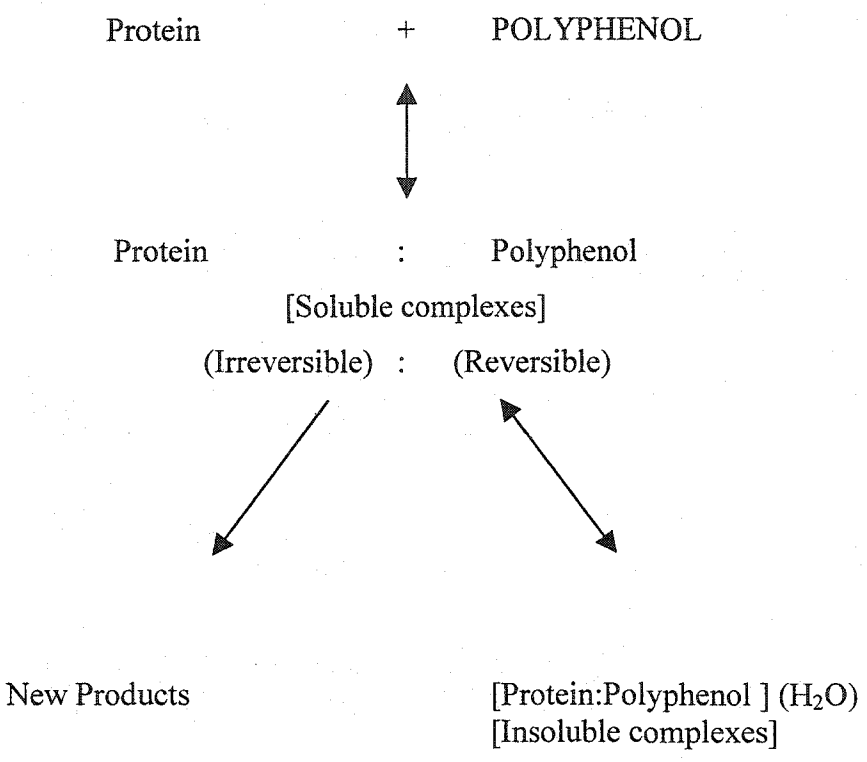
2.2A Formation of protein-phenolic complexes

Hagerman (1989) reported that the presence of bovine serum albumin (BSA) affected the relative antioxidant potency of low molecular weight phenolics, ferulic acid, caffeic acid, (+)- catechin, and this was partly attributed to the binding of phenols to the protein matrix. It has been suggested (Haslam et al, 1999) that hydrogen bonding between the phenolic hydroxyl groups, and the NH- and CO- groups of protein are involved in protein-phenolic interactions. In addition, hydrogen bonding and covalent linkages between oxidized phenols and nucleophilic amino-acid side chains, such as lysine or cysteine, are reported to be involved in binding of chlorogenic acid to sunflower proteins (Sastry and Rao, 1990). It has been reported (Siebert, 1999) that oxidized polyphenols leads to compounds that interact more strongly with proteins. The interaction between phenolic compounds and peptides can also involve formation of α-bonded complexes (Bianco et al, 1997). Hydrophobic interaction between nonpolar regions of the phenolic molecules and nonpolar domains of the protein may be responsible for weak interactions between phenolic compounds and proteins (Bartolome et al, 2000).

The interaction between polyphenols and proteins may be either reversible or irreversible (Figure 2.1) (Haslam et al, 1999). Irreversible complexes usually are formed autocatalytically in the presence of oxygen, or in the presence of polyphenoloxidases (enzymes) (Mole and Waterman, 1987), and can lead to the formation of clearly defined new products, as apparent in the case of black tea processing. Conversely, reversible complexation can take place via various non-covalent forces, such as hydrophobic

interactions, hydrogen bonding, solvation, and desolvation forces (Haslam et al, 1999).

Figure 2.1: Reversible and irreversible protein-polyphenol complexation (Haslam et al, 1999).



2.2.1A Characteristics of proteins and peptides

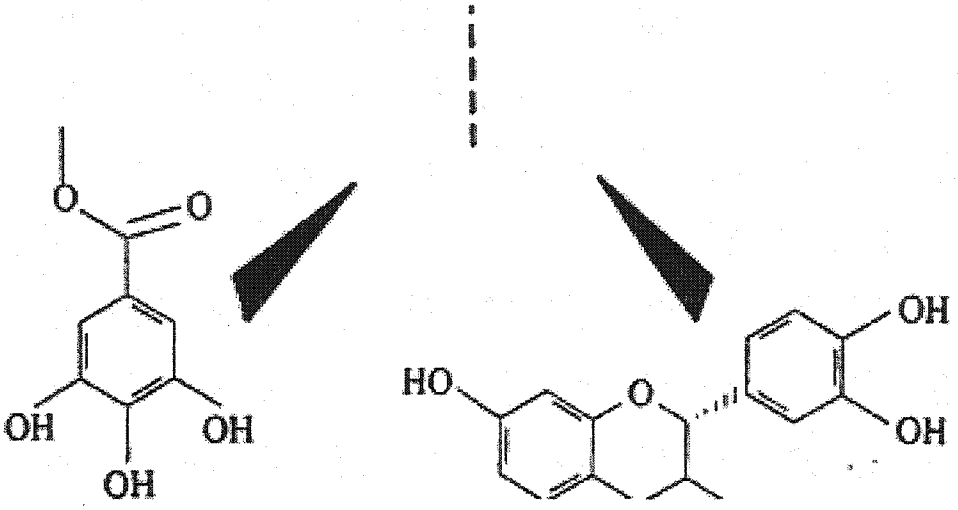
Proteins with high affinity for phenols usually have relatively higher molecular weight, open and lose conformational structure and are rich in proline (Hagerman, 1989). Glycosylated proteins also have enhanced affinity and selectivity towards binding phenols; this may be due to the ability of oligosaccharide portion of the glycoproteins to maintain the protein structure in a relative open conformation (Haslam et al, 1999). Phenols can readily precipitate proteins at or near their isoelectric points; however tightly

coiled globular proteins or those which have random coil conformation and in particular, which were rich in the amino acids proline (proline rich proteins- PRP) did not show the same characteristics. It was also shown that the relative affinities of polypeptides and proteins for the polymeric proanthocyanidin were influenced by their size (Siebert, 1999). The low affinity of small peptides and low molecular weight oligomers of proline for tannin, and the non-linearilty of the increase in affinity which was observed with higher oligomers of proline, suggest that the complexation involved the deployment of multiple binding sites on both substrates (Haslam et al, 1999).

2.2.2A Characteristics of phenols

It has been shown (Haslam et al, 1999) that molecular size, the number and disposition of phenolic nuclei, conformational flexibility, and water solubility are the dominant features in the determination of the strength of binding of a particular phenolic compound to the protein. High resolution NMR studies generally confirm that the aromatic nuclei of polyphenols provide the principal site for association with proteins (Figure 2.2) (Murray et al, 1994). The data obtained from equilibrium dialysis studies with bovine serum albumin (BSA) and a range of simple phenols (resorcinol, catechol, pyrogallol) gave scattered plots which revealed that the affinity of both catechol and pyrogallol for BSA was three times the order of magnitude greater than that of resorcinol; resorcinol, at 20°C had twice the solubility in water of catechol and pyrogallol. This observation supports earlier reports (Haslam et al, 1999) that the 0-dihydroxy and 0-trihydroxy aromatic nuclei of the neutral phenols represents the principal sites for complexation with proteins.

Figure 2.2. Principal aromatic sites in natural phenols for the complexation with proteins (Memanus et al, 1981).



Galloyl esters Flavan-3-ols

2.3A Tannin-protein interaction and its importance

Tannins are a major constituent of plants, including many plants that are consumed widely by humans (Murray et al, 1994). Tannins have been classified into two groups: the hydrolysable tannins, which are esters usually with D-glucose of gallic acid and its derivatives, and the condensed tannins (proanthocyanidins) which are oligomers of the flavan-3-ol skeleton, e.g. epicatechin. Their biological effects are wide-ranging and include inhibition of gut enzymes, inhibition of non-heme iron absorption, influencing the taste and reducing the nutrition value of food, esophageal cancer, and effects of astringency (Haslam, 1989).

Binding of tannin to protein molecules can bring about conformational changes in both the protein and the tannin molecules, thus lowering their solubility (Asguith and Butler, 1986). The lowest solubility of tannin-protein complex occurs at a pH near the isoelectric point of the protein (Hagerman and Butler, 1981). The tannin-protein interaction is also dependent on the initial concentration of both the tannins and proteins. Proteins are completely precipitated in the presence of excess tannins, however, excess of proteins led to the formation of soluble protein-tannin complex (Hagerman and Robins, 1987).

The formation of tannin-protein complexes is not only affected by the composition and structure of proteins but also by the size, length and flexibility of tannin molecules. It has been determined that tannin should have at least three flavanols subunits to be an effective protein precipitating agent; dimers precipitated proteins, but were much less effective, whereas simple flavanols did not precipitate proteins at all (Artz et al, 1987).

The binding of protein to tannin may be due to the formation of multiple hydrogen bonds between the hydroxyl groups of the tannins and the carbonyl group of the proteins (Haslam, 1974). The tannin-protein complex may also be stabilized by other types of molecular interactions such as ionic bonds between the phenolate anion and cationic sites on the protein molecule, and/or covalent links formed as a result of condensation of oxidized phenolic groups of tannins with nucleophilic groups (SH, OH, NH2) of proteins (Loomis, 1974), hydrophobic interaction between the aromatic ring of tannins and hydrophobic region of proteins may also be involved. The 1, 2-di- (or 1,2,3-tri-) hydroxyphenyl residue is considered as the prime binding site of tannins, it is

believed that tannin-protein complexation is usually the result of formation of hydrogen bonds and hydrophobic interactions (Oh et al, 1980).

2.4A Interaction of tannins with salivary proline-rich proteins

The salivary proline rich proteins (PRPs) comprise about 70% of the protein content of saliva, and have been divided into glycosylated, acidic and basic classes (Kauffman and Keller, 1979). Salivary PRPs consist of between 70-80% proline, glutamine and glycine, arranged as 5-15 almost identical repeats of an approximate 18-residue sequence (Wong and Bennick, 1980). It has been suggested that the main function of proline-rich repeats is to bind to polyphenols present in the diet, thus improving the bioavailability of the dietary proteins by protecting them from interactions with tannins (Warner and Azen, 1998). Feeding tannin-rich diet to rats and mice led to an initial weight loss; a normal weight gain resumed after about three days and was correlated with the induction of salivary PRPs synthesis (Mehansho et al, 1987). The high affinity of salivary PRPs for polyphenols has not only been attributed to their open random conformation, but also to prolyl groups which provide "stick patches" on the protein for the phenolic nuclei of the polyphenolic substrate (Murray and Williamson, 1994).

2.5A Interaction of low molecular weight phenolics

There have been relatively few studies dealing with the interaction of low molecular weight phenols and proteins. It has been demonstrated that additives interact with food proteins, leading to a decrease in the perceived intensity of the flavor (Hansen and Heinis, 1991). Certain protein concentrates from sunflower seeds, develop an

undesirable brown color because of protein binding with oxidation products of low molecular weight phenols such as chlorogenic acid (Sastry and Rao, 1990).

More recently, BSA was investigated for its interaction with low molecular weight phenols (Bartolomé et al, 2000). Protocatechuic and caffeic acid exhibited the highest binding for the protein, whereas p-hydroxybenzoic acid exhibited the lowest binding capacity; whereas, p-coumaric acid and (+)- catechin exhibited a negligible value for protein-retained phenols. Similar work with myoglobin and various plant phenols has shown that phenloic compounds effects the chemical and in vitro enzymatic degradation of proteins; the protein-phenol reaction was shown to occur at the lysine side chains and at the indole rings of the tryptophan residues of the myoglobin (Kroll et al, 2001).

2.6A Interaction of phenolic compounds with canola protein

Canola contains relatively high quantities of phenolic compounds. In spite of the nutritional potential of these proteins, their objectionable color and taste limits the use of these proteins in foods. They contain phenolic compounds with bitterness intensity similar to caffeine and readily undergo enzymatic and non-enzymatic oxidation to produce quinones which can then react with protein, causing a dark color in the final product (Gheysuddin et al, 1970). These organoleptic problems were believed to be caused by the presence of phenolic compounds (Sosulski, 1979). Phenolic compounds bind to canola protein products through a mechanism such as hydrogen bonding (Loomis and Battaile, 1966), hydrophobic interaction (Hagerman and Butler, 1978) and ionic bonding in aqueous media.

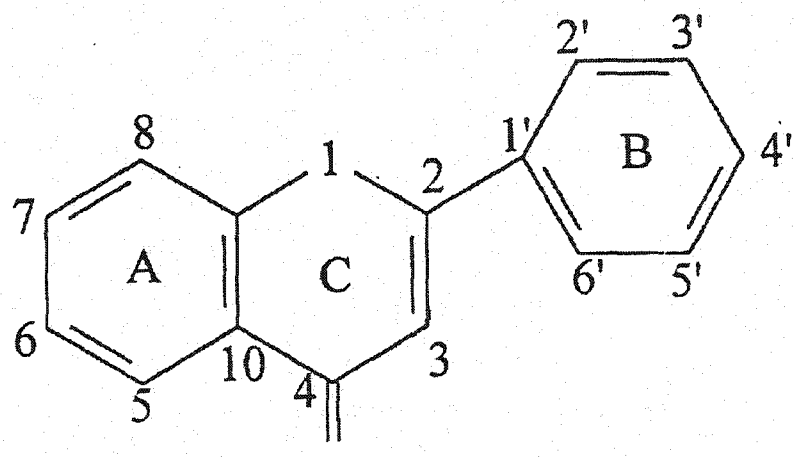
2.1B Interaction between phenolic compounds and soybean proteins

Soybeans are an important source of protein and fat, their composition in terms of moisture, oil, protein and carbohydrates being 8-10, 17-20, 38-40 and 26-29%, respectively. In addition, soybeans contain a variety of phytochemicals, and in particular, isoflavone, a class of phenolic compounds. Soybeans and soy products contain ≈1-3 mg isoflavones (Wang and Murphy, 1994). Isoflavones have received considerable attention in recent years for their potential role in the prevention and treatment of a number of chronic diseases including heart disease, certain forms of cancers, osteoporosis and also for their ability to relieve menopausal symptoms (Kennedy, 1998). Isoflavones are heterocyclic phenolic compounds belonging to a subclass of flavonoids. They are isomeric with flavonoids and differ only in the position of attachment of the aryl-B-ring to the central pyran nucleus, the second benzene ring is attached to 3 instead of 2 position (Figure 2.3). Common isoflavones have the usual 5,7,4′ -or 5,7,3′, 4′- hydroxylation patterns, but there are many rarer isoflavones that have no known counterpart in the flavone series (Harborne, 1967). As of 1994, chemists recognized more than 334 different kinds of isoflavones (Dixon, 1999). Isoflavones are considered to be non-nutritive, as they neither yield any energy nor function as vitamins.

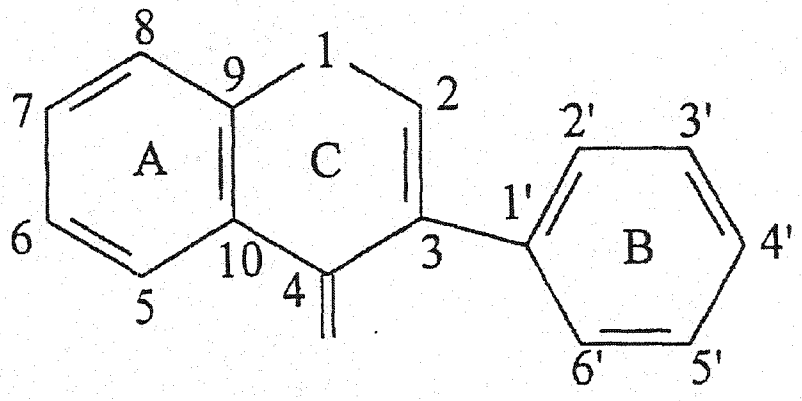
2.2B Chemical structures of soybean isoflavones

Soybeans contain twelve major isomers of naturally occurring isoflavones. They exist in soybeans either as aglucones or free forms (Tekel et al, 1999). The aglucone isoflavones include daidzein, genistein and glycitein; the glucosides of daidzein, genistein

Figure 2.3: Comparative illustration between flavone and isoflavone.



Flavone



Isoflavone

and glycitein are called as daidzin, genistin and glycitin, respectively (Wang and Murphy, 1994). Genistein and its glucoside, genistin, have been recognized for many decades, and it was believed that the primary form of this isoflavone in unprocessed soybeans was the glycoside form, genistin; similarly, daidzein and its aglucone daidzein have long been known as soy constituents. Relative proportion of glycitein and its glucoside, glycitin, are rather low in soy products (Wang and Murphy, 1994). Recent research shows that the predominant fraction of the isoflavone found in soybean and soybean extracts, especially in unprocessed or mildly processed soybean products, occur not as the glucoside form but rather as their 6″-O-malonyl esters (Shen et al, 2000). Chemically, these compounds consists of the glucoside form esterified with a single malonate half-ester, located at the primary (6″-) hydroxyl group of the sugar moiety. The malonyl esters are thermally and chemically unstable and are easily converted during soy products processing, especially via heating, toasting and/or at high pH, to either the free glucoside form or to yet another type of isoflavone derivative, 6″-O-acetyl esters, which are somewhat more stable than the malonyl forms (Wang and Murphy, 1994). The six derivatives of the glocusides which exist in soybean are 6″-O Ac daidzin, 6″-O Mal daidzin, 6″-O Ac genistin, 6″-O Mal genistin, 6″-O Ac glycitin and 6″-O Mal glycitin, "Ac" represents "acetly" and "Mal" represents malonyl (Shen et al, 2000). The general formula of the soybean isoflavones is shown in Figure 2.4. R1, R2, R3 and R4 may be selected from the group consisting of H, OH and OCH3 (Table 2.1) (Figure 2.5) (Wang and Murphy, 1994).

Figure 2.4: General structure of soybean isoflavones.

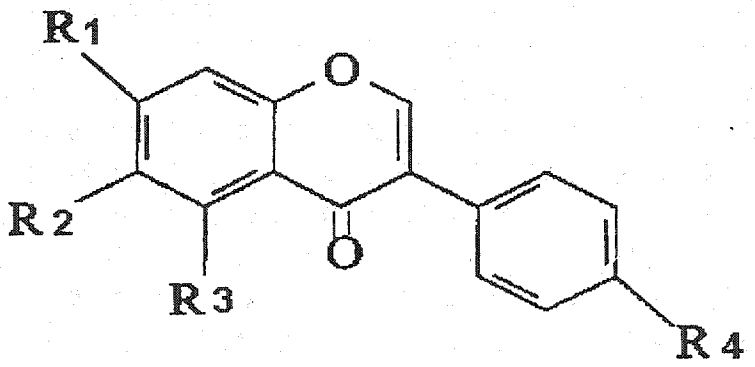
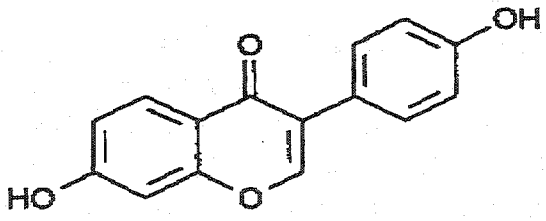


Table 2.1: Soybean isoflavones structural formula table.

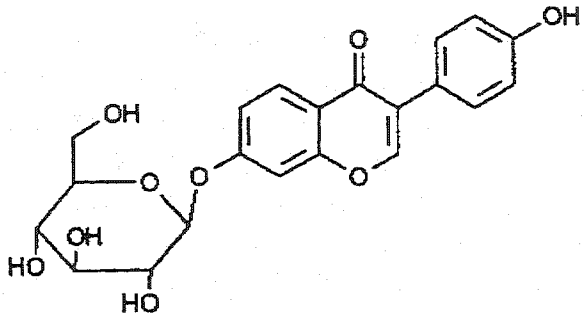
Compound	R1	R2	R3	R4
Daidzein	OH	H	H	OH
Daidzin	H	H	H	OH
6″-O Mal Daidzin	COCH.CO	H	H	OH
6″-O Ac Daidzin	COCH	H	H	OH
Genistein	OH	H	OH	OH
Genistin	H	H	OH	OH
6″-O Mal Genistin	COCH.CO	H	OH	OH
6″-O Ac Genistin	COCH	H	OH	OH
Glycitein	OH	OCH3	H	OH
Glycitin	H	OCH3	H	OH
6″-O Mal Glycitin	COCH.CO	OCH3	H	OH
6″-O Ac Glycitin	COCH	OCH3	H	OH

*Source: Wang and Murphy, 1994

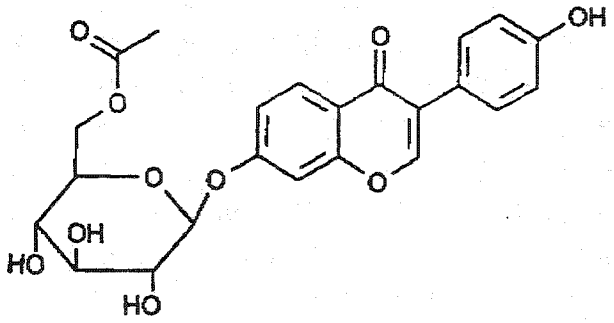
Figure 2.5: Chemical structures of twelve isoflavone isomers.



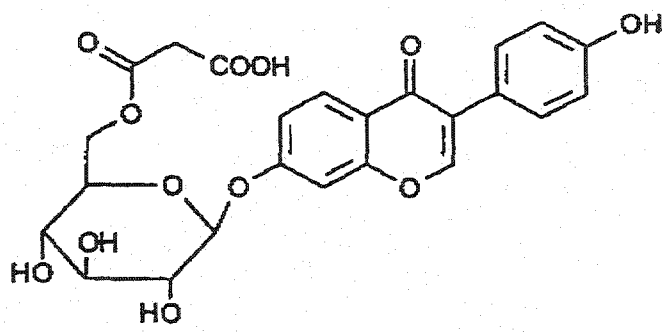
1. Daidzein [4',7-Dihydroxyisoflavone]



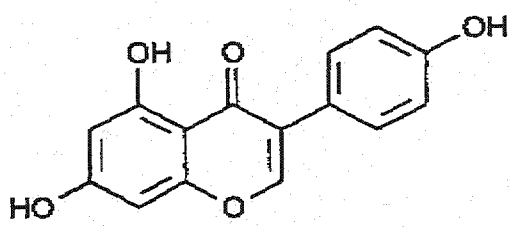
2. Daidzin [4',7-Dihydroxyisoflavone, 7-O-ß-D-glucopyranoside]



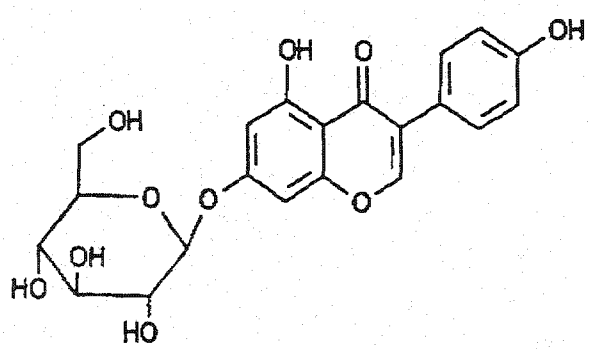
3. 6"-O-Acetyldaidzin



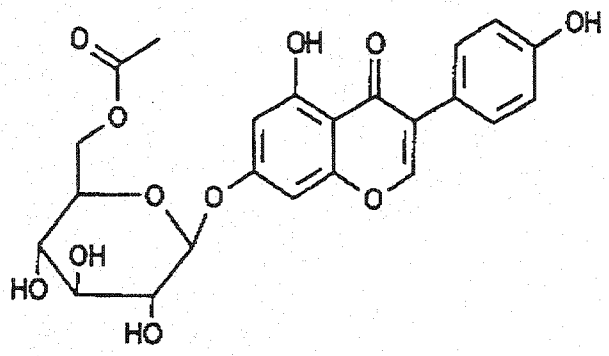
4. 6'-O-Malonyldaidzin



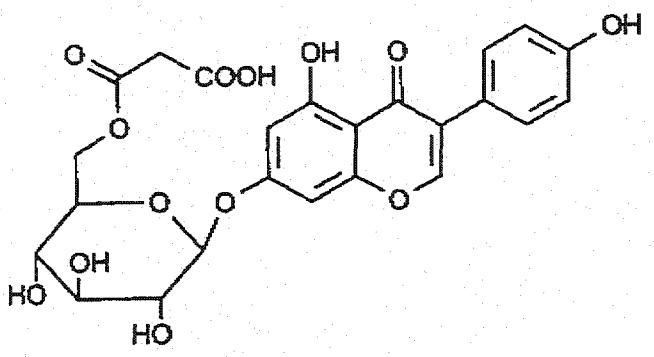
5. Genistein [4',5,7-Trihydroxyisoflavone]



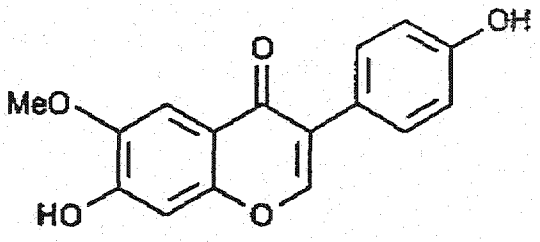
6. Genistin [Genistein, 7-O-ß-D-glucopyranoside]



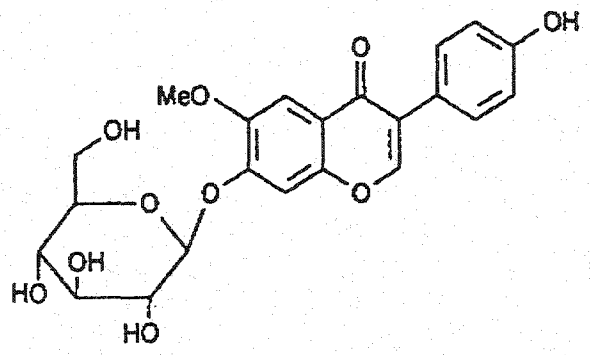
7. 6"-O-Acetylgenistin



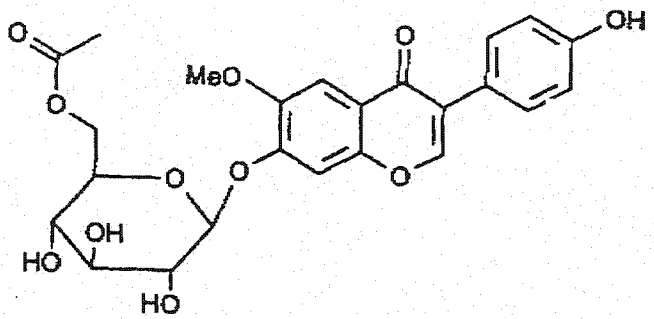
8. 6"-O-Malonylgenistin



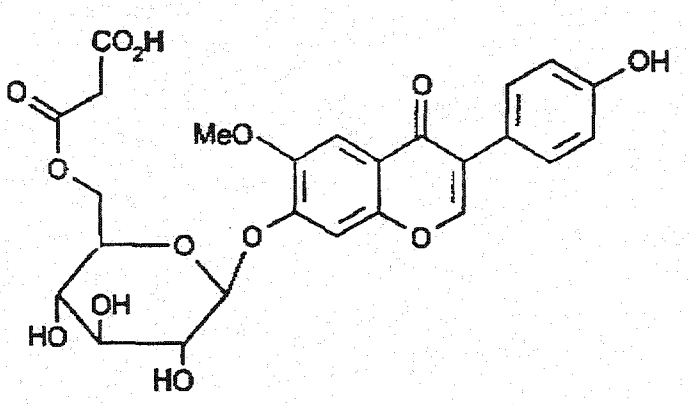
9. Glycitein[4',7-dihydroxy-6-methoxyisoflavone]



10. Glycitin [Glycitein, 7-O-β-D-glucopyranoside]



11. 6"-O-Acetylglycitin



12. 6"-0-Malonylglycitin

2.3B Importance of isoflavones in soybean protein foods

In October 1999, the Food and Drug Administration (FDA) of United States responded to a petition by Protein Technologies International, (Du Pont Company), by authorizing health claims for foods containing soybean protein products. The FDA has tentatively acknowledged that daily intake of 25g, soybean protein included in a diet low in saturated fat and cholesterol, may reduce the risk of coronary heart diseases (CHD). The 25g of soybean protein recommended by FDA contain approximately 45mg of isoflavones, mostly genistein and daidzein (Palevitz, 2000).

Isoflavones have received considerable attention in recent years. It has been hypothesized that isoflavones, which closely resemble estrogenic steroids in structure, bind to Er-β receptor, blocking more potent endogenous steroid, and presumably reduce the risk of hormone dependent tumors (Messina et al, 1994). Isoflavones had stronger binding affinity for Er-β than for Er-α receptor; Er-β is mainly in the brain, vascular tissues and bone, whereas Er-α is found mainly in breast, ovary and endometrium. Due to their similarity to estradiol-17B, isoflavones are also known as phytoestrogens (Price and Fenwick, 1985), however, they are weak estrogens with both oestrogenic and anti-oestrogenic activity with between 1/1,000 and 1/100,000 the activity of estradiol (Messina et al, 1994). Soybean isoflavones have also been reported to influence growth factors that regulate cell proliferation (Akiyamma, 1991), may reduce the risk of cardiovascular risk factors (Potter, 1998), osteoporosis, and may relieve menopausal symptoms (Messina, 1999).

2.3.1B Coronary heart diseases and isoflavones

Coronary heart diseases (CHD) is a leading cause of morbidity and mortality in the United State (Greaves et al, 1999). The dietary intake of soybean protein and soy-based food products has been linked to a reduction of CHD. The lower rates of mortality and morbidity due to CHD in Asian countries, compared with Western countries (Thom et al, 1992), may be due to considerably higher intake of soybean protein products in Asian countries (Adlercreutz, 1990).

Setchell (1985) was the first to suggest that isoflavones contained in soy-protein foods could contribute to the cholesterol-lowering effect, since they have weak estrogenic activity. Evidence supporting this hypothesis has come for several animals studies. Anthony et al, (1997) reported that diets containing intact soy protein isolates (SP+) and soy protein isolates from which isoflavone phytoestrogens had been removed by ethanol extraction (SP-) were fed to non-human primates. Estrogen-like effects were observed in serum lipoproteins in (SP+) fed animals showing reduced cholesterol and elevated HDL levels compared to (SP-) animals. In addition, the mean atherosclerotic plaque size was reduced in (SP+) animals suggesting the possibility of antiatherogenic effects caused by alteration in serum lipoprotein profile.

2.3.2B Isoflavones and cancer

Soybean foods and soybean components have received considerable attention lately for their potential role in reducing cancer risk. In 1990, a National Cancer Institute workshop identified several anti-carcinogens in soybean and recommended that the relationship between soy intake and cancer risk be investigated (Messina and Barnes, 1991), with particular interest on anti-cancer effect of soybeans isoflavones (Kennedy,

1998). Ingram, (1997) assessed the association between intake of isoflavones and breast cancer risk and concluded that increased excretion of some isoflavones is associated with a substantial reduction in breast cancer risk. On the contrary Lee et al, (1991) found a negative association between soy products in premenopausal, but not postmenopausal women in Singapore.

2.4B Adverse effects of isoflavones

In 1940's, several reproductive problems were observed in sheep in Western Australia, including ovarian cysts, irreversible endometriosis and a failure to conceive (Bennetts et al, 1946). The condition was named clover disease and was found to be caused by ingestion of subterranean clover (*Trifolium subterraneum*) which contains high levels of isoflavones (Bradbury and White, 1954). Dietary isoflavones have also been implicated as playing a role in the reproductive failure and liver disease of captive cheetahs in North America (Setchell et al, 1987).

Sheehan (1999) has indicated that soy isoflavone genistein causes estrogenic responses in developing and adult animals including adult humans, his Research suggest that during pregnancy in humans, soy isoflavones could be a risk factor for abnormal brain and reproductive tract development, and that isoflavones may cause thyroid abnormalities, including goiter and autoimmune thyroiditis. In the same way children who received soy formulas as infant were twice as likely to develop autoimmune thyroiditis compared to children who received other types forms of milk (Sheehan, 1999). The reproductive capacity of Asian women, who have consumed large amount of soy products as part of their staple diet for generations, does not appear to be affected by the high levels of isoflavones present in their food (Petrakis et al, 1996).

2.5B Distribution and origin of isoflavones

The first isoflavones to be isolated from soybean was genistin, which was obtained in crystalline form from a 90% methanol extract of soybeans, and acid hydrolysis, and was shown to yield its aglycone, genistein (Walter, 1941). Isoflavones have also been identified in other legumes (Axelson et al, 1984) respectively. Some phenolic compounds including isoflavones from plant sources are listed in (Table 2.2).

Table 2.2: Nonessential but bioactive phenolic compounds from plant source.

Compounds	Plant source
Phenolics	fruits, vegetables, soybeans, cereals
Elagic acid	vegetables
Curcumic	curry spice
Coumarin	vegetables, citrus fruits
Flavones	fruits, vegetables
Quercetin	berries, tomatoes, potatoes
Tangeretin	citrus fruit
Rutin	citrus fruit
Isoflavone	***flax seed, lentils, soybean***
Genistein	***soybean***
Daidzein	***soybean***

*Source: Swanson (1998)

2.6B Analysis of isoflavones

Various chromatographic techniques have been employed for the quantitative and qualitative analysis of soybean isoflavones. However, High Performance Liquid Chromatography (HPLC) is the method of choice for the analysis of most natural products. This form of chromatography is fast, most important reproducible, requires little sample and can be used for both qualitative and quantitative analysis as well as preparative work. A number of HPLC methods have been developed during the past decade for the determination of isoflavones (Mullner and Sontag, 1999). HPLC with reversed phase (C18) column can be directly used for the analysis of isoflavones in free or conjugated forms without any derivatization step. This method has been used for the analysis of isoflavone content in soybean foods (Wang and Murphy, 1994), legumes (Franke et al, 1994), and soy bean infant formula (Murphy et al, 1998).

2.7B Analysis of protein-phenolic interactions

Some of the methods used to investigate interactions, for a range of proteins and phenolic compounds are; a) ^{1}H and ^{13}C NMR spectroscopy b) microcalorimetry c) equilibrium dialysis d) enzyme kinetics and inhibition (Haslam et al, 1999). However, examining these effects, we have selected three different analytical techniques: polyacrylamide gel electrophoresis (PAGE), differentical scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR). These techniques have been found useful for studying various parameters on proteins in aqueous solutions. The objective is to evaluate the potential of mentioned techniques in studying protein-phenol interactions and to conclude regarding the mode of interaction between these compounds.

CHAPTER NO. 3

DETECTING PROTEIN-PHENOLIC INTERACTION USING POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

3.1 MATERIALS AND METHODS

3.1.1 Materials

Bovine serum albumin (BSA) (product A-2153) and β-Lactoglobulin (β-Lg) (product L-0130) were obtained from Sigma Chemical Co (St. Louis, MO, USA) and used as received. Cryoprecipitated soybean glycinin previously prepared in our laboratory (Ramadan, 2000) and stored at –20°C, were used. 3,4,5-trihydroxybenzoic acid (gallic acid, product G-7384) and 5,7-dihydroxy 4′-methoxy isoflavone (biochanin A, product D-2016) were purchased from Sigma Chemical Co (St. Louis, MO, USA). 3,4-dihydroxy cinnamic acid (caffeic acid, product 11493-0050) and *p*-hydroxycinnamic acid (*p*-coumaric acid, product 12109-0250) were purchased from Acros Organics (New Jersey, USA). The phenolic standards were stored at room temperature.

3.1.2 Preliminary experiment

The BSA solution (0.05%) was incubated at 35°C, 45°C and 55°C in a 15ml centrifuge tube with the gallic acid (0.05%) at a ratio of 1:1 in a water bath, for 2h. The solutions were prepared in phosphate buffer pH 3, 5, 7 and 9, ionic strength (0.01M), dilute acid and base were used to adjust the solutions to the desired. BSA with buffer only at pH 3, 5, 7 and 9 were used as controls in the experiment.

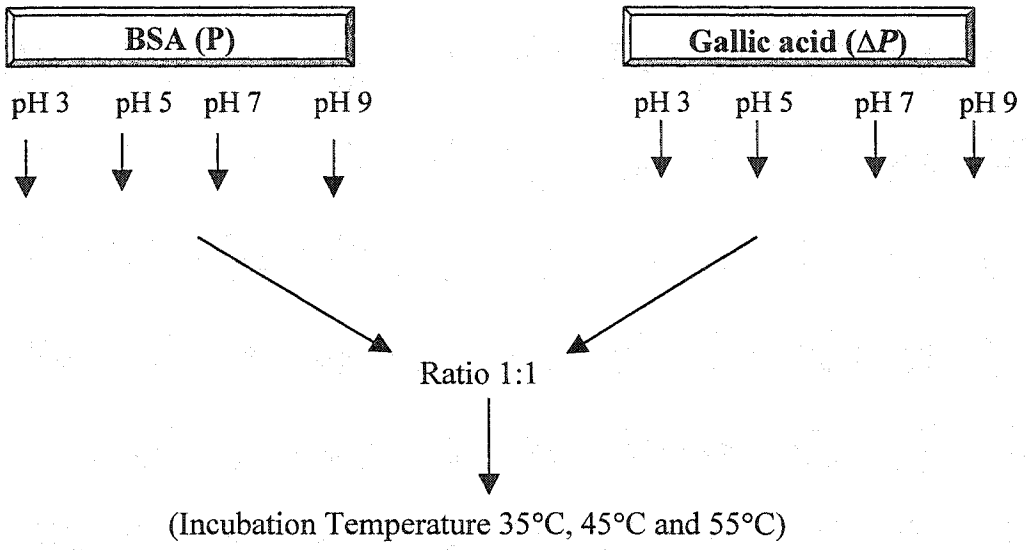
3.1.3 Preparation of solutions for preliminary experiment

Solutions were prepared from BSA in phosphate buffer at pH 3, 5, 7 and 9 by dissolving 6mg BSA in 6ml of each buffer in 15ml centrifuge tube. The gallic acid solutions were prepared by dissolving 3mg in 3ml of the respective buffer. Gallic acid was added to heated (100°C) buffer solution for 5min to facilitate solubility and then cooled to room temperature before incubating with BSA. BSA only was used as controls with the corresponding buffer. From each solution of BSA and gallic acid, 1ml was mixed at 1:1 ratio in a 15ml centrifuge tube. The final concentration for each reactant in the solution was (0.05% W/V). For the control, each centrifuge tube contained 1ml BSA and 1ml of corresponding buffer. The samples were incubated in a water bath at the desired temperatures (35°C, 45°C and 55°C) for 2h, then cooled to room temperature. A summary of the preliminary experiment is presented in Figure 3.1.

3.1.4 Preparation of solutions for protein-phenolic interactions

The solutions of proteins (BSA, β-Lg and soy glycinin) and phenolic standard (gallic acid, caffeic acid, *p*-coumaric acid and biochanin A) were prepared as described in Section 3.1.3. BSA was incubated with the four phenolic compounds; β-Lg and soybean glycinin was incubated with gallic acid and biochanin A. Compared to the preliminary experiment with BSA, the concentration was increased three times for β-Lg, and eight times for soy glycinin, compared to the concentration of BSA, and pH 3 was eliminated. Due to high concentration of soybean glycinin used, it was not possible to completely solubilize this protein in the buffer solution. The temperature and time of incubation were similar to those described in Section 3.1.3.

Figure 3.1. Schematic design of preliminary experiment.



P: Protein (BSA)
ΔP: Phenol (Gallic acid)

3.1.5 Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970) using a Mini-Protein II electrophoresis cell unit. All chemicals were purchased from Bio-Rad (Mississauga, Ontario, Canada, electrophoresis grade).

3.1.6 Sample preparation for SDS-PAGE

From the incubated solutions 262μL (containing 0.131mg BSA) was diluted with 300μL of sample buffer (3% SDS, 0.7 M 2-mercaptoethanol, 25 mM Tris-HCl pH 6.8, 1% glycerol and 0.05% bromophenol blue) in a microcentrifuge tube. Samples were heated at 95°C for 5min in a water bath. The final concentration of the protein was reached 0.23mg/ml (0.023%).

3.1.7 Concentration of gels and their preparation

Separating gels of 10% concentration and 6.5% concentration stacking gels were used. Separating gels contained 0.1% (W/V) SDS, 16% (V/V) glycerol and 0.375 M Tris-HCl (pH8.8); polymerization was initiated by the addition of 0.25% (W/V) ammonium persulphate (APS) and 0.05% N, N, N', N' tetamethylethylene diamine (TEMED). The 6.5% stacking gel included 0.1% SDS and 0.25 M Tris-HCl (pH 6.8). Polymerization was catalyzed by the addition of 0.1% APS and 0.1% (V/V) TEMED. After mixing the appropriate amounts of the reagents, the gel solutions were poured between 7 × 8 mm glass sandwich plates for polymerization. Before pouring the stacking gel, combs were mounted on the top of the gels to form 10 sample wells. The holding capacities of

individual sample wells were approximately 25μL. After polymerization, the combs were removed and the sample (15μL) was applied to the gels. The upper buffer chamber was then immediately filled with approximately 115ml of running buffer (50 mM Tris HCl, pH 8.3, SDS 0.1% and 1.384 M Glycine), until the buffer reached a level halfway between the short and long plates. The remainder of the buffer was poured into the lower buffer chamber so that, at least, the bottom 1cm of the gels was covered.

3.1.8 Sample loading

BSA solutions and BSA-gallic acid and solutions for Section 3.1.3 (Figure 3.1) (15μL) were loaded into the sample wells using a Hamilton syringe. The following broad range molecular weight protein standards were also loaded on the gels (10μL): myosin (Mw 200kDa), β-galactosidase (Mw 116.5kDa), phosphorylase B (Mw 97.4kDa), serum albumin (Mw 66.2kDa), ovalbumin (Mw 45kDa), carbonic anlydrase (Mw 31kDa), trypsin inhibitor (Mw 21.5kDa), lysozyme (Mw 14.4kDa) and aprotinin (Mw 6.5kDa).

3.1.9 Running conditions

The electrophorectic run was performed at a constant current of 15milliamps (mA) per gel. The voltage and power limits were 350V and 15W respectively. To maintain the temperature at 20°C, the bottom part of the electrophoresis unit was immersed in a water tub. The power supply was terminated when the tracking dye front (Bromophenol blue) reached the end of the gel; this took 1h and 17min.

3.1.10 Protein fixing, staining and destaining solutions

At the completion of the run, the gels were removed from the glass plates and immersed in a fixing solution (20% V/V methanol, 10% V/V acetic acid, and 70% distilled water) for 2h. The fixing solution was discarded and the protein bands were stained with 10% (V/V) acetic acid, 20% methanol (V/V) and 0.1% (W/V) Coomassie Brilliant Blue R250 for 10h. Destaining was accomplished by placing the gels in the fixing solution repeatedly until the background color was completely removed. The gels were stored in 7% acetic acid in a refrigerator until they were photographed.

3.1.11 Native-PAGE electrophoresis

Native electrophoresis was carried out according to the method described by Davis (1964) with some modifications. Electrophoresis was carried out on Mini Protein II electrophoresis cell unit (Bio-Rad, Hercules, CA).

3.1.12 Sample preparation

A total of 612µL from the incubated stock solution, containing 0.306mg of protein was mixed with 300µL of sample buffer (0.3 M Tris-HCl, pH 8.8, 10% glycerol and 25mg/ml bromophenol blue) to give a final protein concentration of 0.33mg/ml approximately 0.033%.

3.1.13 Gel concentration and preparation

A 10% separating and 6.5% stacking gel were used. Polymerization was initiated by the addition of 0.25% (W/V) ammonium persulphate (APS) and 0.05% TEMED for

separating gel and 0.1% (W/V) APS and 0.1% TEMED respectively. Gel preparation and sample loading were similar as described in the previous Section 3.1.7.

3.1.14 Electrophoresis conditions

The running conditions were similar to those described for SDS-PAGE, except that SDS was not the part of running buffer. A constant current of 7.5 mA per gel was applied. The time of the electrophorctic run was 2h and 45min for BSA and β-Lg, and 3h and 30min for soybean glycinin. The power limits and temperature was similar to that described in Section 3.1.9.

3.1.15 Protein fixing, staining and destaining solutions

Proteins fixing, staining and destaining was performed as described in Section 3.1.10.

3.2 RESULTS AND DISCUSSIONS

3.2.1 Effect of pH on color changes of phenolic compounds

Visual color changes were observed for the protein-phenol solutions at the different pH and incubation temperatures; no such changes were observed with the solution containing protein alone, suggesting that the color changes were due to the phenolic compounds. At 45°C, the proteins-gallic acid solutions changed to light green at pH 7.0 and dark green at pH 9.0; the color was intense at incubation temperature of 55°C. Friedman and Juergens (2000) reported that resonance, hydrogen-bonding, hydrated structure and colors of phenolic compounds are strongly influenced by pH. Our observation suggest that gallic acid was unstable at pH 9, based on visual color changes.

Protein-caffeic acid solutions incubated for 2h, at 45°C also changed color to reddish pink at pH 7 and 9.0, the change in the color was more intense at 55°C. The stability of caffeic acid is strongly influenced by pH in the range of 7-11 (Friedman and Juergens, 2000). No changes in color were observed for the solutions of proteins-*p*-coumaric acid at any incubation temperature and pH. *P*-coumaric acid, with a single hydroxyl group (Figure 3.2), was found to be more stable at high pH (Friedman and Juergens, 2000). No color changes were observed for the proteins-biochanin A solution. Biochanin A has a complex multiring aromatic structure (Figure 2.3) and is more stable to pH changes than the monoring phenolic compounds (Friedman and Juergens, 2000); this explains the observation that no color changes were observed for biochanin A at any pH range and incubation temperatures.

3.2.2 Preliminary experiment with BSA-gallic acid

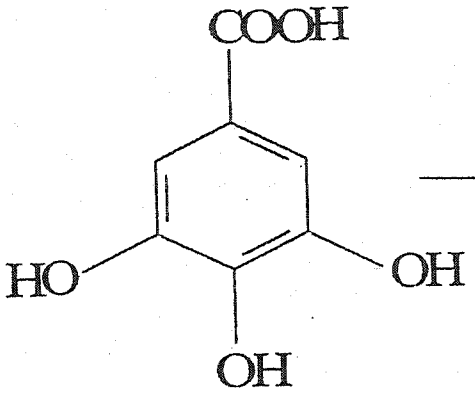
The SDS-PAGE results of BSA-gallic acid at 35°C, 45°C and 55°C and pH 3, 5, 7 and 9, are shown in Figure 3.3. There was no observed difference between the migration of BSA and BSA-gallic acid. In addition, there was no observed effect of pH on migration of BSA and BSA-gallic acid. On the basis of the information obtained in this preliminary experiment, pH 3.0 was not used in the remaining experiments.

3.2.3 Native-PAGE of proteins and phenolic acids

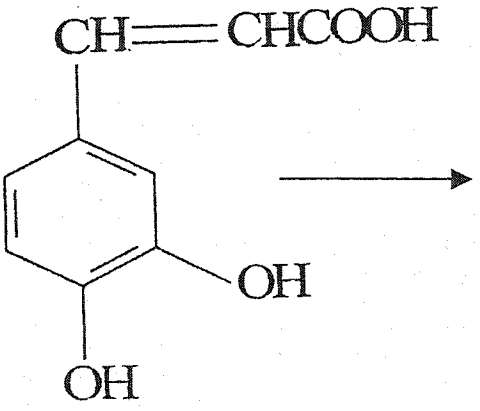
3.2.3.1BSA-phenolic compounds

Figure 3.4 shows the native-PAGE electropherogram of BSA-gallic acid and

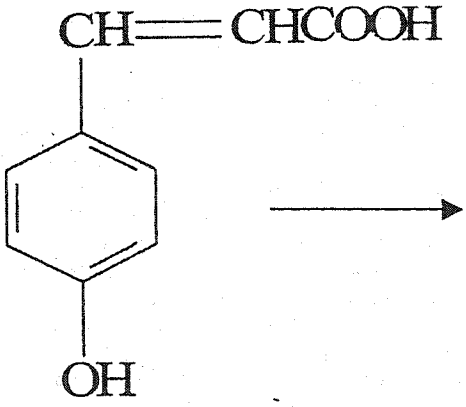
Figure 3.2: Molecular structure of: a) gallic acid b) caffeic acid and c) *p*-coumaric acid.



a) Gallic acid

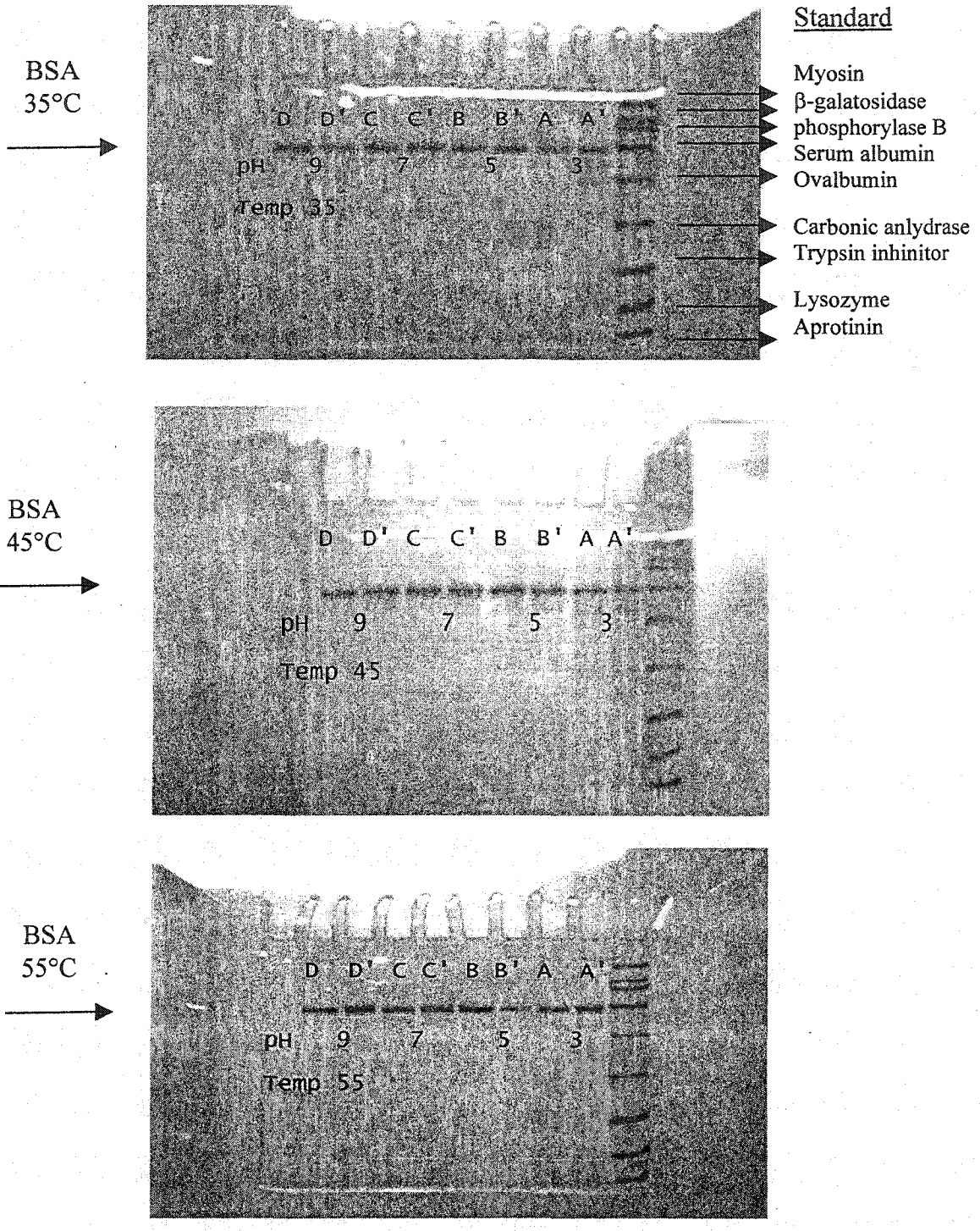


b) Caffeic acid



c) *P*-coumaric acid

Figure 3.3: Electropherogram (SDS-PAGE) of BSA at pH 3, 5, 7 and 9 incubated at 35°C, 45°C and 55°C. A′, B′ and C′: BSA-gallic acid and A, B and C: BSA alone.



BSA. BSA-gallic acid showed relatively higher migration than BSA alone at all pHs and all temperatures (Table 3.1). The maximum difference in relative mobility (Rf) was between BSA and BSA-gallic acid incubated at 45°C and 55°C at pH 7 and 9. The only difference in migration at pH 5 was observed at incubation temperature of 45°C; the Rf shifted from 0.35 to 0.36. The difference in relative mobilities can explain the increase in net negative (–) charge on the native BSA molecule, as a result of gallic acid, as the migration of the protein in native-PAGE is dependent on the charge of the protein molecule. BSA alone incubated at 55°C, at pH 7 and 9 (Figure 3.4, B and C) showed loss of band intensities with the corresponding appearance of slower migration bands; these bands are very likely aggregation bands of BSA. BSA-gallic acid did not show the slower migration bands (Figure 3.4, B′ and C′), suggesting that the gallic acid inhibited the aggregation of BSA. BSA starts unfolding with subsequent denaturation and aggregation; these changes are affected by the pH of the medium. Some proteins are known to be relatively stable with in a narrow pH range (4-7), however, exposure to pH outside this ranges often cause denaturation and subsequently aggregation of the proteins (Boye et al, 1996). The absence of aggregation bands of BSA-gallic acid is the indication of the protective effect against aggregation by the gallic acid. It should be mentioned that the unknown protein bands in Figure 3.4 is a protein impurity in the BSA standard and not an aggregation band. Inhibition of aggregation suggests that the hydroxyl groups of gallic acid can bind simultaneously at more than one site on the BSA molecule, this can prevent the unfolding of the BSA molecule.

Figures 3.5 shows BSA and BSA-caffeic acid incubated at 34°C, 45°C and 55°C. There were differences in mobility between BSA and BSA-caffeic acid (Table 3.2), for

Figure 3.4: Electropherogram (native-PAGE) of BSA at pH 5, 7 and 9 incubated at 35°C, 45°C and 55°C. A′, B′ and C′: BSA-gallic acid and A, B and C: BSA alone.

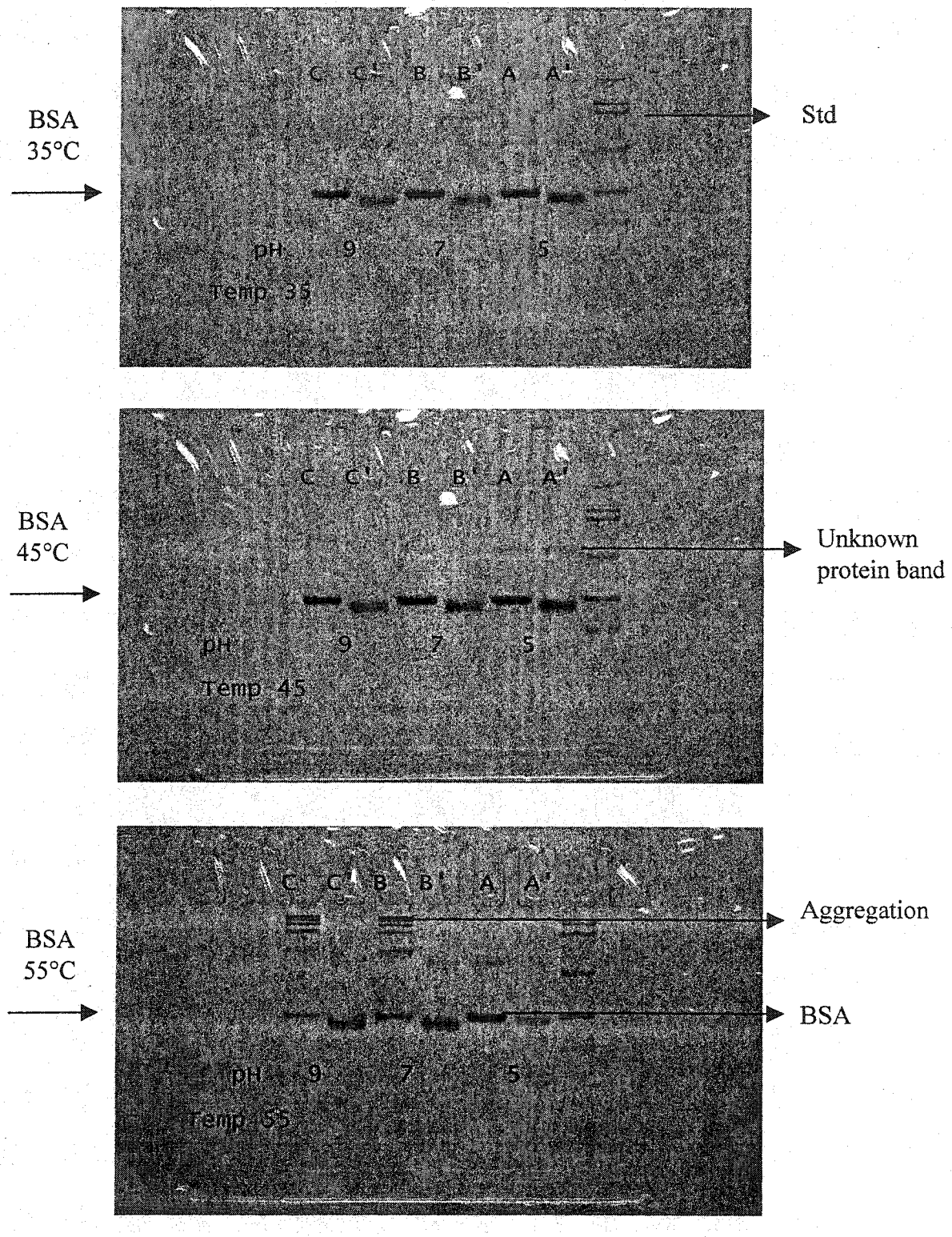


Table 3.1: Relative mobility (Rf, native-PAGE) of BSA incubated with gallic acid at 35°C, 45°C and 55°C.

pH	Compounds	Rf		
		35°C	45°C	55°C
pH 5.0	BSA-gallic acid (A′)	0.41	0.36	0.41
	BSA (A)	0.41	0.35	0.41
pH 7.0	BSA-gallic acid (B′)	0.43	0.38	0.43
	BSA (B)	0.40	0.35	0.40
pH 9.0	BSA-gallic acid (C′)	0.44	0.38	0.44
	BSA (C)	0.41	0.35	0.41

Figure 3.5: Electropherogram (native-PAGE) of BSA at pH 5, 7 and 9 incubated at 35°C, 45°C and 55°C. A′, B′ and C′: BSA-caffeic acid and A, B and C: BSA alone.

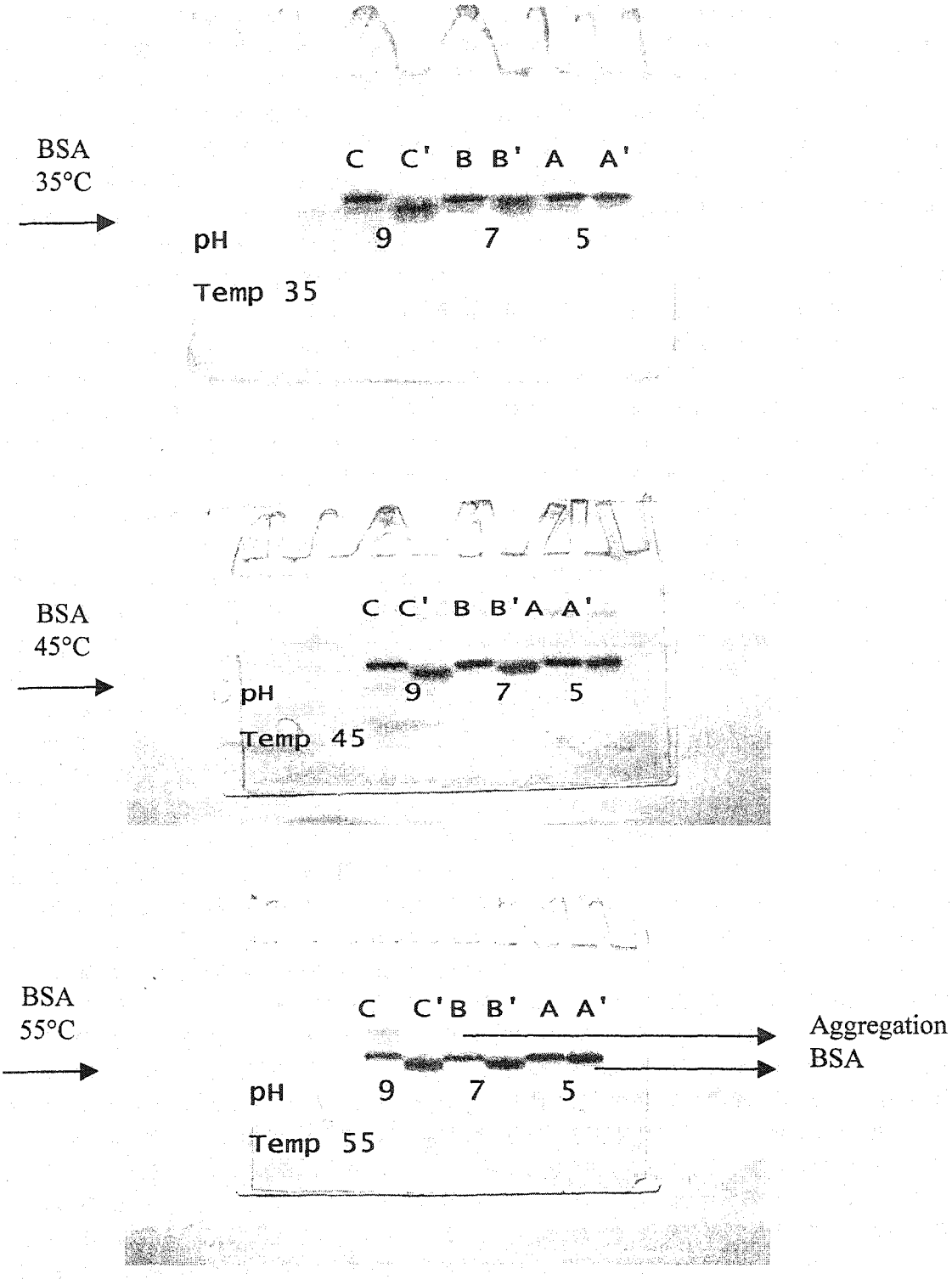


Table 3.2: Relative mobility (Rf, native-PAGE) of BSA incubated with caffeic acid at 35°C, 45°C and 55°C.

pH	Compounds	Rf		
		35°C	45°C	55°C
pH 5.0	BSA-caffeic acid (A′)	0.42	0.47	0.48
	BSA (A)	0.42	0.47	0.48
pH 7.0	BSA-caffeic acid (B′)	0.44	0.49	0.51
	BSA (B)	0.42	0.46	0.48
pH 9.0	BSA-caffeic acid (C′)	0.45	0.5	0.52
	BSA (C)	0.42	0.46	0.48

Figure 3.6: Electropherogram (native-PAGE) of BSA at pH 5, 7 and 9 incubated at 35°C, 45°C and 55°C. A′, B′ and C′: BSA-*p*-coumaric acid and A, B and C: BSA alone.

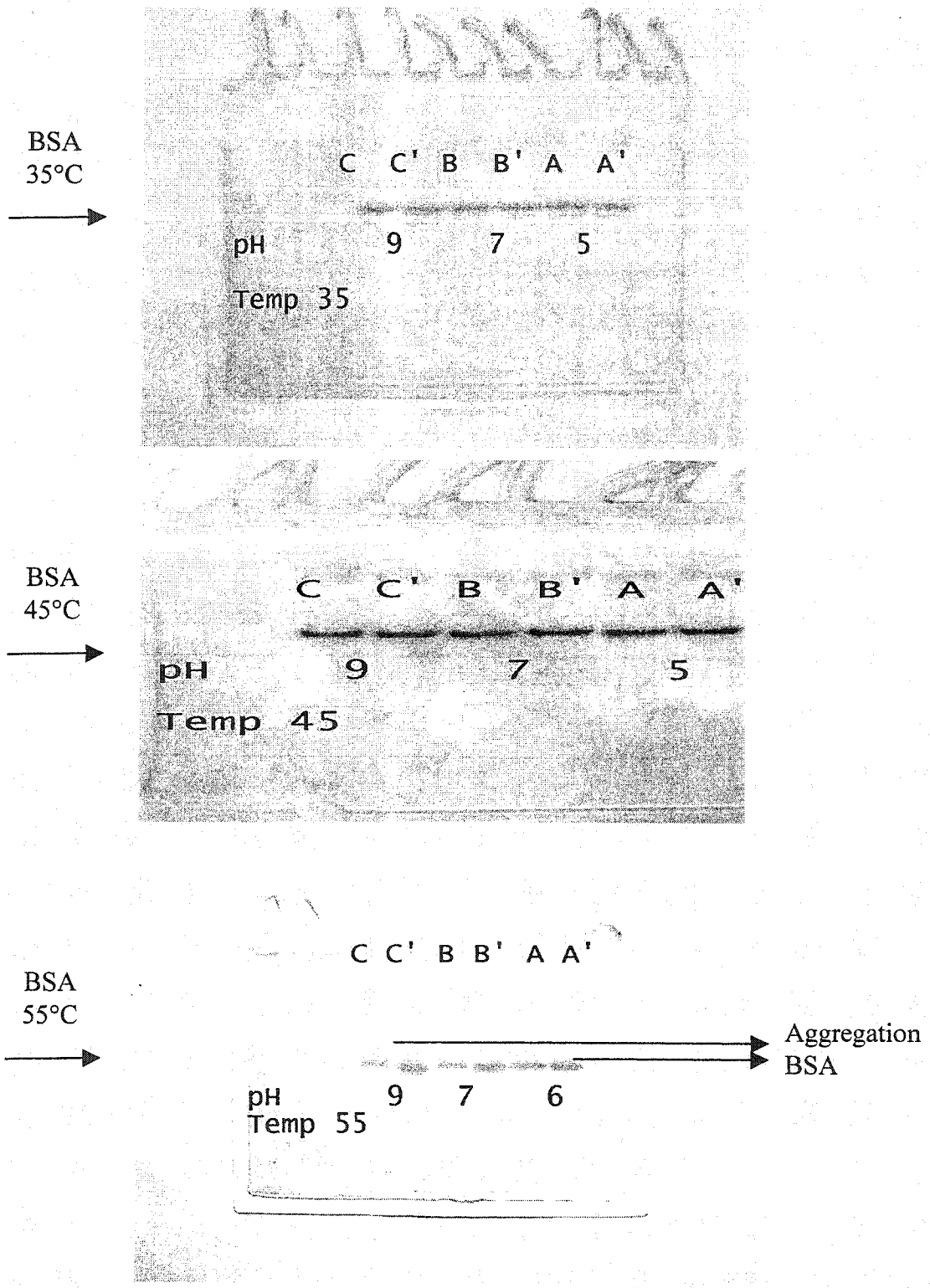


Table 3.3: Relative mobility (Rf, native-PAGE) of BSA incubated with *p*-coumaric acid at 35°C, 45°C and 55°C.

pH	Compounds	Rf		
		35°C	45°C	55°C
pH 5.0	BSA- *p*-coumaric acid (A′)	0.44	0.41	0.44
	BSA (A)	0.44	0.41	0.44
pH 7.0	BSA- *p*-coumaric acid (B′)	0.44	0.41	0.45
	BSA (B)	0.44	0.41	0.44
pH 9.0	BSA- *p*-coumaric acid (C′)	0.44	0.42	0.44
	BSA (C)	0.44	0.41	0.46

Figure 3.7: Electropherogram (native-PAGE) of BSA at pH 5, 7 and 9 incubated at 35°C, 45°C and 55°C. A′, B′ and C′: BSA-biochanin A and A, B and C: BSA alone.

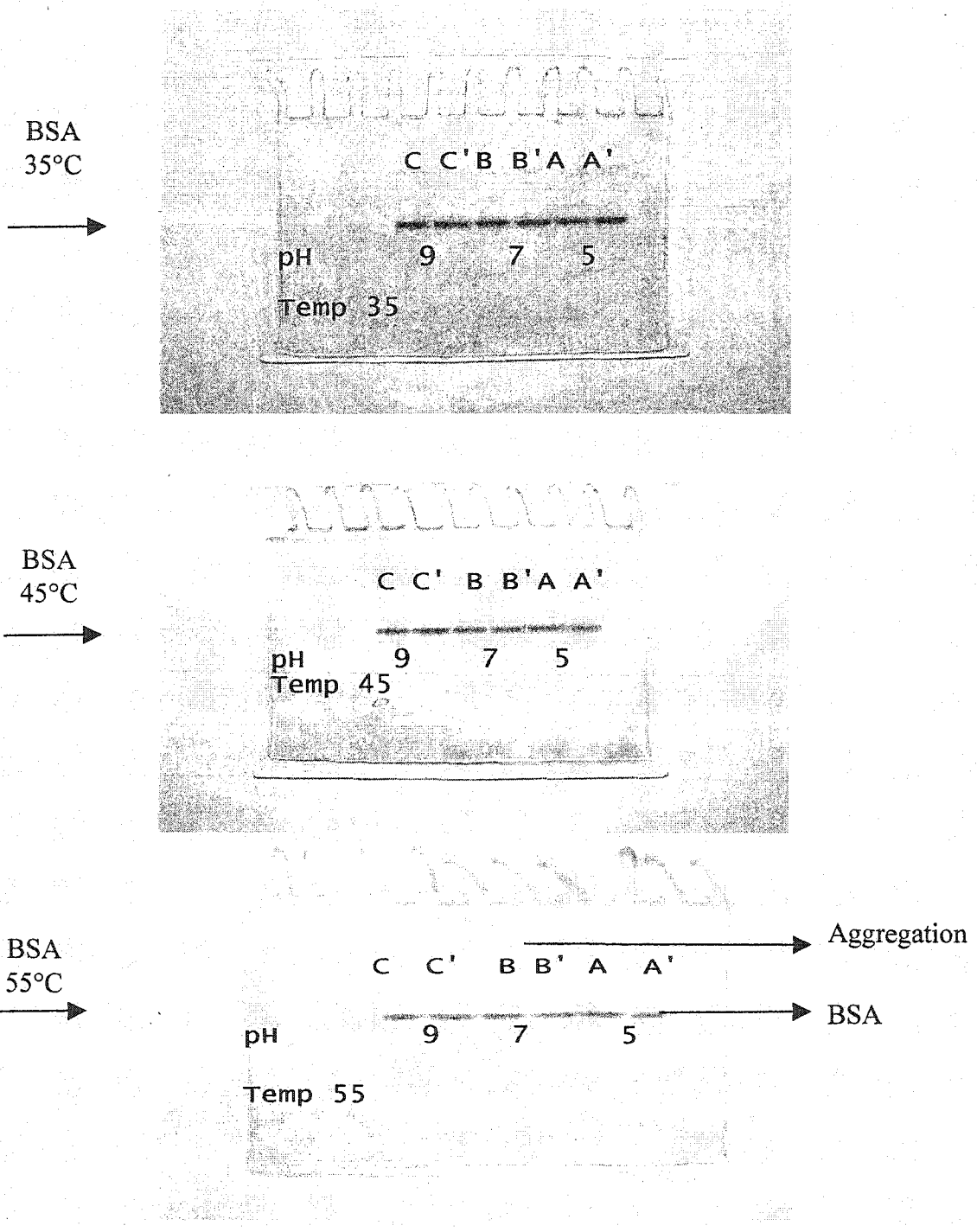


Table 3.4: Relative mobility (Rf, native-PAGE) of BSA incubated with biochanin A at 35°C, 45°C and 55°C.

pH	Compounds	Rf		
		35°C	45°C	55°C
pH 5.0	BSA- biochanin A (A')	0.45	0.46	0.45
	BSA (A)	0.45	0.46	0.45
pH 7.0	BSA- biochanin A (B')	0.45	0.46	0.45
	BSA (B)	0.45	0.46	0.45
pH 9.0	BSA- biochanin A (C')	0.45	0.46	0.45
	BSA (C)	0.45	0.46	0.45

pH 7 and 9 and all incubation temperatures. The Rf for BSA, at pH 9 in the presence of gallic acid, increased from 0.42 to 0.45 at 35°C; from 0.46 to 0.5 at 45°C, and from 0.48 to 0.52 at 55°C temperature. The effect of caffeic acid on the aggregation bands of BSA at 55°C were similar to gallic acid. Table 3.3 shows the relative mobility of BSA and BSA-*p*-coumaric acid. The Rf for BSA, at incubation temperature of 55°C, increased from 0.44 to 0.45 at pH 7 and 0.44 to 0.46 at pH 9, respectively. *P*-coumaric acid also showed inhibition of aggregation bands at 55°C, however, overall difference was not as significant compared to both gallic and caffeic acid (Figure 3.6).

Figure 3.7 shows the electropherogram of BSA and BSA-biochanin A; there was no marked difference between their relative mobilities (Table 3.4). The Rf calculated for both BSA and BSA-biochanin A incubated at 45°C, was slightly higher than at 35°C and 55°C. Though, aggregation of BSA at 55°C was inhibited in the presence of biochanin A.

3.2.3.2β-Lg with gallic acid and biochanin A

Figure 3.8 shows the electropherograms of β-Lg-gallic acid and β-Lg alone at 55°C. β-Lg show two bands; designated as variant A and variant B. β-Lg-gallic acid showed substantial differences in migration than the ones alone. For β-Lg-gallic acid migration was greater to an extent that the bands diffused and the two variants could not be distinguished. No difference was observed between β-Lg and β-Lg-biochanin A. The slower migration band appearing above the two variants is attributed to dimer of β-Lg; not the aggregation band since β-Lg has a denaturation temperature above 67°C (Boye and Alli, 2000).

Figure 3.8: Electropherogram (native-PAGE) of β-Lg at pH 5, 7 and 9 incubated at and 55°C with gallic acid (I) and biochanin A (II). A′, B′ and C′: β-lg-gallic acid and A, B and C: β-Lg alone.

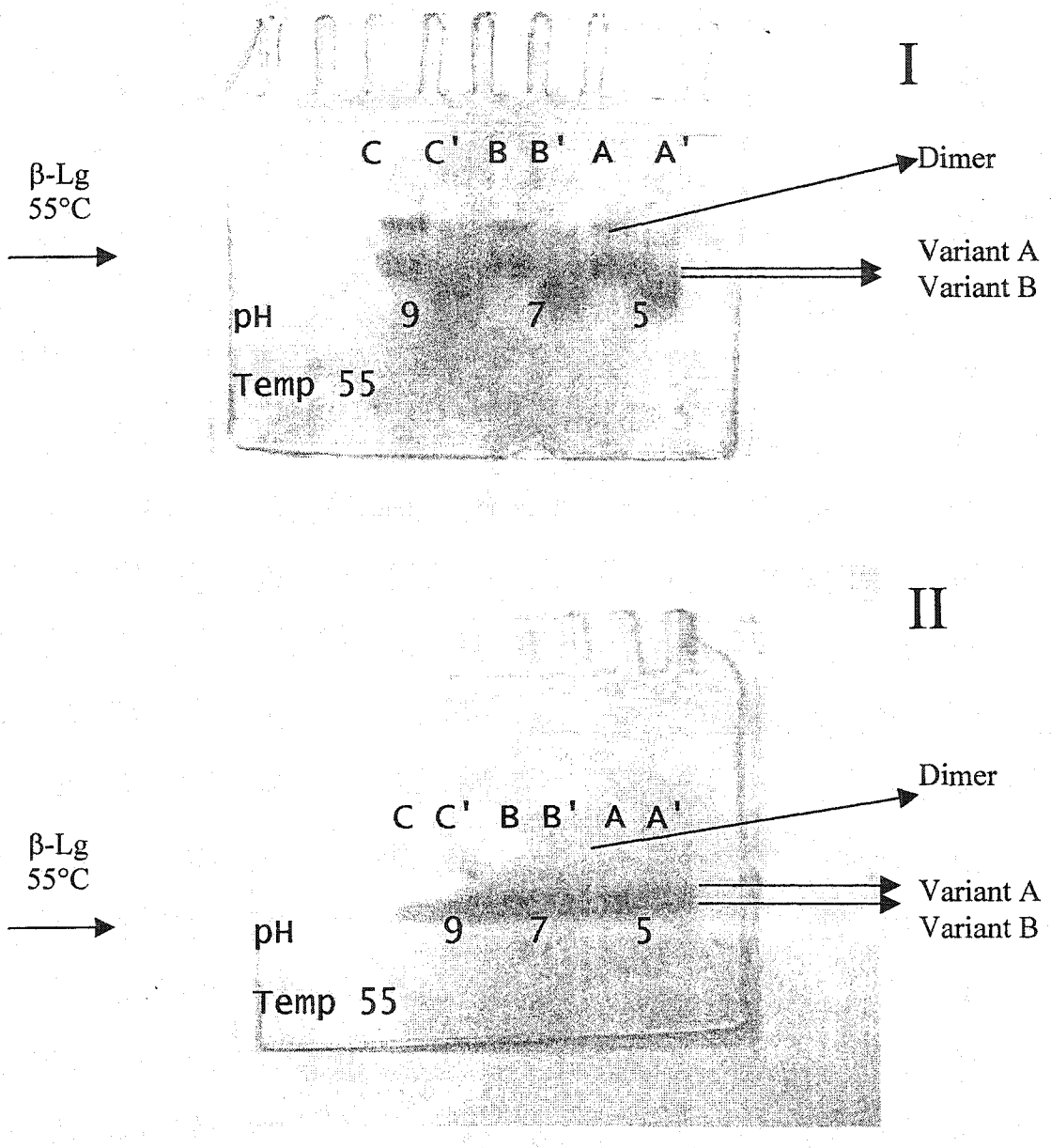
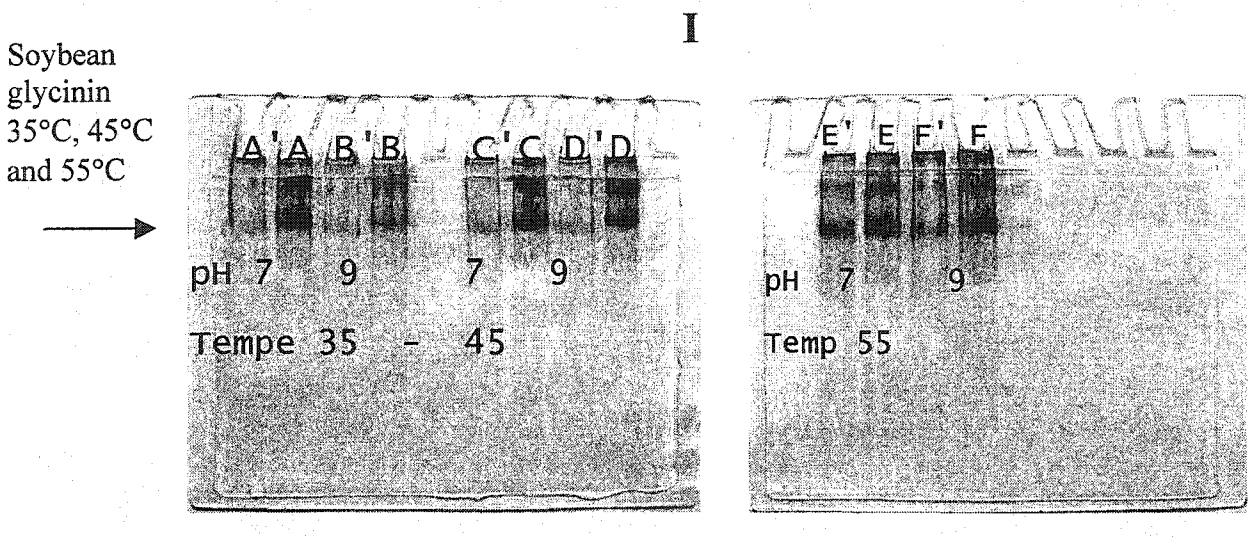
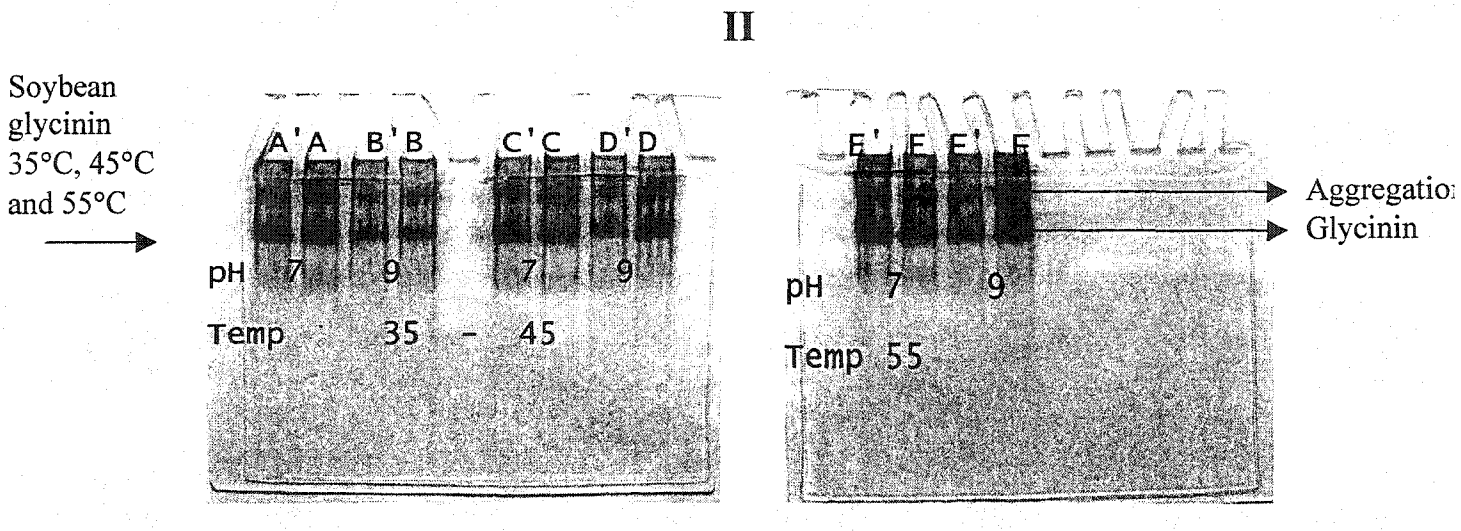


Figure 3.9: Electropherogram (native-PAGE) of soybean glycinin at pH 7 and 9 incubated at 35°C, 45°C and 55°C with gallic acid (I) and biochanin A (II). A′, B′, C′, D′, E′ and F′: soybean glycinin-gallic acid and A, B, C, D, E and F: soybean glycinin.





3.2.3.3Soybean glycinin with gallic acid and biochanin A

The electropherogram of soybean glycinin, glycinin-gallic acid and glycinin-biochanin A is shown in Figure 3.9. The results show two discrete bands; one representing glycinin and the other aggregate of glycinin. There was no noticeable difference in the relative migration between glycinin, glycinin-gallic acid and glycinin-biochanin A. The intensities of glycinin treated with gallic acid was less compared to glycinin band alone. The difference in the intensities can be a result of glycinin-gallic acid complexation, which can have resulted in precipitation of the glycinin. However, no difference was observed for the glycinin treated with biochanin A.

3.3 Conclusion

Potein-phenol binding was measured for a range of phenols and proteins. Native-PAGE was used successfully to identify effect of phenolic compounds on protein characteristics. Protein aggregation at 55°C was found to be inhibited by the presence of phenolic compounds. This protective effect was obtained with the four phenolic compounds investigated. On the basis of the observation on the effect of migration of BSA, it appears that inhibition can occur without changes on the charge properties of the BSA.

CHAPTER NO. 4

STUDYING PROTEIN-PHENOLIC INTERACTION USING DIFFERENTIAL SCANNING CALORIMETRY (DSC) AND FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

4.1 MATERIALS AND METHODS

4.1.1 Materials

Bovine serum albumin (BSA), β-lactoglobulin (β-Lg), and cryoprecipitated soy glycinin, 3,4,5 trihydroxybenzoic acid (gallic acid) and 5,7-dihydroxy4′-methoxy isoflavone (biochanin A) used in Section 3.1.1 of Chapter 3 were used.

4.1.2 Sample preparation for DSC

Untreated proteins (BSA and β-Lg, 20%, without gallic acid and biochanin A) were dissolved in phosphate buffer (pH 5, 7 and 9) ionic strength (0.01M). Soybean glycinin was dissolved in pH 7 and 9 buffer only. Proteins (20mg/50μl buffer) and gallic acid and biochanin A (1mg/50μl buffer) solutions were mixed to give final protein concentration of 20% containing 1% gallic and biochanin A. The gallic acid and biochanin A solutions were heated (100°C) in water bath for 7min for solubilization then cooled to room temperature, before reacting with protein solutions.

4.1.3 Differential Scanning Calorimetry

A Differential scanning calorimeter equipped with TC 11 processor (Mittler TA 3000, Mettler Instrument Corporation, Greitensee, Switzerland) was used to study the effect of gallic acid and biochanin A on the denaturation characteristics of BSA, β-Lg and soybean glycinin. Each 12μl of solution were placed in preweighed DSC pans, which

was same for both BSA, β-Lg and for soybean glycinin 70μl (medium pressure DSC pans). The samples were placed in the DSC and scanned from 30°C to 90°C for BSA and β-Lg and 40°C to 120°C for soy glycinin at a programmed heating rate of 10°C/min. For each run, a sample pan containing the buffer used for dissolving the protein, was used as reference. After heating, the samples were allowed to cool to room temperature in the DSC and the heating cycle was repeated under the same experimental conditions. The DSC was calibrated by using indium standards. All DSC experiments was done in duplicate.

4.1.4 Sample preparation for FTIR

BSA and soybean glycinin (10% W/V) samples were dissolved in deuterium oxide containing 0.01M phosphate buffer at pH 7 and 9. Gallic acid and biochanin A were dissolved in phosphate buffer pH 7 and 9 (1mg/50μl); the phosphate buffer was initially freeze dried and water replaced with deuterium oxide. Proteins (10mg/50μl buffer) and gallic acid and biochanin A (1mg/1μm buffer) solutions were mixed together to have a final protein concentration of 10% containing 1% gallic acid and biochanin A.

4.1.5 Fourier Transform Infrared Spectroscopy

FTIR analysis was performed on BSA and soybean glycinin to investigate the effect of gallic acid and biochanin A on the secondary structure of the proteins. An infrared spectrum was recorded with a 8210E Nicolet FTIR spectrometer equipped with a deuterated triglycine sulfate detector. A total of 512 scans were averaged at 4 cm^{-1} resolution. Wavelength accuracy was with in ±1 0.01cm^{-1}. The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA, USA) the samples were held

in an IR cell with a 50μm path length and CaF^2 windows. The temperature of the sample was regulated by placing the cell in a holder employing an Omega temperature controller (Omega Engineering, Laval, QC, Canada). The temperature was increased in 5°C increments, and the cell allowed to equilibrate for 3min prior to data acquisition. Deconvolution of the observed infrared spectra was performed using the Nicolet FTIR software, Omnic 1.2a. All FTIR experiments were done in duplicate.

4.2 RESULTS AND DISCUSSIONS

4.2.1 Effect gallic acid and biochanin A on thermal characteristics of BSA

The effects of pH on DSC characteristics of BSA with and without gallic acid are shown in Figures 4.1 and 4.2 and Table 4.1. At pH 5 the denaturation temperature (Td) peak of BSA was at 61.8±0.2°C, which was increased to 62.0±0.1°C for BSA-gallic acid. At pH 7, Td for BSA was at 58.2±0.1°C and 62.7±0.1°C for BSA-gallic acid. At pH 9, Td for BSA was at 57.8±0.3°C and 62.6±0.2°C for BSA-gallic acid. The enthalpies (ΔH) for BSA alone were 1.6±0.02 J/g, 1.1±0.04 J/g and 1.4±0.02 J/g at pH 5, 7 and 9, respectively, however, enthalpies for BSA-gallic acid increased to 1.7±0.02 J/g, 1.8±0.03 J/g, and 1.6±0.02 J/g at pH 5, 7 and 9, respectively. This data suggest that BSA-gallic acid showed greater thermal stability, than BSA at pH 7 and 9, but not at pH 5. The transition temperature of each domain in the order in which the domains unfold within a protein, has been reported to change with environment such as pH (Boye et al, 1999). The higher thermal denaturation temperature of BSA-gallic acid indicates higher thermal stability. This is consistent with the electrophoresis results, which demonstrated that

Figure 4.1: Effect of gallic acid on denaturation temperature of BSA.

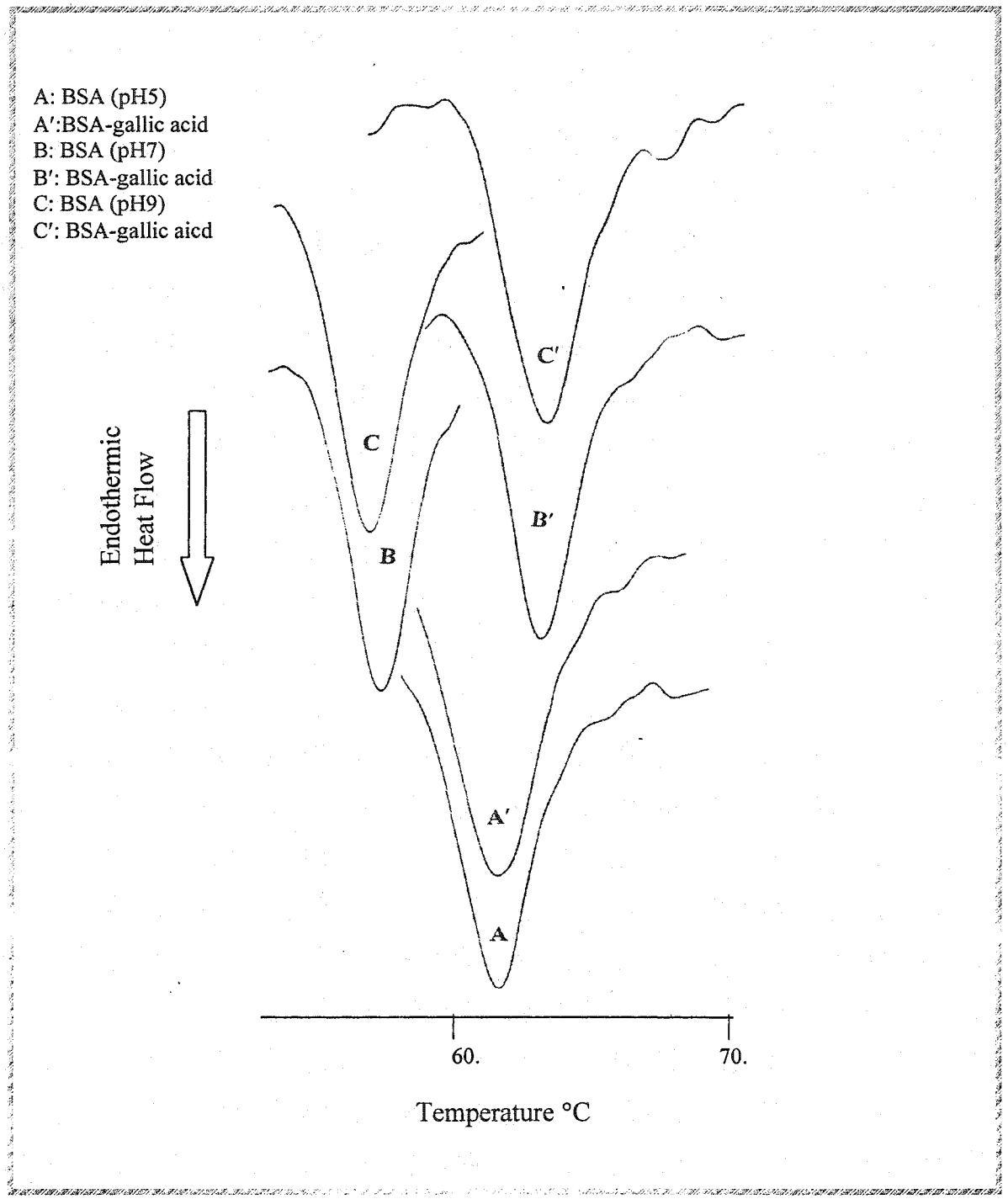


Figure 4.2: Effect of gallic acid on the denaturation temperature of BSA.

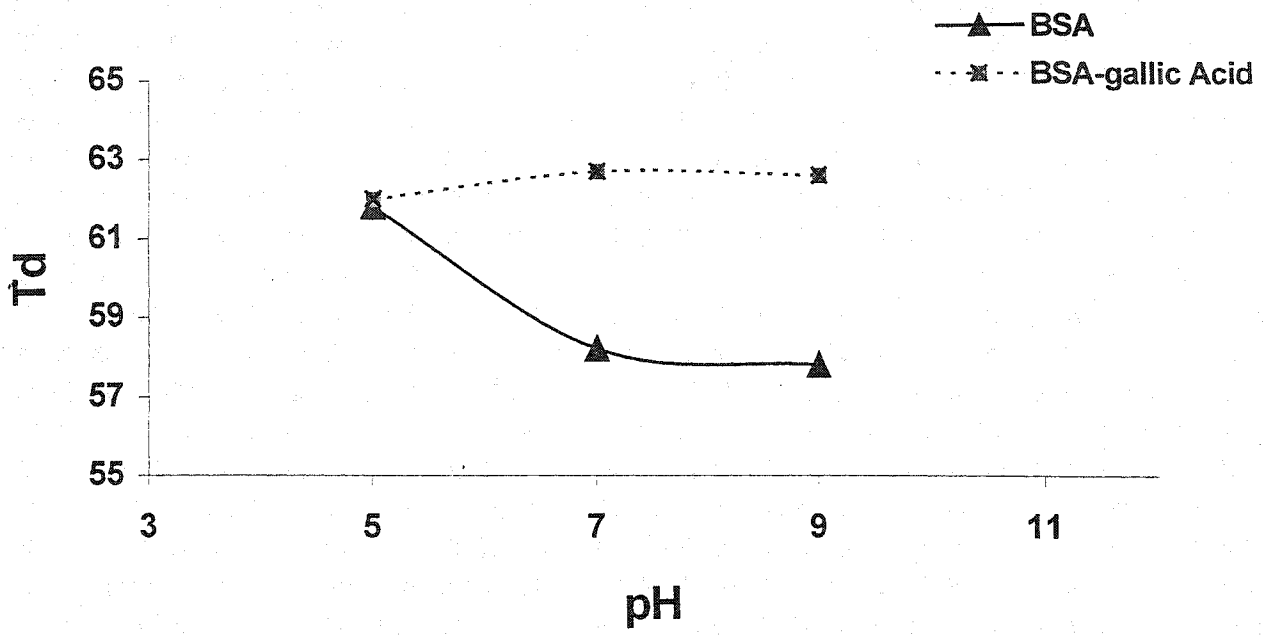


Figure 4.3: Effect of biochanin A on denaturation temperature of BSA.

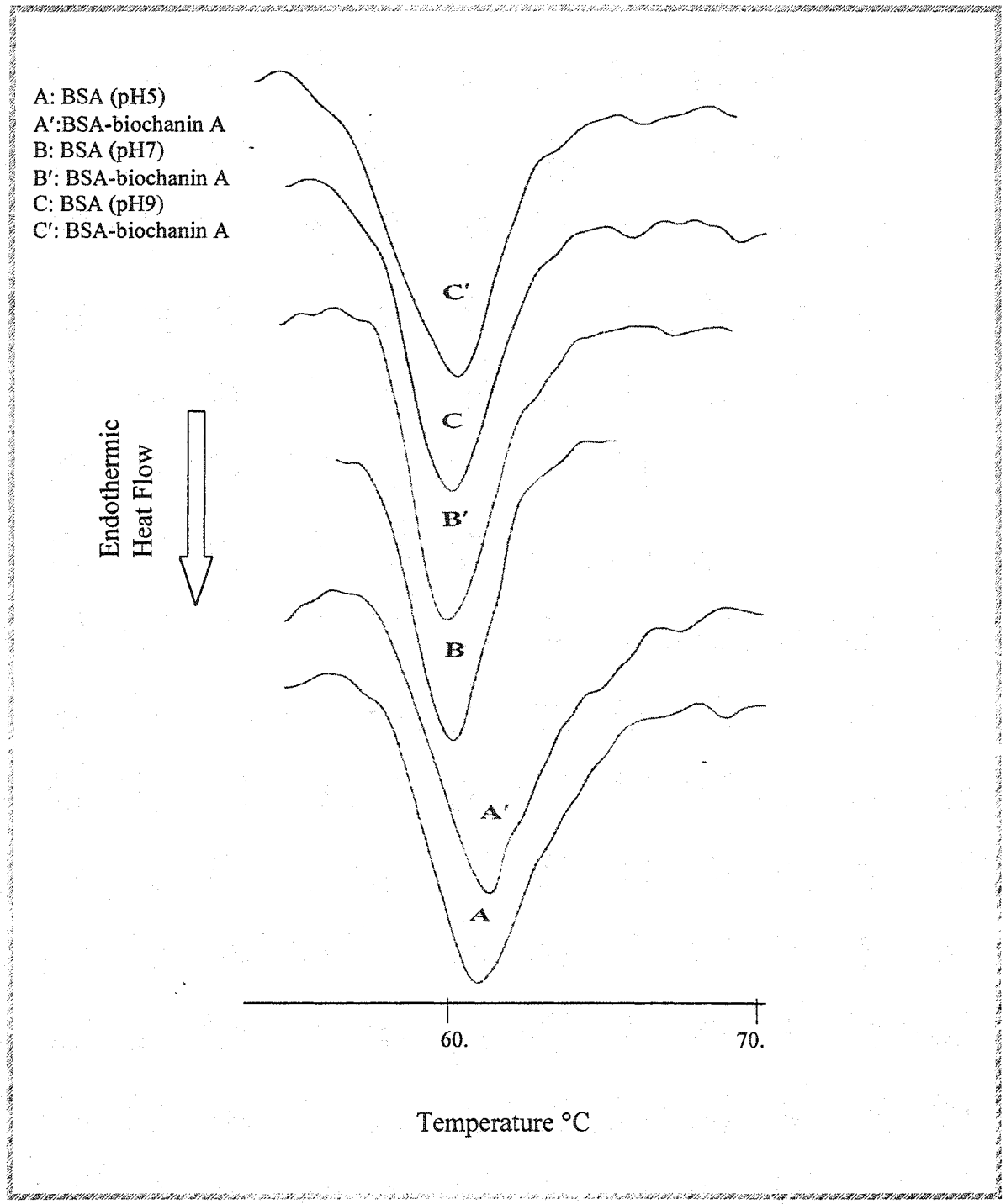


Figure 4.4: Effect of biochanin A on denaturation temperature of BSA.

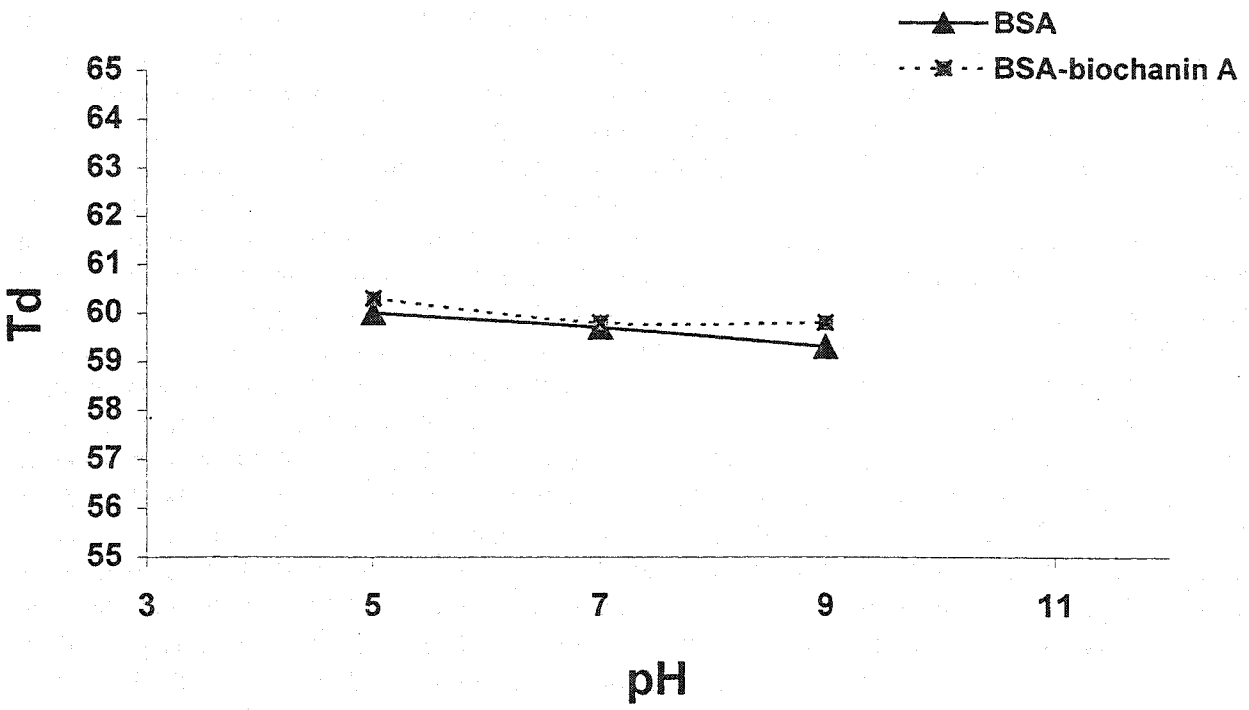


Table 4.1: Effect of gallic acid and biochanin A on denaturation temperature (Td) and enthalpy (ΔH) of BSA.

Treatment (pH)	Denaturation Temperature (Td °C)	Enthalpy (ΔH)
BSA		
pH 5	61.8 ±0.2°C	1.6±0.02 J/g
pH 7	58.2±0.1°C	1.1±0.04 J/g
pH 9	57.8±0.3°C	1.4±0.02 J/g
BSA-gallic acid		
pH 5	62.0±0.1°C	1.7±0.02 J/g
pH 7	62.7±0.1°C	1.8±0.03 J/g
pH 9	62.6±0.2°C	1.6±0.02 J/g
BSA-biochanin A		
pH 5	60.3±0.1°C	1.3±0.02 J/g
pH 7	59.8±0.1°C	1.1±0.03 J/g
pH 9	59.8±0.2°C	1.2±0.02 J/g

gallic acid protected BSA against denaturation and aggregation at 55°C and pH 7 and 9.

Figures 4.3 and 4.4 show the thermograms and relative differences of BSA and BSA-biochanin A at pH 5, 7 and 9, respectively. The denaturation temperature (Td) of BSA was lower than that of BSA-biochanin A, suggesting the higher thermal stability of BSA-biochanin A. At pH 9 the peak of denaturation shifted from 57.8±0.3°C to 59.8±0.2°C and enthalpies (ΔH) from 1.4±0.02 J/g to 1.2±0.02 J/g. Data suggest that BSA-gallic acid showed maximum stability at pH 9 (Table 4.1).

4.2.2 Effect of gallic acid and biochanin A on thermal characteristics of β-Lg

Figures 4.5 and 4.6 show the thermograms and the relative difference of β-Lg and β-Lg-gallic acid, respectively. Denaturation temperatures (Td) at 71.8±0.3°C, 70.9±0.5°C and 69.8±0.5°C were observed for β-Lg and enthalpies (ΔH) of 1.7±0.03 J/g, 1.3±0.03 J/g and 1.1±0.04 J/g at pH 5, 7 and 9, respectively (Table 4.2). For β-Lg-gallic acid Td and ΔH were 77.8±0.3°C, 76.9±0.2°C, 75.7±0.5°C for pH 5, 7 and 9, and 2.0±0.03 J/g, 1.6±0.01 J/g, 1.9±0.03 J/g, respectively. This result shows higher thermal stability of β-Lg-gallic acid compared with β-Lg at all pH.

Figures 4.7 and 4.8 show the effects of biochanin A on the thermal properties of β-Lg. The denaturation temperatures of β-Lg and β-Lg-biochanin A at pH 5, 7 and 9, were 71.8±0.2°C, 71.9±0.1°C, and 70.9±0.1°C, 70.5±0.1, and 69.8±0.3°C, 69.4±0.2°C, respectively; with corresponding enthalpies of 1.7±0.02J/g, 1.6±0.02 J/g, and 1.2±0.04 J/g, 1.2±0.03 J/g, and 1.3.±0.02 J/g, 1.2±0.02 J/g, respectively (Table 4.2). There was only slight difference between β-Lg and β-Lg-biochanin A.

Figure 4.5: Effect of gallic acid on denaturation temperature of β-Lg.

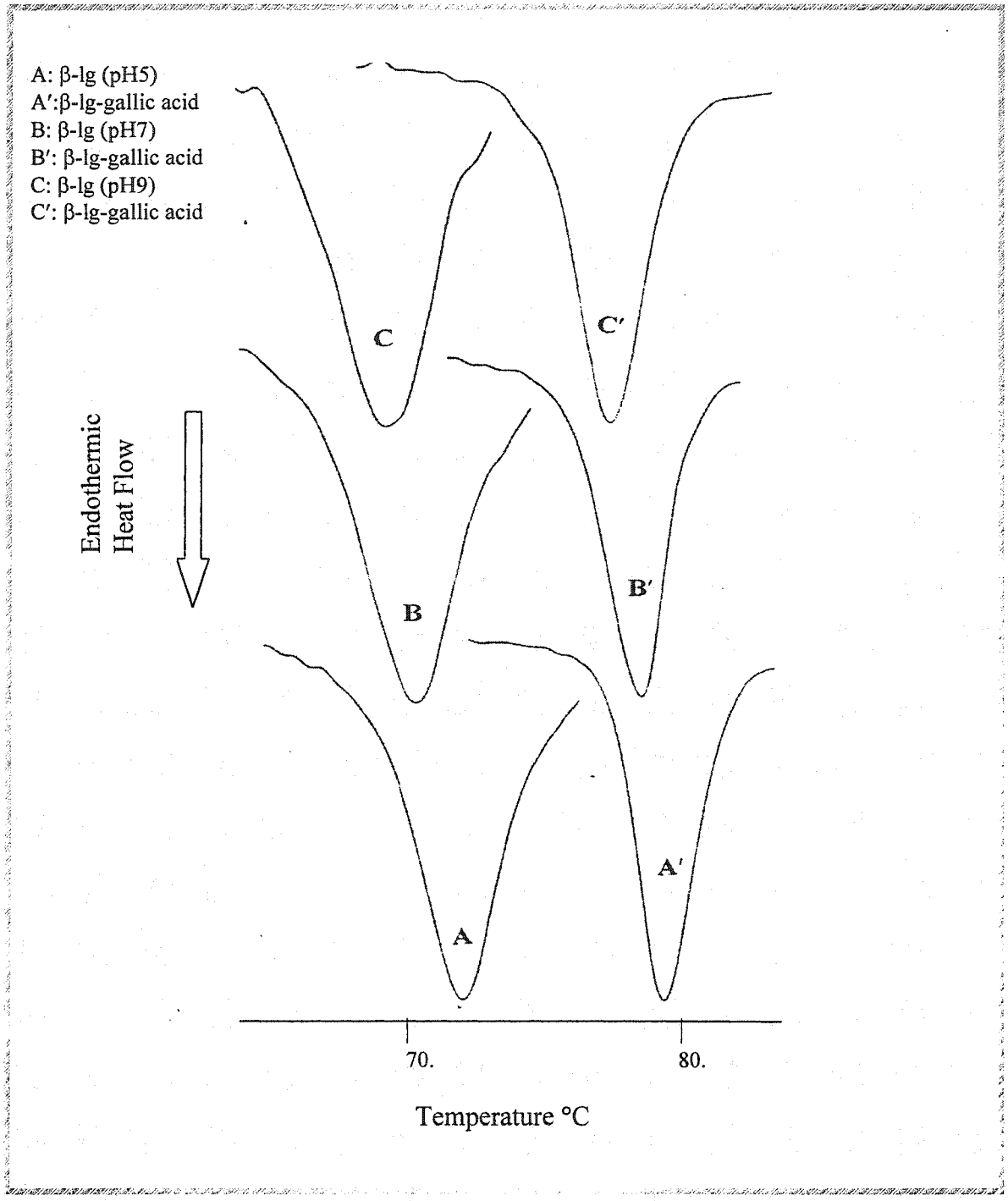


Figure 4.6: Effect of gallic acid on denaturation temperature of β-Lg.

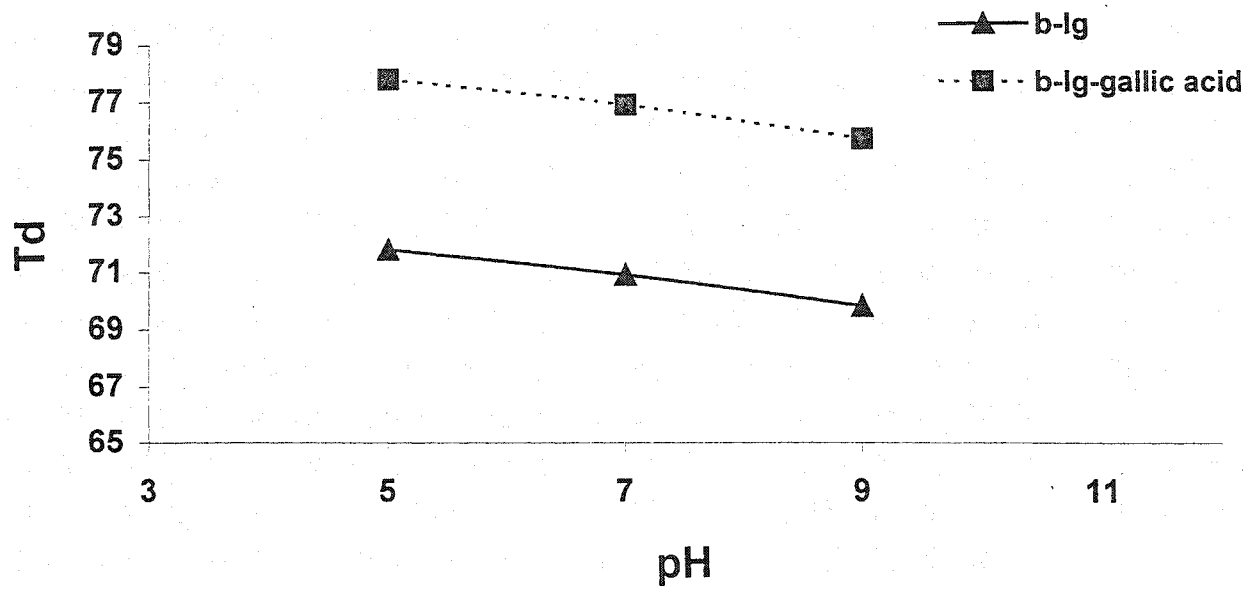


Figure 4.7: Effect of biochanin A on denaturation temperature of β-Lg.

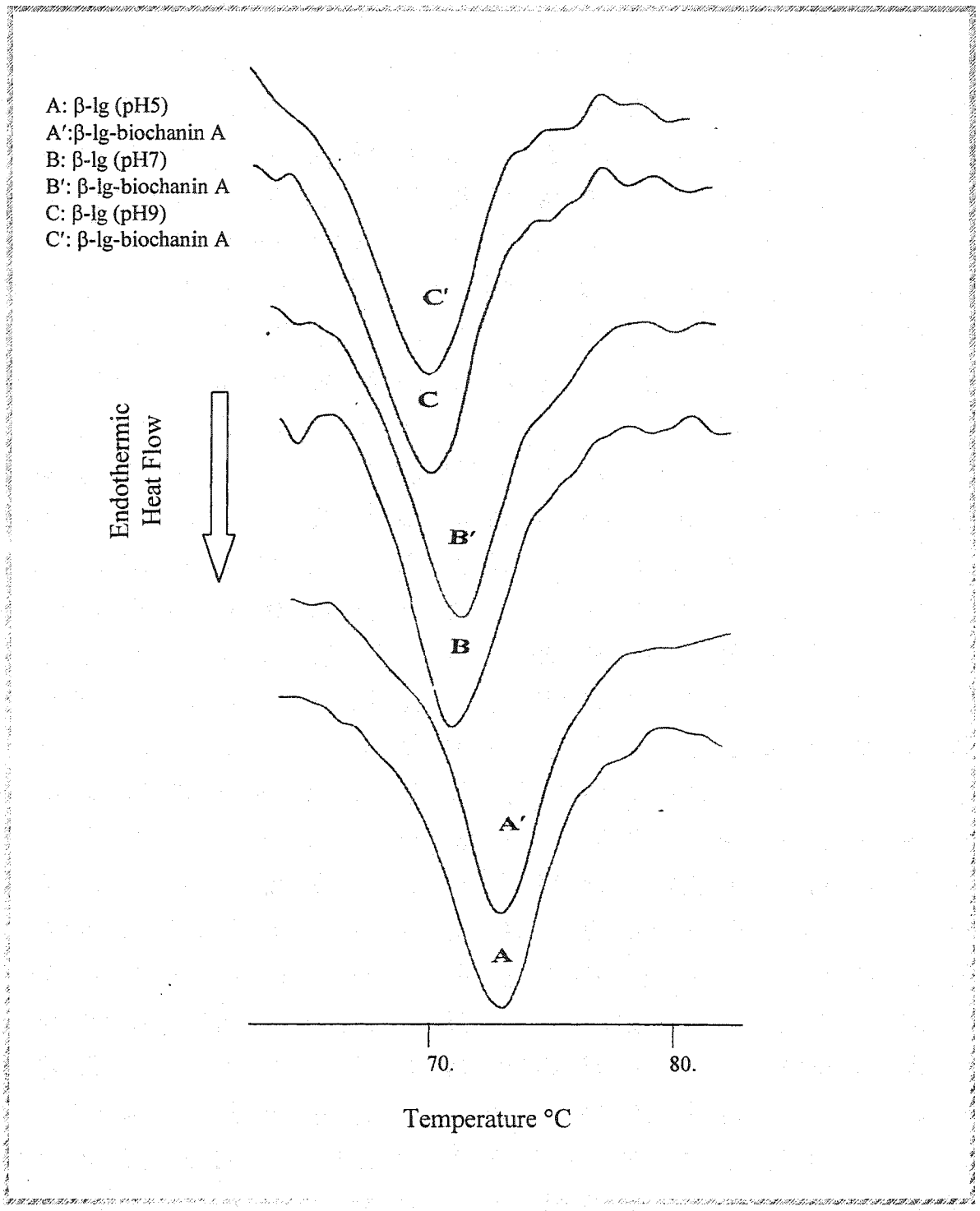


Figure 4.8: Effect of biochanin A on the denaturation temperature of β-Lg.

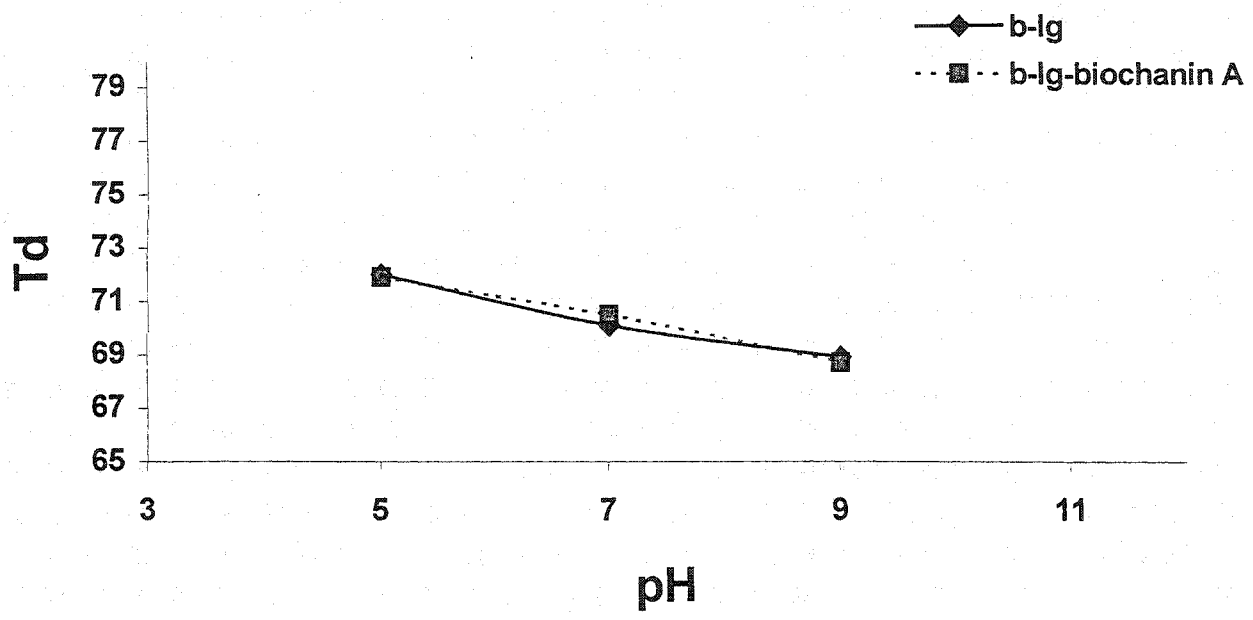


Table 4.2: Effect of gallic acid and biochanin A on denaturation temperature (Td) and enthalpy ΔH of β-Lg.

Treatment (pH)	Denaturation Temperature (Td °C)	Enthalpy (ΔH)
β-Lg		
pH 5	71.8 ±0.2°C	1.7±0.02 J/g
pH 7	70.9±0.1°C	1.2±0.04 J/g
pH 9	69.8±0.3°C	1.3±0.02 J/g
β-Lg-gallic acid		
pH 5	77.8±0.1°C	1.8±0.02 J/g
pH 7	76.9±0.1°C	1.6±0.03 J/g
pH 9	75.7±0.2°C	1.9±0.02 J/g
β-Lg-biochanin A		
pH 5	71.9±0.1°C	1.6±0.02 J/g
pH 7	70.5±0.1°C	1.2±0.03 J/g
pH 9	69.4±0.2°C	1.2±0.02 J/g

4.2.3 Effect of gallic acid and biochanin A on thermal properties of soybean glycinin

The thermal properties of soybean glycinin and glycinin-gallic acid at pH 7 and 9 are shown in the DSC thermogram (Figures 4.9). Soybean glycinin exhibited only one endothermic transition peak confirming the homogeneity of the protein. The denaturation temperature (Td) for glycinin-gallic acid was higher by 3°C (92.5±0.1°C to 96.0±0.1°C) at pH 7, and by 2.7°C (92.4±0.3°C to 95.1±0.2°C) at pH 9, respectively, compared to glycinin alone (Table 4.3). The enthalplies (ΔH) for glycinin and glycinin-gallic acid were 2.1±0.04 J/g and 3.1±0.03 J/g at pH7, and 2.4±0.02 J/g and 3.0±0.02 J/g at pH 9, respectively. The higher denaturation temperature reflects higher thermal stability of glycinin-gallic acid, compared to glycinin alone.

Figure 4.10 shows the denaturation temperatures (Td) of glycinin and glycinin-biochanin A; the Td values were similar (92.5±0.1°C and 92.7±0.1°C at pH 7, and 92.4±0.2°C to 93±0.1°C at pH 9, respectively). The ΔH for glycinin and glycinin-biochanin A were 2.1 ±0.04 J/g and 2.2±0.03 J/g at pH 7, and 2.4±0.02 J/g and 2.2±0.02 J/g at pH 9 (Table 4.3).

Both Td and ΔH of soybean glycinin increased in the presence of gallic acid indicating conformational structural changes of soybean glycinin, possibly result in the formation of protein-phenolic complexes through hydrogen bonding and hydrophobic interaction, these conformational changes were not observed in the presence of biochanin A.

Figure 4.9: Effect of gallic acid on thermal denaturation of soybean glycinin.

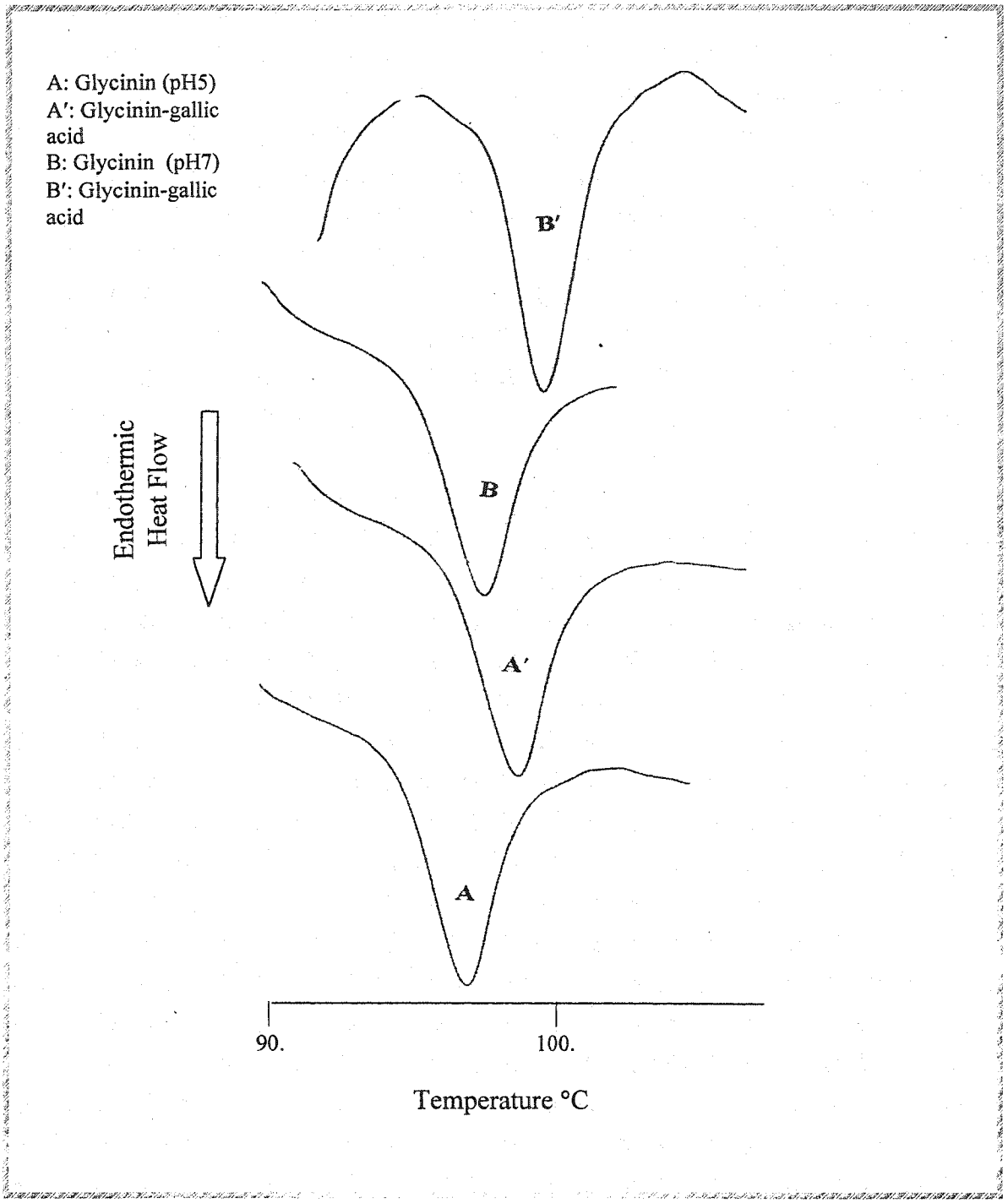


Figure 4.10: Effect of biochanin A on denaturation temperature of soybean glycinin

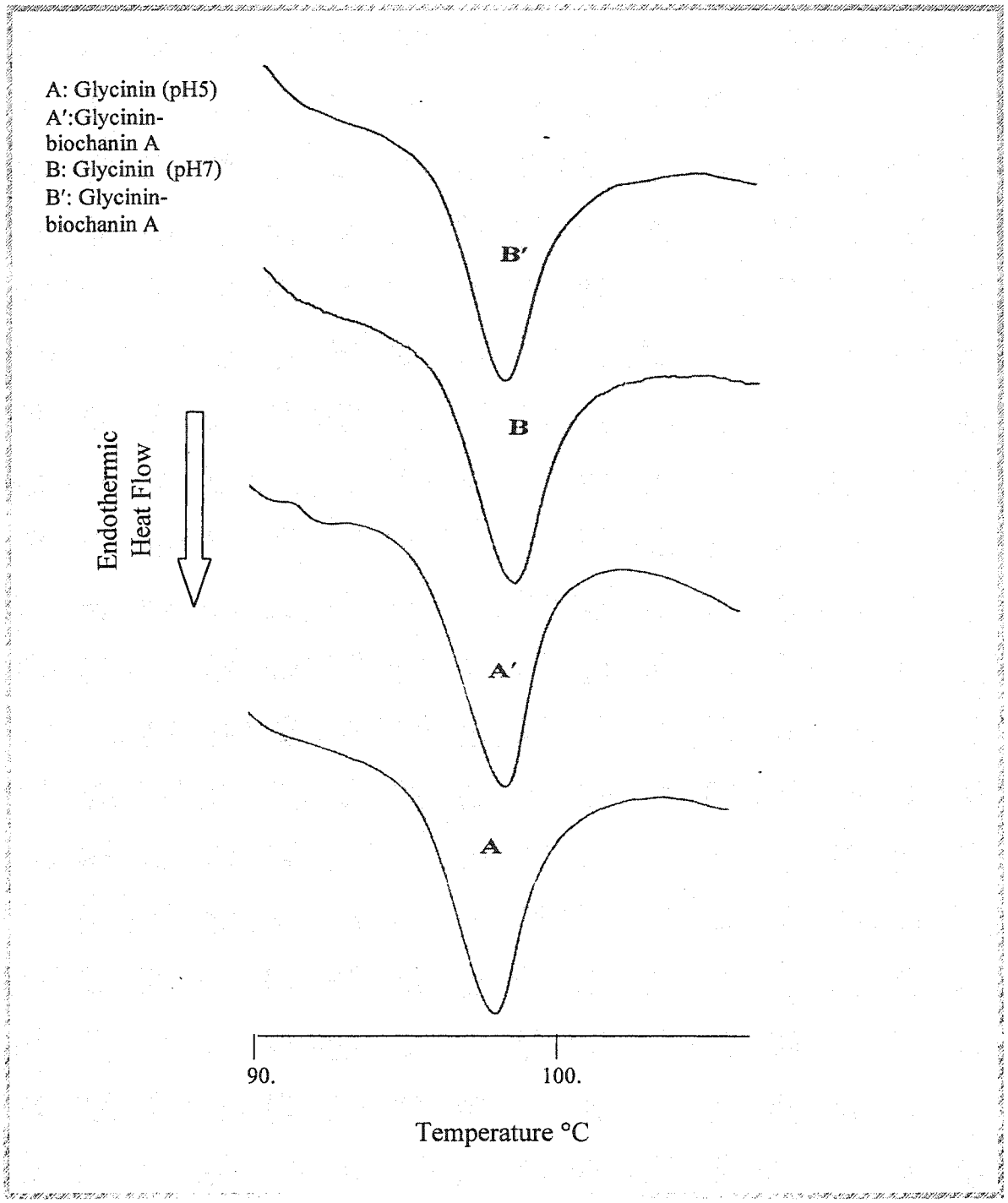


Table 4.3: Effect of gallic acid and biochanin A on denaturation temperature (Td) and enthalpy (ΔH) of soybean glycinin.

Treatment (pH)	Denaturation Temperature (Td °C)	Enthalpy (ΔH)
Glycinin		
pH 7	92.5±0.1°C	2.1±0.04 J/g
pH 9	92.4±0.3°C	2.4±0.02 J/g
Glycinin-gallic acid		
pH 7	95.6±0.1°C	3.1±0.03 J/g
pH 9	95.1±0.2°C	3.0±0.02 J/g
Glycinin-biochanin A		
pH 7	92.7±0.1°C	2.2±0.03 J/g
pH 9	93.0±0.2°C	2.2±0.02 J/g

4.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

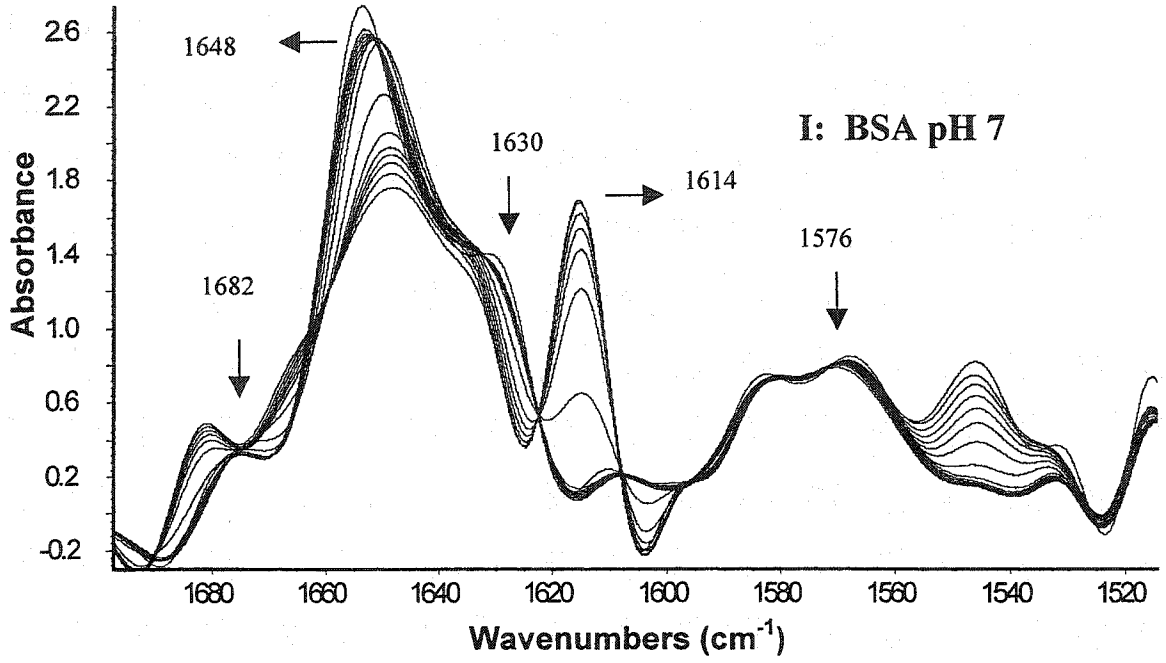
4.2.4.1Effect of gallic acid and biochanin A on secondary structure of BSA and soybean glycinin

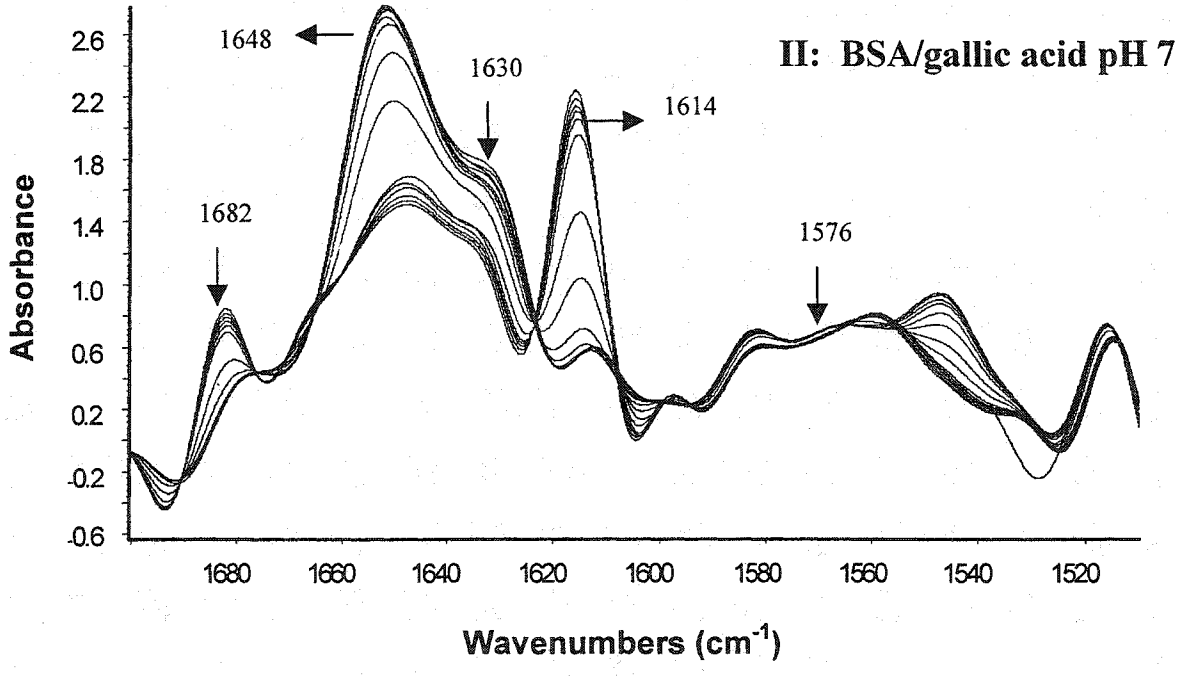
The deconvolved infrared spectra of BSA (10%) recorded immediately after dissolving BSA, BSA-gallic acid and BSA-biochanin A in deuterated phosphate buffer (pH 7 and 9), heated from 25°C to 95°C are shown in Figure 4.11, I, II and III.

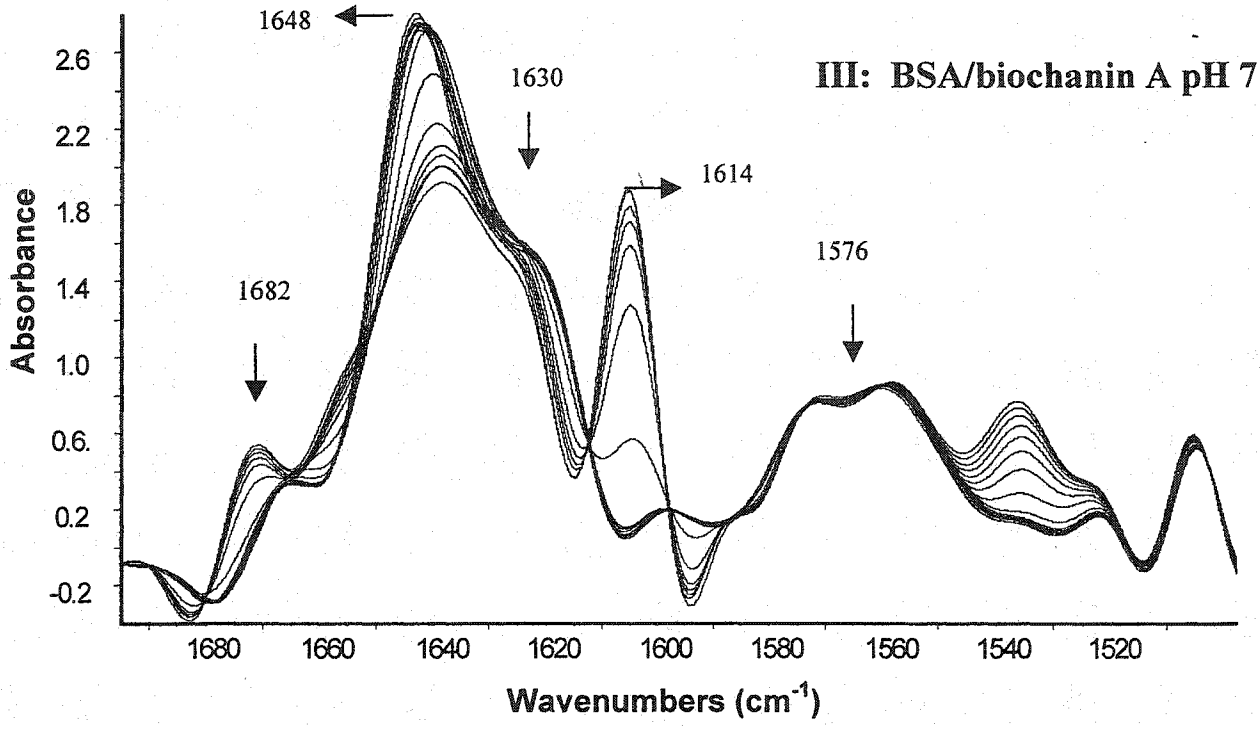
Various regions in the IR spectra provide specific infromation regarding the secondary structure of the protein. An intense peak at 1654 cm^{-1} in the infrared spectrum of BSA is attributed to α-helical conformation, a shoulder at 1670 cm^{-1} attributes to turns, the bands at 1630 cm^{-1} is attributed to β-sheets (Susi and Byler, 1988), whereas the 1618 and 1684 cm^{-1} bands have been attributed to intermolecular hydrogen bonded β-sheet structure (Clark et al, 1981). The Amide II absorption region in the infrared spectra occurs between 1551-1520 cm^{-1} and is attributed to the N-H stretching. H-D exchange results in a shift of the amide II band by approximately 100 cm^{-1} to lower wave numbers. Hydrogen-deuterium exchange can give an indication as to the extent of protein unfolding (Hvit and Nielsen, 1966).

To investigate the effect of gallic acid and biochanin A on the hydrogen-bonding network of BSA, the integrated intensity in the region between 1684-1674 cm^{-1} was plotted as a function of increasing temperature. The plot (Figure 4.12) shows that, starting at 55°C at both pH 7 and 9, there was an increase in the band intensity, the band is attributed to intermolecular hydrogen bonded β-sheet structure. However biochanin A had no effect at on the thermal denaturation of BSA.

Figure 4.11: Deconvolved infrared spectra of BSA (I) BSA-gallic acid (II), and BSA-biochanin A (III) at pH 7: 1682 and 1614 (intermolecular hydrogen bonded β-sheet), 1648 (α-helix), 1630 (β-sheet) and 1576 (hydrogen-deuterium exchange).







To monitor the changes occurring in the α-helical domains of BSA, the integrated intensity in the region between 1658-1643 cm^{-1} was plotted as a function of temperature at pH 7 and 9 (Figure 4.13, I and II). The plot shows, starting at 55°C for both pH 7 and 9, that there was a decrease in integrated intensity of the α-helical. BSA-gallic acid showed the fastest rate of decrease in the α-helical band intensity at pH 9.

A plot of the integrated intensity of the 1621-1608 cm^{-1} band (attributed to intermolecular hydrogen bonded β-sheet resulting from aggregate formation after protein denaturation), as a function of increasing temperature is shown in Figure 4.14, I and II. Integrated intensity increased, starting at 55°C; the highest rate of increase was observed with BSA-gallic acid at pH7.

Figure 4.12: Plot of integrated intensity (spectral region 1684-1674cm^{-1}) versus temperature °C of BSA with and with out gallic acid and biochanin A at pH 7 (I) & 9 (II). ♦ BSA-pH 7 & 9 (alone), ■ BSA-gallic acid, ▲ BSA-biochanin A.

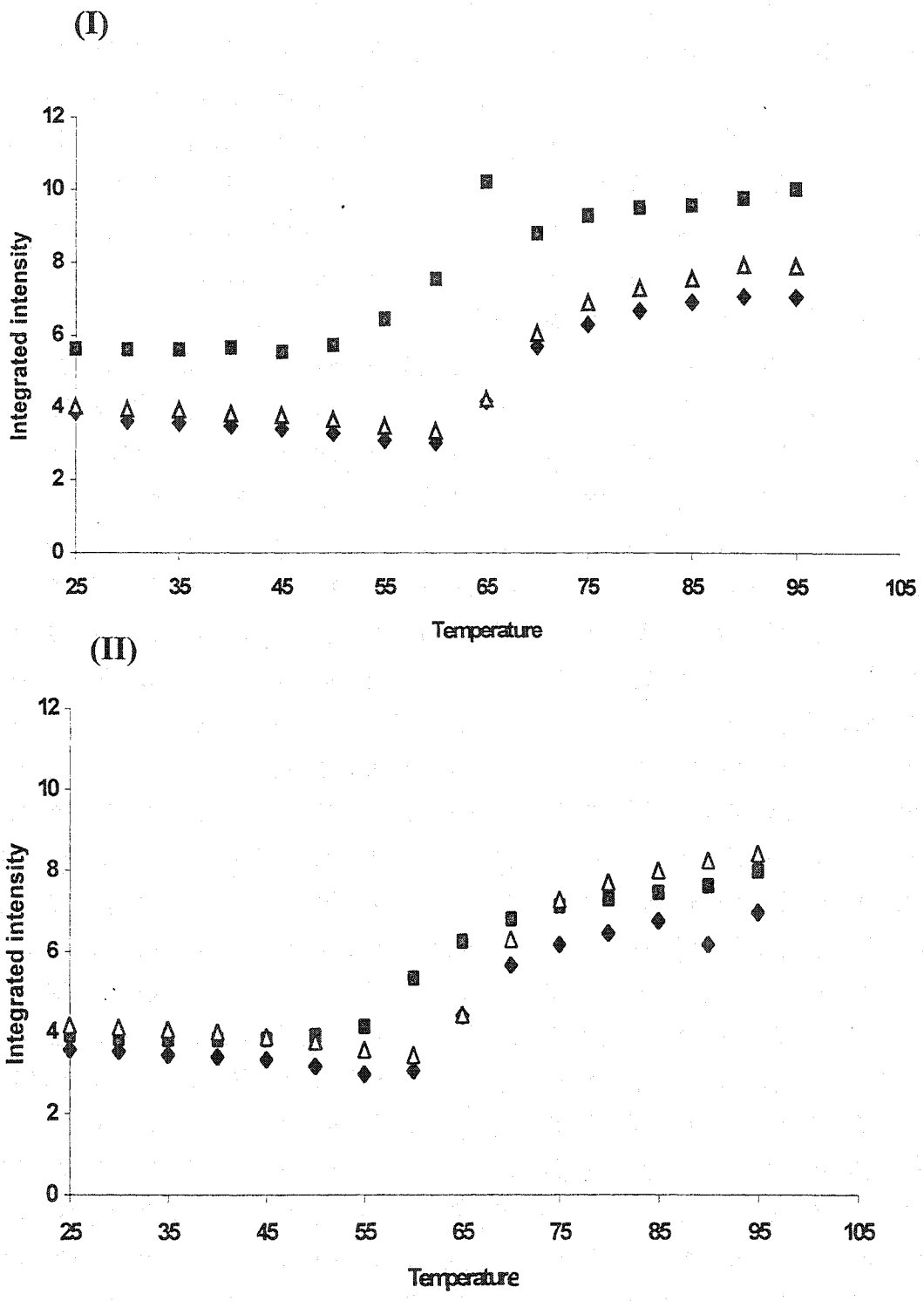


Figure 4.13: Plot of integrated intensity (spectral region 1658-1643cm^{-1}) versus temperature °C of BSA with and with out gallic acid and biochanin A at pH 7 (I) & 9 (II). ♦ BSA-pH 7 & 9 (alone), ■ BSA-gallic acid, ▲ BSA-biochanin A.

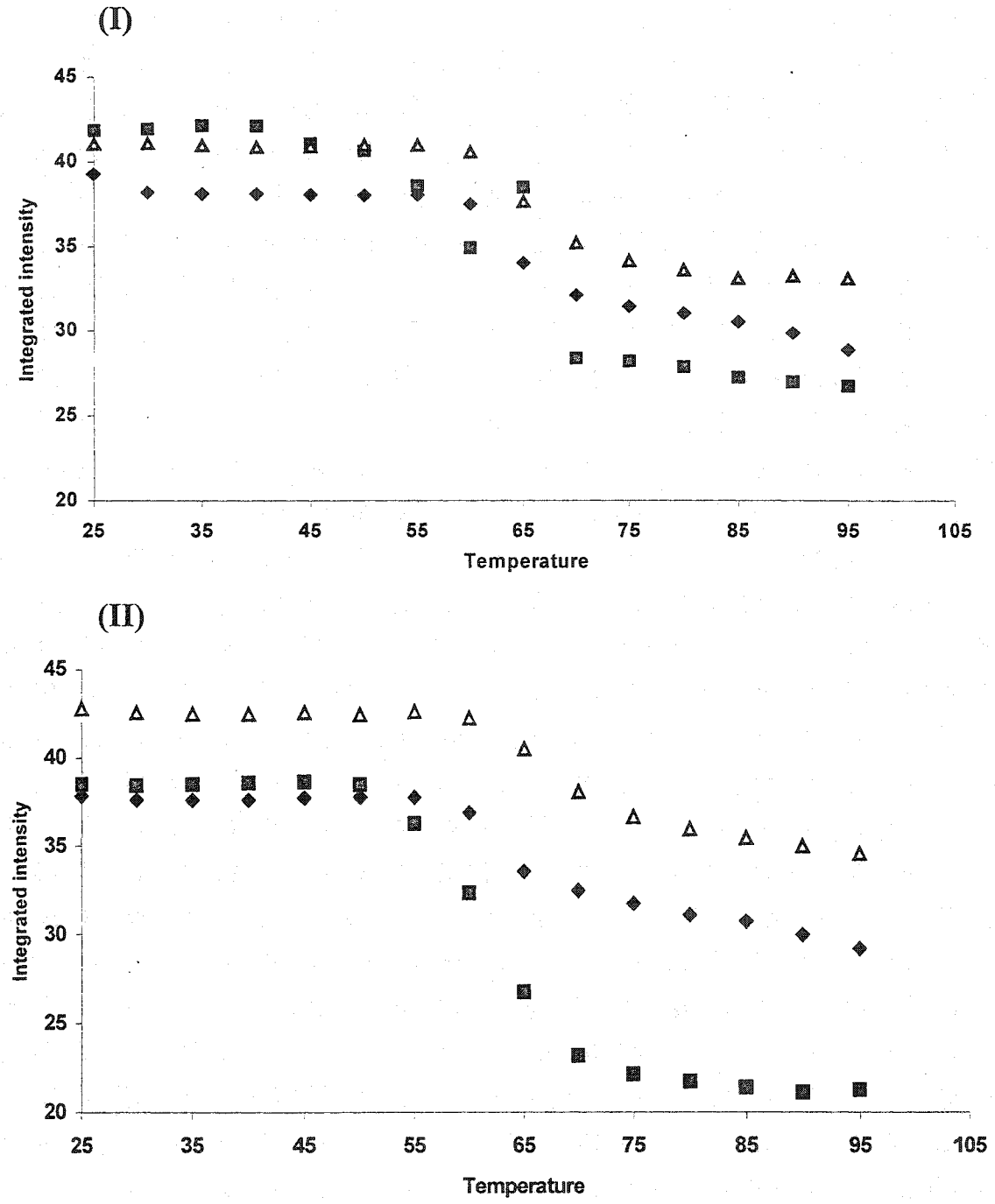


Figure 4.14: Plot of integrated intensity (spectral region 1621-1608cm^{-1}) versus temperature °C of BSA with and with out gallic acid and biochanin A at pH 7 (I) & 9 (II). ♦ BSA-pH 7 & 9 (alone), ■ BSA-gallic acid, ▲ BSA-biochanin A.

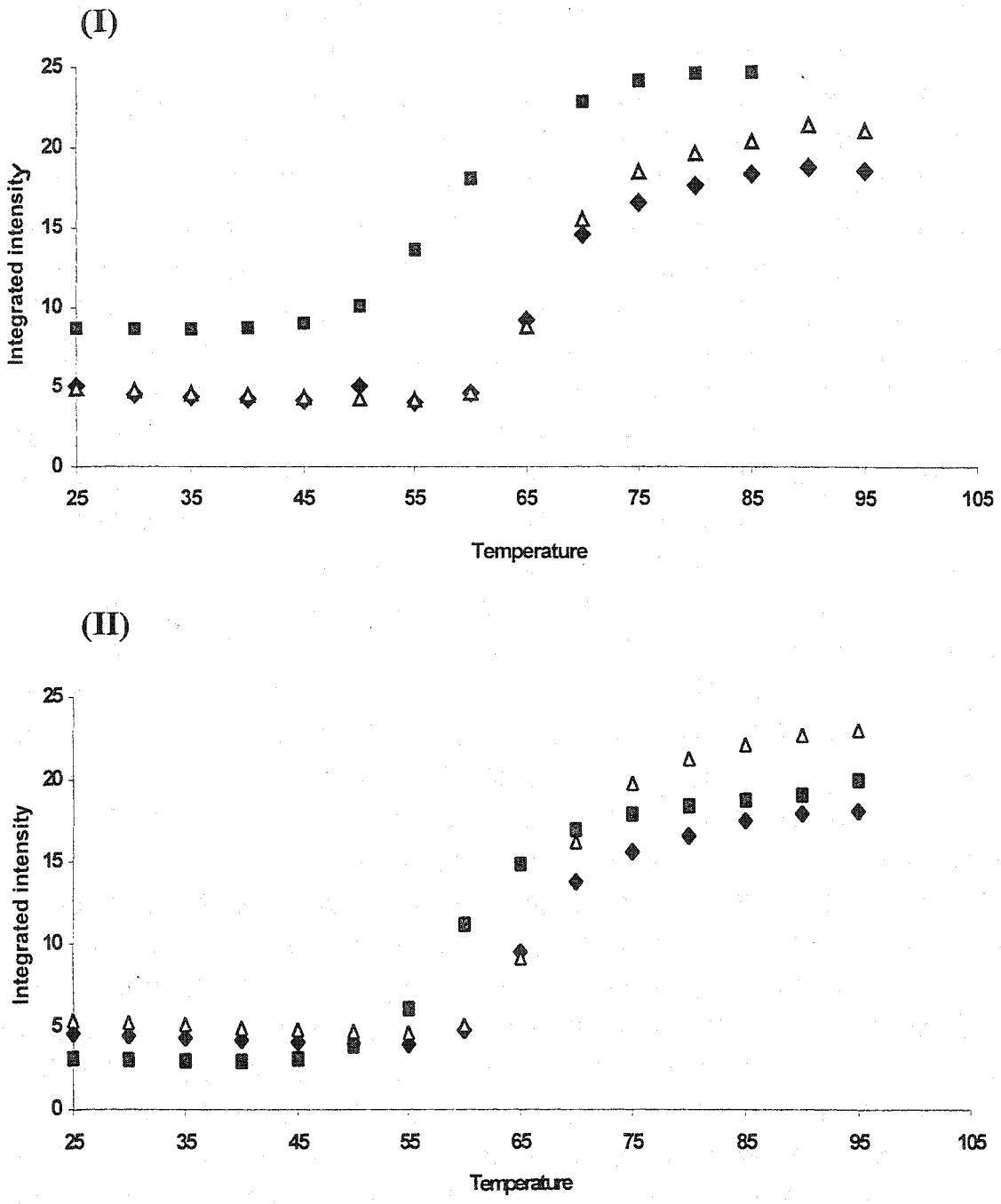
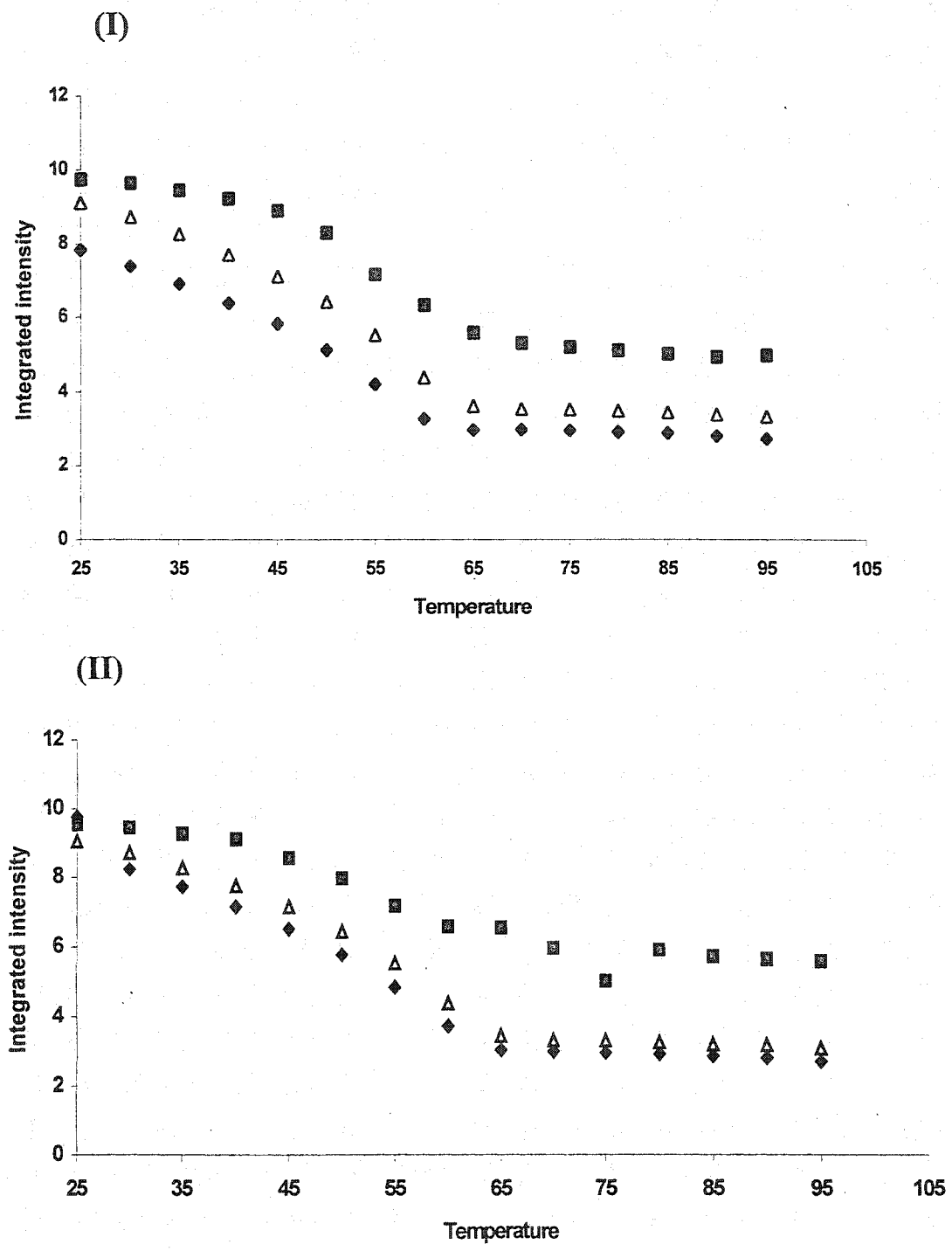


Figure 4.15: Plot of integrated intensity (spectral region 1551-1539cm^{-1}) versus temperature °C of BSA with and with out gallic acid and biochanin A at pH 7 (I) & 9 (II): ♦ BSA-pH 7 & 9 (alone), ■ BSA-gallic acid, ▲ BSA-biochanin A.



To observe the effect of gallic acid and biochanin A at pH 7 and 9 on the rate of H-D exchange in BSA, the integrated intensity of the amide II band was plotted as a function of increasing temperature (Figure 4.15, I and II). BSA-gallic acid at pH 7 showed the highest band intensity, the band intensity remained constant after 65°C suggesting completion of H-D exchange. BSA-biochanin A showed a similar pattern of H-D exchange as BSA at pH 9, suggesting that biochanin A had little effect on the unfolding of BSA.

5. GENERAL CONCLUSION

Results from this research indicate that phenolic compounds can bind to proteins, and this can be demonstrated by native polyacrylamide gel electrophoresis. The pH and the temperature of the protein-phenolic compound have considerable effect on the interaction. phenolic acids with single aromatic ring showed much greater interaction than multi-aromatic ring isoflavone. BSA-phenolic acid showed considerable difference in electrophoretic migration, and prevented protein aggregation when compared to BSA. Similar effects were not found with BSA-isoflavone.

Thermal analysis using differential scanning calorimetry demonstrated the higher thermal stability of the protein-phenolic compounds, compared with the proteins alone. Fourier Transform Infrared spectroscopy showed differences in the secondary structure of the protein-phenolic compounds when compared with the protein; in addition FTIR confirmed the higher thermal stability and the inhibition of aggregation as a result of interactions of phenols to the proteins.

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