ISOLATION AND CHARACTERIZATION OF A HIGH GELLING PROTEIN FROM SOYBEAN

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Suggested short title: **A HIGH GELLING SOYBEAN PROTEIN**

ABSTRACT

Defatted soybean was subjected to isolation procedures as follows: NaOH extraction / acid precipitation (NaOH-IE); water extraction / acid precipitation (H_2O-IE); NaOH extraction / cryoprecipitation (NaOH-CP) and citric acid extraction / cryoprecipitation (CA-CP). NaOH-CP and CA-CP each resulted in precipitation of one protein mainly consisting of glycinin and β–conglycinin, respectively. The proteins were subjected to polyacrylamide gel electrophoresis (PAGE), SDS-PAGE, size exclusion-chromatography (SEC) and differential scanning calorimetry (DSC) for characterization. PAGE, SDS-PAGE, SEC and DSC confirmed the homogeneity of NaOH-CP. The gelation properties and structural characteristics of NaOH-CP were investigated; the minimum protein concentration required for formation of a thermally-induced gel from NaOH-CP was 8%. With cold-set gelation, in the presence of 30 mM $CaCl_2$, the minimum protein concentration required for formation of a self-supporting gel was 4%. At 100 mM $CaCl_2$, cold-set gelation of 10% protein concentration gave gel of strength 7.4 N compared to the 7.8 N gel strength obtained with thermally-induced gel at 16% protein concentration. The effects of heating on the NaOH-CP structure, in the presence of varying concentrations of NaCl and reducing agents, were examined by Fourier transform infrared (FTIR) spectroscopy and DSC. FTIR spectroscopy revealed that at 25°C the NaOH-CP protein showed four bands at 1633, 1650, 1667, and 1689 cm^{-1}. At 85°C, the temperature associated with gelation, the four bands decreased in intensity with the simultaneous increase in the intensity of a band at 1644 cm^{-1} as result of the protein unfolding, furthermore two new bands at 1616 and 1685 cm^{-1} appeared. the latter two bands are attributed to intermolecular β–sheet formed during gelation. The NaOH-CP did not exhibit any aggregation band between 25-100°C in the presence of NaCl. The DSC studies revealed that the protein denatured at

approximately 102 °C (0.5 M NaCl) and 106 °C (1.0 M NaCl). Small amount of intermolecular β–sheet was observed in the presence of SDS, while in the presence of 2-mercaptoethanol, significant amount of intermolecular β–sheet was observed. DSC showed that low concentrations of urea increased T_d with decreasing ΔH, however high concentrations of urea completely denatured NaOH-CP structure.

RESUME

Des fèves de soja sans matières grasses ont été soumises à une série de procédures d' isolation. La première méthode consistait en une extraction au NaOH suivie d'une précipitation acidique (NaOH-PA). La seconde était basée sur une extraction aqueuse suivie d'une précipitation acidique (H2O-PA). La troisième méthode comportait une extraction au NaOH et une précipitation cryogénique (NaOH-PC), et la dernière consistait en une extraction à l'acide citrique et une précipitation cryogénique (AC-PC). NaOH-PC et AC-PC ont toutes deux produit une protéine contenant principalement de la glycinine et de la β-conglycinine. Afin de les caractériser, les protéines ont été étudiées par électrophorèse sur gel de polyacrylamide (PAGE), SDS-PAGE, chromatographie par exclusion de taille (SEC) et par calorimétrie à balayage differentiel (DSC). L' homogénéité de NaOH-PC a été confirmée par PAGE, SDS-PAGE, SEC et DSC.

Les caractéristiques structurales et les propriétés des gels de NaOH-PC ont été étudieés. La concentration minimale en protéine nécessaire à la formation par le chaleur des gels à partir de NaOH-PC est de 8%. Un gel de concentration minimale en protéine de 4% a été produit à froid et en présence de 30 mM $CaCl_2$. A 100 mM $CaCi_2$, la mise en gel froid à une concentration protéique de 10 % a donné un gel avec une fermeté de 7.4 N, proche de celle de 7.8 N obtenue pour un gel induit thermiquement d'une concentration protéique de 16%.

Les effets du chauffage sur la structure de NaOH-PC à différentes concentrations de NaCl ou d'agents réducteurs ont été examinés par spéctroscopie infrarouge à transformée de Fourier (FTIR) et par DSC. La fonction amide de la protéine produite par NaOH-PC et préparée dans du D_2O absorbe spécifiquement à quatre fréquences soit 1633, 1650,1667 et 1689 cm^{-1}, et ce à température ambiante. A 85 °C, temperature associée a l'apparition d'un gel, les quatre bandes ont diminué en intensité parallèlement à l'augmentation de l'intensité de la bande à 1644 cm^{-1} attribuée à la stucture non définie de la protéine et deux nouvelles bandes sont apparues à 1616 et 1685 cm^{-1}. Ces deux bandes sont attribuées au feuillet β intermoleculaire formé pendant la mise en gel. En présence de NaCl, NaOH-PC ne présentait aucune agglutination entre 25 et 100 °C. Les études du DSC ont révelé que les protéines se dénaturaient a une température approximative de 102 °C (0.5 M NaCl) et 106 °C (1.0 M NaCl). De petites quantités de feuillets β intermoléculaires ont été observées en présence de SDS, tandis qu'en présence de 2-mercaptoethanol, une proportion significative de feuillets β intermoléculaires a été observée. Des études par DSC ont montré que de faibles concentrations en urée augmentent T_d et diminuent ΔH; Cependant, de fortes concentrations en urée dénaturent totalement la structure de la proteine NaOH-PC.

ACKNOWLEDGMENTS

I take this opportunity to express my deepest gratitude and sincere thanks to Professor Inteaz Alli, my thesis supervisor, for his valuable advice and suggestion, his patience and constant support through my work. He was always there to encourage me, bring up new ideas, spent an incredible amount of time getting me on my way; I would like also to thank his family for their friendship.

I wish to express my sincere thanks to Dr S. Kermasha for making his laboratory facilities available to me, to Dr A. Asmail for his advice and comments during FTIR work. I also extend my thanks to my friends in Dr Alli laboratory and all members of the Food Science Department.

I would like to express especial thanks to Mrs. Nooralein Chapman for her invaluable comments on the manuscript and to Mr. Abdirashid for helping me in the statistical analysis. The French translation of the abstract by Isabella and Aline is also appreciated.

My study was fully funded by the Ministry of Higher Education in Libya, which was administered by the Canadian Bureau for International Education (CBIE). I would like to take this opportunity to express my sincere appreciation.

Deepest gratitude goes to my dear wife Hanan for her patience, understanding and moral support and to my son Mohamed and my daughter Aya for creating very nice environment. I would like to extend a very special note of appreciation to my brothers and sisters for their support and prayers. Finally, this thesis is dedicated to the memory of my parents. I miss you still.

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ABBREVATIONS

CA-CP	Citric acid extraction / cryoprecipitation
ΔH	Change in enthalpy
Da	Dalton
D_2O	Deuterium oxide
DSC	Differential Scanning Calorimetry
FTIR	Fourier Transform Infrared spectroscopy
H_2O-IE	Water extraction / acid precipitation
kDa	Kilodalton
MW	Molecular Weight
NaN_3	Sodium azide
NaOH-IE	Sodium hydroxide extraction / acid precipitation
NaOH-CP	Sodium hydroxide extraction / Cryoprecipitation
NEM	N-ethylmaleimide
PAGE	Polyacrylamide Gel Electrophoresis
PG	Propylene Glycol
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEC	Size-Exclusion Chromatography
SEM	Scanning Electron Microscopy
T_d	Peak Temperature denaturation
WHC	Water Holding Capacity
WPC	Whey protein concentrate
WPI	Whey protein isolate

CHAPTER 1

INTRODUCTION

Soybean has been an integral part of the diet of people in the Far East for more than 5000 years (Rhee 1994). The natural seeds of soybean contain 35-40% protein, 15-20% oil and from 20-25% sugar depending on the variety (Messina 1995). It is still considered to be the world's most inexpensive source of protein and provides a high quality edible oil; these two products are utilized in numerous food, feed and industrial applications.

From a nutritional perspective, soy products may hold many advantages over animal products, for instance, soybean oil is low in saturated fats and is therefore less likely to cause heart disease (Bakhit *et al.* 1994). In addition, soybean contains a number of minor constituents (some previously considered to be antinutritional factors); these include trypsin inhibitors, phytate, saponins, and isoflavones. Several of these compounds are now believed to have beneficial biological effects in the diet, such as lowering blood cholesterol or prevention of cancer (Anderson and Wolf 1995).

Soy protein has been classified based on sedimentation characteristics. The conventional nomenclature for soy protein fractions is the 2S, 7S, 11S and 15S fractions (Yamauchi *et al.* 1991); the major fractions are the 7S fraction (7S globulin or conglycinin) and 11S fraction (11S globulin or glycinin). Because of their heterogeneity, these fractions have often been termed the 7S and 11S protein-rich fractions (Schmidt and Morris 1984). The technical problems which

have limited soy protein utilization in food applications have been related to the complexity of these proteins, their susceptibility to denaturation, and their variability due to differences in processing conditions (Morr 1979).

A number of technological processes have been developed with a view to improving the quality and lowering the cost of soybean proteins; these processe can influence nutritional value and effect other biological factors (Rackis *et al.* 1975). Isolated soy proteins have found widespread application in formulated food products of many types and are also used directly as nutritional supplements. The general process for the manufacture of isolated soy protein includes aqueous extraction of soluble proteins and carbohydrates from defatted soybean; separation of the insoluble residue, followed by isoelectric precipitation of protein, and drying of the protein (Johnson and Kikuchi 1988). This method results in the formation of heterogeneous proteins, some of which do not resolubilize, in addition to imparting off-flavors. These disadvantages limit the use of acid precipitated soy proteins in many food applications (Kinsella *et al.* 1985). Due to these limitations, several alternative methods have been studied. Among these is cryoprecipitation, which is used for initial purification of the 11S ultracentrifugal component, the major portion of soybean globulin; cryoprecipitated proteins have potential as functional and nutritional ingredients in foods in spite of the fact that comparability lower yields are associated with cryoprecipitation (Bau *et al.* 1978). Nevertheless the relatively simple technique

can be scaled up as desired and may be used for isolation of glycinin-rich protein on a commercial scale (Kinsella 1979)

The application of soybean proteins as ingredients of foods depends on their functional properties (Nakamura *et al.* 1984). The gel forming ability induced on heating of soybean proteins, is one of the most important functional properties. The ability of gels to act as a matrix for holding water, lipids, sugars, flavors and other ingredients is useful in many food applications and in new product development (Utsumi and Kinsella 1985).

The process of protein gelation involves two stages involving initial denaturation or unfolding of protein molecules followed by subsequent aggregation. The protein molecule unfolds as thermal denaturation occurs and the quantity of highly bonded water increases (Mangino 1984). Due to protein-protein interactions, a three-dimensional network forms to entrain water molecules, leading to gel formation (Jayaprakasha and Brueckner 1999). Many factors affect the formation and properties of protein gels; these include temperature, pH, concentration of protein, salts and free sulfhdryl groups in the system (Taylor *et al.* 1994; Hoalr *et al.* 1995).

The present work was conducted to (a) prepare soy protein using a procedure which minimize the structural and functional changes of the protein; (b) examine acid and alkaline extraction followed by cryoprecipitation as an extraction procedure for soy protein of high functionality, (c) investigate the mechanism associated with cryoprecipitation of soy proteins, (d) increase the

yield of 11S obtained from cryoprecipitation, and finally (e) investigate the gel characteristics of thermally induced 11S gels and cold-set 11S gels.

CHAPTER 2

LITERATURE REVIEW

2.1 Molecular Structure of Soy Proteins

Soy protein is a mixed or heterogeneous protein made up of several different individual proteins as indicated by the listing of the major protein fractions in Table 2.1. The range of molecular sizes of the soy proteins has been demonstrated by ultracentrifugation (Yamauchi *et al.* 1991). The water-extractable proteins of defatted soybean meal give a typical ultracentrifuge pattern in which four major fractions are resolved. The fractions are designated 2, 7,11 and 15-S (Table 2.1) based on their sedimentation rates (Wolf 1970). Approximately 80 % of soybean proteins have molecular weights greater than 100 kDa, implying a high degree of structural order (Kilara and Harwalkar 1996). The major proteins are the globulins 7S (β-conglycinin) and 11S (glycinin); these two proteins account for about 70 % of the protein in the seed and play important roles in several food systems because of their high nutritional value and functional properties (Hill and Breidenbach 1974; Puppo *et al.* 1995). The 7S fraction constitutes approximately a third of the total protein and contains globulins and enzymes such as lipoxygenase and amylase (Nielsen 1985a). The 11S fraction (glycinin) which accounts for approximately 30% of the total protein, has a hexameric structure with each subunit consisting of an acidic and a basic polypeptide linked by a single disulfide bond (Wolf *et al.* 1992).

Table 2.1: Components of ultracentrifuge fractions of water extractable soybean proteins

Fractions	% of total	Components	Mol. wt (Da)
2S	22	Trypsin inhibitors	8000-21500
		Cytochrome C	12000
7S	37	Hemagglutinin	110000
		Lipoxygenase	102000
		β–amyalse	61700
		7-S Globulins	180000-210000
11S	31	11-S Globulin	350000
15S	11		≈600000

Source: Wolf (1970).

2.2 7S Globulin

The 7S globulin, a major component of the 7S fraction (Table 2.1), is one of the major storage proteins in soybean. It can be separated into three individual proteins designated β-conglycinin, γ-conglycinin and basic 7S globulin (Hirano *et al.* 1987). Koshiyama and Fukushima (1976) identified β–conglycinin as the major component of 7S globulin, while γ-conglycinin represents only 3%. Basic 7S globulin represents 3.6 % (Yamauchi *et al.* 1991); it is a glycoprotein having a higher isoelectric point (pH 9.05 - 9.26) than β-, and γ-conglycinin (Yamauchi *et al.* 1984). Basic 7S globulin is composed of four subunits each consisting of a high molecular weight polypeptide (26 kDa) and a low molecular weight polypeptide (16 kDa) linked by a disulfide bond (Utsumi *et al.* 1997).

2.2.1 Quaternary Structure of β–conglycinin

β-conglycinin is composed of three subunits designated as α', α and β subunits (Kilara and Sharkasi 1986) with variable apparent molecular weights. Thanh and Shibasaki (1977) reported molecular weights of 57000, 57000, and 42000 Da respectively, on the basis of SDS-PAGE but Sathe *et al.* (1987) reported higher molecular weights of 79070, 69500, and 52480 Da for these subunits.

The α', α, and β subunits are glycoproteins (Thanh and Shibasaki 1977) with one oligosaccharide unit attached to the aspartic acid residue at the N-terminal end of the molecule (Yamauchi *et al.* 1975; Kilara and Sharkasi 1986). No difference in carbohydrate content was observed between the α', and α subunit, although they contained twice as much carbohydrate as the β-subunit. The α', α, and β-subunits of β-conglycinin exhibited isoelectric points of 4.9, 5.2, and 5.7, respectively (Thanh and Shibasaki 1977). The three subunits interact to produce six isomeric forms (B_1-B_6). Figure 2.1 shows the molar ratio of the α', α, and β subunits in each of the six isomers (Kilara and Harwalkar 1996). Thanh and Shibasaki (1978) suggested that the six isomers can be classified into three groups: group A (B1-B2) has two β subunits per molecule of β-conglycinin; group B (B3-B4), one β subunit; and group C (B5-B6), no β subunits.

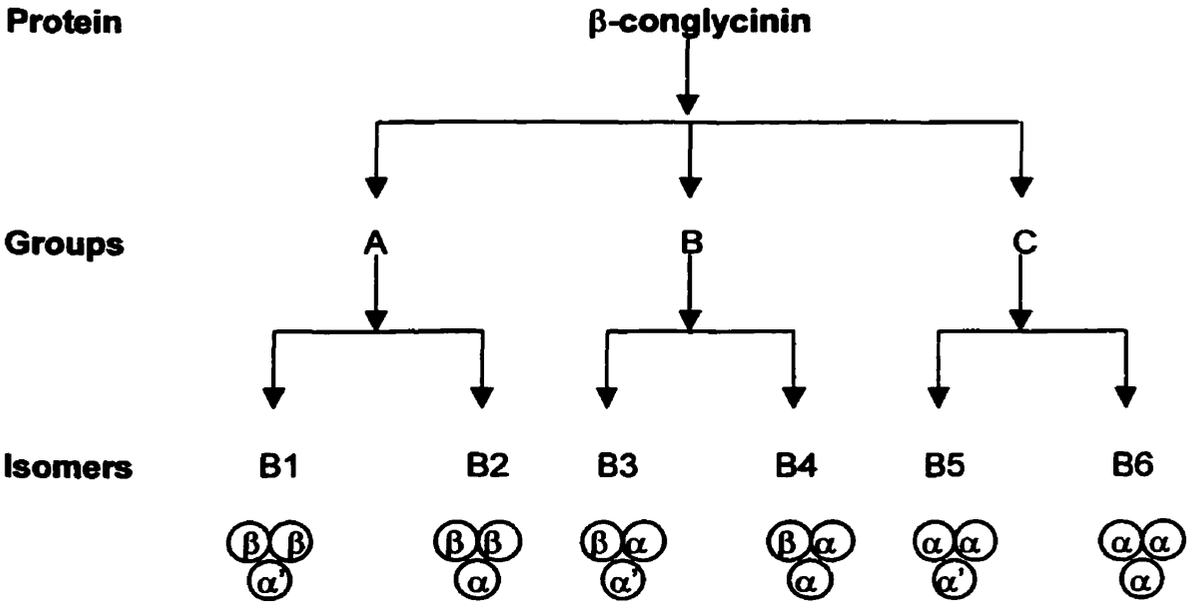


Figure 2.1: Molar ratio of subunits in each of isomers of 7S protein from soybean. Kilara and Harwalkar (1996)

2.2.2 Secondary and Tertiary Structure of β-conglycinin

Relatively little information on the structural conformation of β-conglycinin can be found in the scientific literature. β-conglycinin considered to be a compactly folded protein with high degree of unordered regions; the α-helix, β-structure and random coil contents of the secondary structure are 5, 35, and 60%, respectively (Lewis and Chen 1979).

2.2.3 Primary Structure of β-conglycinin

β-conglycinin is a glycoprotein containing 3.8-5.4% carbohydrate (Koshiyama 1969; Lewis and Chen 1979); the carbohydrate moiety consists of 38 mannose and 12 glucosamine residues per molecule of protein (Thanh and Shibasaki 1978). The estimated molecular weight is in the range 150-210 kDa for the monomeric 7S form and 370 kDa for the dimeric 9S form (Wolf 1971; Thanh and Shibasaki 1978; Lewis and Chen 1979; Sathe 1991).

The N-terminal sequences of the α', α, and β subuints have been determined (Figure 2.2).

					5						10				
α'	NH_2-Val	Glu	Glu	Glu	Glu	Glu	(	)	(	)	Glu	(	)	(	)	Ile
α	NH_2-Val	Glu	Lsy	Glu	Glu	(Ser)	Glu	(Glu)			Glu	(Glu)		Ile	Pro	
β	NH_2-Leu	Lys	Val	Agr	Glu	Asp	Glu	Asn			Asn	Pro		Tyr	Leu	

Figure 2.2: N-terminal sequences for the α', α, and β subunits. Nielsen (1985a)

The α', α subunits were quite homologous; however, the β subunit has N-terminal leucine and exhibits a completely different N-terminal sequence.

The amino acid composition of β-conglycinin and its subunits are given in Table 2.2 showing that the subunits are rich in aspartate, glutamate, leucine, and arginine. However, methionine and cysteine are low in the α' and α subunits and absent in the β subunit. The α and β subunits differ in histidine content; this

Table 2.2. Amino acid composition of β-conglycinin and its subunits

Amino acid	α' Subunit	α Subunit	β Subunit	β-conglycinin
Asx	11.8	12.6	14.1	14.1
Thr	2.7	2.1	2.8	2.8
Ser	7.0	6.9	7.6	6.8
Glx	23.4	23.5	18.1	20.5
Pro	7.1	6.4	4.7	4.3
Gly	5.6	4.8	5.2	2.8
Ala	4.0	4.5	5.7	3.7
Val	3.4	3.4	4.1	5.1
Met	0.6	0.4	0.0	0.2
Ile	3.7	4.5	4.4	6.4
Leu	7.0	8.3	9.6	10.2
Tyr	2.1	1.5	2.6	3.6
Phe	4.7	5.0	6.1	7.4
His	3.6	1.1	1.8	1.7
Lys	7.0	6.0	5.8	7.0
Arg	6.9	8.0	7.2	8.8
Cys	0.2	0.4	0.0	0.3
Trp	ND[a]	ND[a]	ND[a]	ND[a]

Source : Nielsen (1985a)
[a] ND, Not determined

may contribute to the differences in their isoelectric points. The content of hydrophobic amino acids (alanine, valine, leucine, and phenylalanine) is higher in the β subunits while the content of basic amino acids (lysine and arginine) in β subunit is lower than that in the α' and α subunits (Thanh and Shibasaki 1977; Nielsen 1985a).

2.3 Glycinin (11S fraction)

The 11S fraction, glycinin is considered to be a single protein, which constitutes of 25 – 35 % of the total protein in soybean (Liu 1997). It is a heterogeneous oligomeric protein consisting of six subunits, which are devoid of sugar. Each glycinin subunit consists of two polypeptide components, one with an acidic and other with a basic isoelectric point. The two polypeptide components are linked by a single disulfide bond (Zarins and Marshall 1990; Petruccelli and Anon 1995).

2.3.1 Quaternary Structure of Glycinin

Monomeric glycinin consists of six subunits: three acidic (A_1, A_2, A_3), and three basic subunits (B_1, B_2, B_3). The acidic and basic subunits alternate in the same layer and are held together by hydrophobic and disulfide bonds (Rhee 1994). Staswick *et al.* (1984) reported that a single disulfide bond is involved in linking each acidic and basic subunit. Dimeric glycinin is made up of 12 subunits. The dimer is composed of two identical annular-hexagonal monomers stacked on top of each other yielding a hollow, oblate cylinder of 110 X 110 X 75 A°. Some or all of the subunits are nonspherical, resulting in a partial blocking of the central hole (Figure 2.3) (Peng *et al.* 1984).

Several researchers, (Kitamura and Shibasaki 1975; Kitamura *et al.* 1976; Mori *et al.* 1979; Staswick *et al.* 1981) reported four to six types of acidic subunits, and four to five types of basic subunits.

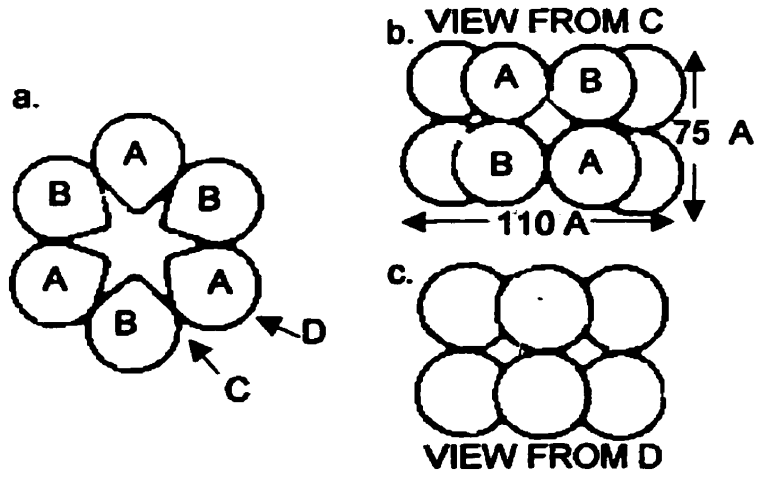


Figure 2.3: Schematic of a glycinin dimer showing the position of the acidic (A) and basic (B) subunits. View from above the central hole. Peng *et al.* (1984)

2.3.2 Secondary and Tertiary Structure of Glycinin

The secondary structure of glycinin was predicted to be 25 % α- helix structure, 25 % β-sheet structure, 42 % turns and 8 % unordered forms on the basis of molecular modeling of its amino acid sequence (Argos *et al.* 1985). Recently, Abbott *et al.* (1996) reported Fourier transform infrared (FTIR) spectra of glycinin as 24 % α- helix structure, 30 % β-sheet structure, 31 % turns, and 12 % unordered structure, their data also indicated that glycinin has the same secondary structure in solution and in hydrate solids. However, Dev *et al.* (1988)

concluded that the secondary structure of glycinin consisted mainly of β–sheets, turns and unordered structure with very little α–helix structure.

The ordered structure of glycinin can be affected by several factors. Alkali treatment decreased ordered structure, especially the β-sheet structure. Adding ethanol to the alkali denatured glycinin, increased the ordered structure (particularly α- helix) and decreased the random coil content (Ishino *et al.* 1980). Acetylation of glycinin decreased the β-sheet structure and increased the random coil content at 90% acetylation (Yamauchi *et al.* 1979). The observed similarity in the amounts of α–helix and β–sheet secondary structures may indicate that their interior conformations are very similar or highly conserved (Marcone 1999).

Examination of the tertiary structure of 11S globulins revealed that substantial differences exist in the exposure, mobility, and arrangement / proximity of aromatic amino acids on 11S globulin surfaces (Marcone 1999). In fact, Marcone and Yada (1998a,b) has shown the tertiary structure to be highly variable even within a single specie.

2.3.3 Primary Structure of Glycinin

Wolf and Briggs (1959) estimated the molecular weight of 11S protein with sedimentation, diffusion, and light-scattering measurements and concluded that the molecular weight was 350 kDa. Koshiyama (1972) reported that the

molecular weight of 11S (glycinin) was 309-332 kDa by three separate methods. Badley *et al.* (1975) reported a value of 320 kDa and Kitamura and Shibasaki (1975) reported molecular weight of 362-373 kDa based on the intermediary subunit molecular and the subunit molecular weight. Despite variation in reported molecular weight due to estimating method, the average reported value is 350 kDa (Peng *et al.* 1984).

The molecular weights of glycinin subunits vary according to the method that is used. Badely *et al.* (1975) reported that the molecular weight of the basic subunits are 19600 Da and the acidic subunits are almost twice as large at 34800 Da. Ochiai-Yanagi *et al.* (1977) reported molecular weight of 28000 Da and 18000 Da for acidic and basic subunits, respectively. Draper and Catsimpoolas (1977) obtained 42000 Da for the acidic subunits and 19000 Da for the basic subunits. Sateh *et al.* (1987) indicated that acidic subunits have a molecular weight of 34670 Da, and that the basic subunits have a molecular weight of 19230 Da. Table 2.3 shows some structural properties of glycinin.

The amino acid composition of glycinin is low in methionine but high in lysine. The proportion of hydrophobic amino acids (Ala, Val, Ile, Leu, and Phe) and hydrophilic amino acids (Lys, His, Arg, Asp, and Glu) are 23.5% and 46.7% respectively (Peng *et al.* 1984). Table 2.4 shows that the acidic subunits have higher contents of glutamic acid, proline, and cystine than the basic subunits (Kilara and Harwalker 1996). The isoelectric points of the basic subunits range between 8.0-8.5 and the acidic subunits between 4.7-5.4 (Kinsella 1979).

Table 2.3: Structural properties of glycinin

Molecular weight (Da)	
Gel filtration	302000 ± 33000
Sedimentation equilibrium	317000 ± 15000
Sedimentation diffusion	322000 ± 15000
From subunit size	326000 ± 35000
Gel electrophoresis	350000 ± 35000
Number of subunits	
Intermediary subunits	12: 6 acidic (A) and 6 basic (B)
Urea or SDS treated	A_1B_3, A_2B_3, A_3B_2, $2A_4B_4$
Urea or SDS + β–ME	A_1A_2, $2A_3$, $2A_4B_1$, B_2, $2B_3$
Molecular weight of acidic subunits	
A_1, A_2, A_4, A_5	38000
A_3	45000
Molecular weight of basic subunits	
B_1, B_2, B_3, B_4	≈ 21000
N-terminal amino acids	
Acidic subunits	
A_1	Phe
A_2	Leu
A_3	Ile
A_4	Ile
Basic subunits	
B_1-B_4	Gly

Source: Kilara and Harwalkar (1996)

Table 2.4: Amino acid composition[a] of glycinin subunits

	Acidic Subunits				Basic Subunits			
Amino Acid	A_1	A_2	A_3	A_4	B_1	B_2	B_3	B_4
Asp	36.8	42.1	45.5	50.8	25.5	24.3	19.2	20.7
Thr	12.0	12.3	15.5	11.8	8.1	9.1	6.2	5.4
Ser	18.3	16.4	27.1	23.5	13.5	12.4	12.1	12.4
Glu	85.3	86.4	91.6	92.6	22.5	22.7	24.8	21.0
Pro	24.0	21.3	33.9	27.3	10.5	10.8	10.2	9.1
Gly	31.0	29.9	29.5	22.4	11.1	10.4	13.4	16.1
Ala	14.4	18.1	10.9	6.2	15.6	14.3	12.4	11.2
Val	11.9	15.3	17.4	12.1	11.4	10.8	17.0	19.2
Leu	20.1	20.0	21.8	14.0	17.9	17.4	18.1	18.1
Ile	17.6	15.3	12.2	10.4	9.2	9.8	7.0	7.3
Try	7.3	6.6	5.6	4.4	2.8	2.5	5.8	8.4
Phe	12.2	12.3	12.0	7.7	8.6	9.1	6.0	5.7
Ilis	6.0	2.6	14.1	9.5	2.1	2.7	4.8	4.2
Lys	21.2	14.9	14.8	18.8	5.9	5.9	7.0	6.5
Arg	18.1	22.7	22.2	28.4	8.9	9.9	10.9	12.5
Met	3.6	5.8	2.4	1.4	2.3	2.7	0.0	1.3
Cys	4.5	4.3	3.6	0.7	1.7	1.5	0.2	1.5

[a] Number of residues per protein molecule

Source: Moreira *et al.* (1979)

N-terminal analysis of the purified glycinin revealed that glycinin has at least 11 polypeptide chains, 8 of which end in glycine, 2 in phenylalanine, and 2 in either leucine or isoleucine (Catsimpoolas *et al.* 1967),

2.4 2S Fraction

The 2S fraction accounts for 20 % of the extractable protein (Nielsen, 1985a), contains several trypsin inhibitors, cytochrome C, and unidentified proteins (Vaidehi and Kadam 1989).

2.4.1 Trypsin Inhibitors

Trypsin inhibitors are proteins with molecular weight ranging from 8000-21500 Da (Vaidehi and Kadam 1989). Five or more trypsin inhibitors are reported for soybeans but only two, the Kunitz and the Bowman-Brik inhibitors, have been purified and studied in detail (Kinsella *et al.* 1985). Raw soybean meal contains 1.4 % Kunitz inhibitor and 0.6 % Bowman-Brik inhibitor (Vaidehi and Kadam 1989).

2.4.1.1 Kunitz Inhibitor

Kunitz inhibitor was first crystallized from soybean in 1946 (Kunitz, 1946). It has a molecular weight of 21500 Da (Snyder and Kwon 1987). The primary structure of the Kunitz inhibitor (Figure 2.4) was determined by Koide and Ikenaka (1973);

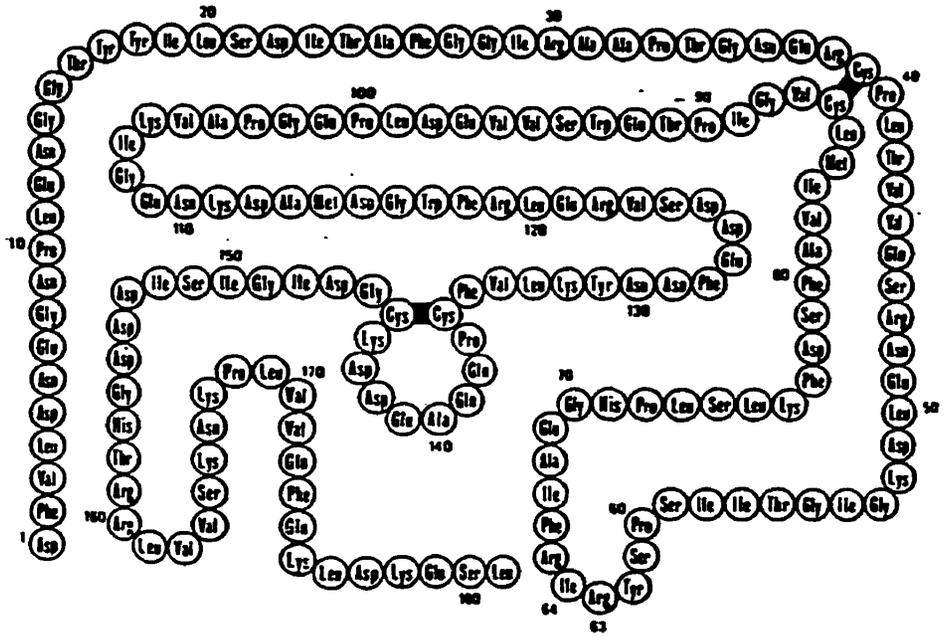


Figure 2.4: Primary structure of Kunitz inhibitor from isolated soybean (Koide and Ikenake 1973)

it consists of 181 amino acid residues. Intramolecular bonding in the Kunitz inhibitor is relatively simple because there are only two sulfide bridges located at residues 39-86 and 136-145 (Wolf 1977). The active site of the Kunitz inhibitor is arginine-isoleucine residue, at position 63-64 (Snyder and Kwon 1987). On the basis of the sequence and theoretical considerations, Koide and Ikenaka (1973) predicated that this molecule should have very little α-helix structure and exist largely in the form a random coil.

2.4.1.2 Bowman-Birk Trypsin

This group of protease inhibitors found in soybean was discovered by Bowman, and purified and characterized by Birk (Snyder and Kown 1987).

Odani and Ikenaka (1973) reported the complete amino acid sequence (Figure 2.5). It is a single polypeptide chain with 71 amino acids including seven disulfide bonds (Liener and Kakade 1980), with molecular weight around 8000 Da. This inhibitor is the smallest protein found in soybean to date (Wolf 1977).

The active site for trypsin is at residue lysine16-serine17 while that for chymotrypsin consists of residue leucine44-serine45 (Synder and Kown 1987). The secondary structure of Bowman-Birk trypsin inhibitor has been reported by a number of workers. Steiner and Frattali (1969) concluded, from a circular dichroism study, that Bowman-Birk trypsin inhibitor had low α–helix structure and recently Wu and Sessa (1994) reported 61 % β–sheet structure, 38 % unordered structure, 1 % turn and 0 % α–helix structure.

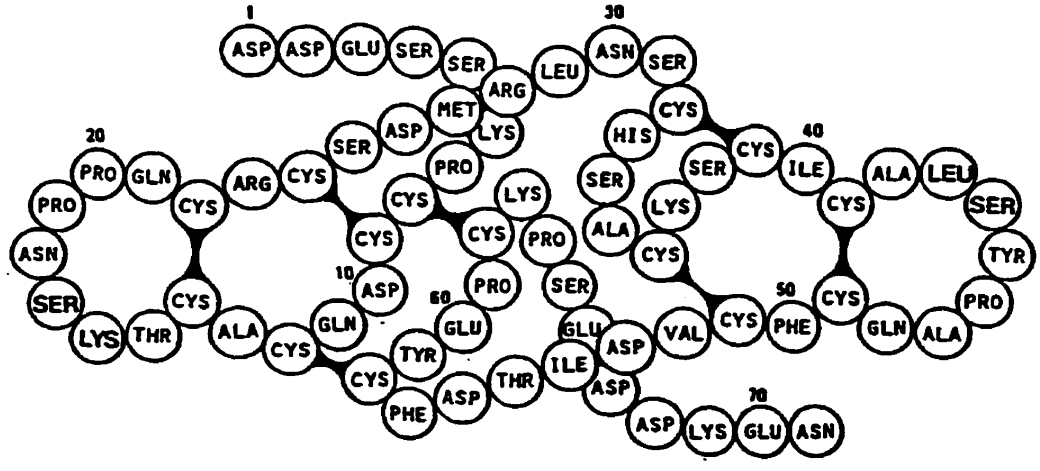


Figure 2.5: Primary structure of Bowman-Birk trypsin inhibitor from isolated soybean (Odani and Ikenaka 1973)

2.5 Isolation of Soybean Proteins

2.5.1 Extraction / Isoelectric Precipitation

Figure 2.6 shows the commercial procedure used for isolating soy protein. This involves extraction of defatted soybean with water or dilute alkali (pH 7-10), followed by centrifugation of the slurry to separate the insoluble residue to obtain a dispersion containing soluble protein and nonprotein materials. A major portion of the protein is then precipitated at pH 4.5, collected by centrifugation, and washed to remove soluble nonprotein materials such as carbohydrates and salts. The isolated soy protein is dried and may then be neutralized (Johnson and Kikuchi 1988; Waggle *et al.* 1989). The disadvantages of this method include denaturation of protein upon exposure to alkali and acid treatment, high ash content, and alteration of protein solubility after rehydration (Wolf 1978; Nash *et al.* 1979). The denaturated protein is formed mostly from the 2S and 7S fractions. This protein is tan to brown in color and binds nonprotein material (oxidized lipids and off-flavors) (Anderson and Warner 1976). The presence of acid sensitive protein, in addition to imparting off-flavors, limits the use of acid precipitated proteins in many foods (Kinsella *et al.* 1985).

Thanh and Shibasaki (1976) fractionated β–conglycinin and glycinin based on differing solubilities. The β–conglycinin precipitated at pH values between 4.0 and 5.6, whereas the glycinin precipitated between 4.4 and 6.8.

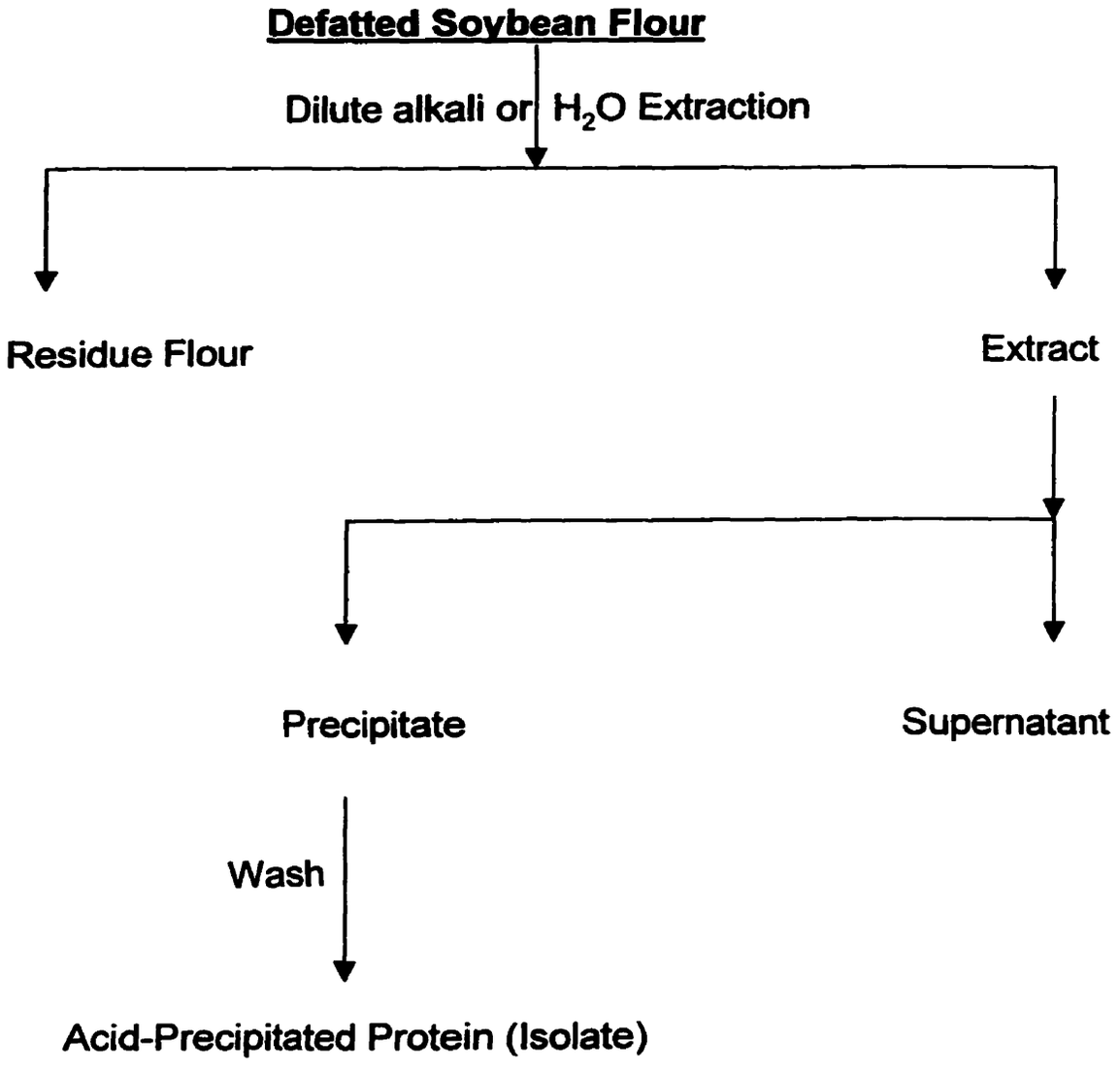


Figure 2.6: Schematic of isoelectric precipitation
Johnson and Kikuch (1988)

In the pH 6.1 - 6.6 region, the β–conglycinin dissolves while most of the glycinin precipitates (Liu 1997). The method of Thanh and Shibasaki (1976) includes extraction of defatted soybean with 0.06 M Tris buffer (pH 8) containing 0.01 M 2-mercaptoethanol at room temperature for 1 h. The extract is adjusted to pH 6.4 with 2 N HCl. The glycinin is collected by centrifugation. β–conglycinin is separated from the supernatant by adjusting to pH 4.8. Howard *et al.* (1983) extracted defatted soybean with an aqueous solution of 0.03-0.06 M sodium chloride and 0.5-0.8 mM sodium bisulfate followed by centrifugation. The extract was adjusted to pH 5.5 with HCl and the precipitated protein separated by centrifugation. The supernatant was adjusted to pH 4.5 and additional precipitated protein was separated from the whey protein and sugars. The protein that precipitated above pH 5.5 consists of about 90 % glycinin, whereas the remaining protein that precipitated at pH 4.5 consists of about 70 % β–conglycinin and 30 % glycinin.

Another procedure, described by Lehnhardt *et al.* (1983), involves extraction of defatted soybean at pH 8.0, clarification of the extract, precipitation of the protein at pH 4.3 with HCl, and separation of the crude protein from the whey protein and sugars. The crude protein was resuspended in an aqueous medium of 0.1 M sodium chloride and 7.5 mM sodium bisulfate. The suspension was brought to pH 5.3 with NaOH. At this pH, the β–conglycinin dissolved and the remaining insoluble protein was comprised of mostly glycinin.

2.5.2 Extraction / Cryoprecipitation

The term plant cryoprotein was introduced by Ghetie and Buzila (1962) to designate proteins that precipitate when aqueous extracts of seeds are cooled (2 - 4 °C). Factors affecting cryoprecipitation include temperature, pH, NaCl, sucrose, $CaCl_2$, and protein concentration (Wolf and Sly 1967). Cryoprecipitation occurs only when the total protein concentration of the extract is greater than 1% or when the protein concentration in the extract exceeds 4 mg / ml (Peng *et al.* 1984).

The cryoprecipitation procedure (Figure 2.7) consists of extraction of defatted soybean with water at room temperature; the insoluble residue is removed by centrifugation. The extract is then cooled to near 0 °C, a precipitate is formed and can be removed by centrifugation (Wolf and Sly 1967). The precipitate obtained from this procedure contains mainly the glycinin fraction. Other investigators have modified this procedure to extract both major soybean proteins (7S and 11S); for example, Davidson *et al.* (1979) have used the procedure with some modifications, to extract separately, the glycinin fraction and β–conglycinin fraction.

2.5.3 Extraction / Ammonium Sulfate Precipitation

This method involves extraction of soybean proteins using water followed by salting out of the extracted proteins by addition of ammonium sulfate which

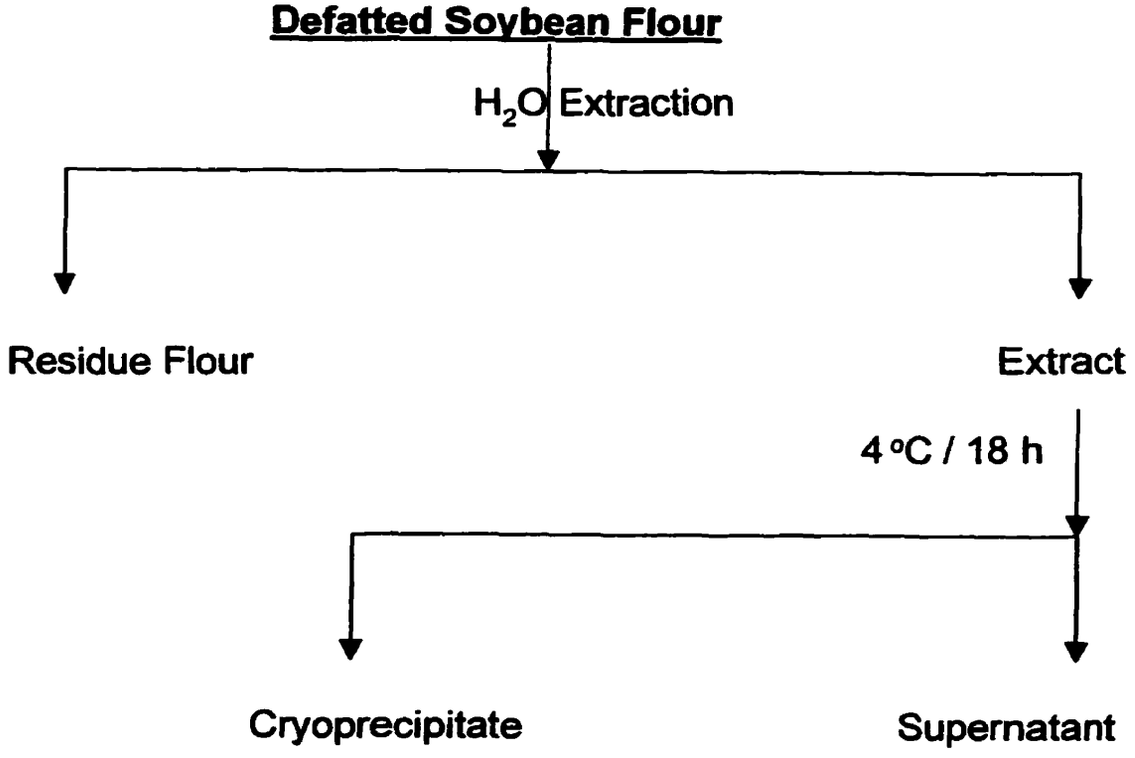


Figure 2.7: Schematic of cryoprecipitation
Wolf and Sly (1967)

allows the fractionation of the β-conglycinin and glycinin (Wolf *et al.* 1962). Dialysis of ammonium sulfate purified glycinin against standard phosphate buffer in the cold for 7 days, resulted in the formation of small amounts of β-conglycinin. Other problems associated with this method are the incomplete separation of the different fractions, the large losses of proteins, and the aggregation of protein molecules, which occurs during precipitation (Kilara and Harwalkar 1996).

2.5.4 Extraction Using Hydrogen Peroxide / Isoelectric Precipitation

In this procedure (Lawhon *et al.* 1981), ground soybeans were extracted with water containing hydrogen peroxide (to inactivate lipoxygenase) at 60 °C and pH 9.0. The slurry was fed into a three-phase centrifuge to separate protein solution, oil emulsion, and residual fiber. The emulsion was broken to recover oil, and the aqueous phase was used to produce protein by acid precipitation. Residual oil level in the spray-dried proteins was about 8 – 10 %.

2.5.5 Extraction / Membrane Processing

Extraction was made with tap water (10:1 solvent-to-flour ratio by weight) adjusted to pH 9 with sodium hydroxide (Lawhon *et al.* 1977). After centrifugation, the insoluble residue was resuspended in one-half the original amount of water at the same pH to more completely remove solubilized

components. Supernatants from the first and the second centrifugation were mixed and pasteurized by heating followed by prefiltration before membrane processing. After pasteurization and prefiltering, the extract was pumped to the membrane system. The extract was concentrated to one-fifth of the starting solution followed by spray-drying the concentrated solution (Lawhon *et al.* 1977).

2.6 Functional Properties of Soy Proteins

Functional properties have been defined as those physicochemical properties of proteins which affect their behavior in food systems during preparation, processing, storage, consumption and contribute to the quality and sensory attributes of food systems (Kinsella 1976). Pour-El (1981) defined functionality as any property of a food or food ingredient, except its nutritional properties, that influences its utilization, this definition has for many years, served well as a general description of the subject, but today it may require modification. The reason for this, is that foods claiming nutritional or health benefits are being described as functional foods (Hall 1996).

Functional properties of proteins in food applications can be classified into three main groups (a) hydration properties: the protein-water interaction, such as water absorption and retention, solubility and viscosity; (b) protein-protein interaction: gelation and dough formation; (c) surface properties: relate primarily to surface tension, emulsification, and foaming characteristics (Cheftel and Cug 1983). From the food manufacturers viewpoint, functional properties are used

for producing or stabilizing certain structures in foods. The specific applications are generally known as emulsification, foaming, gelation, fat or water binding and viscosity (Patel and Fry 1987).

Soy protein isolates are employed in comminuted meats and in dairy foods in which emulsifying, thickening, and gelling properties are of prime importance (Kinsella *et al.* 1985). The functional properties of soy proteins are affected by several intrinsic and extrinsic factors. The intrinsic factors include size, shape, amino acid composition and sequence, net charge and distribution pattern of charges, and hydrophobicity / hydrophilicity ratio, secondary, tertiary and quaternary structural arrangements and inter- and intra-peptide cross-links (disulfide bonds). The extrinsic factors include the method of extraction and isolation, pH of medium, temperature of extraction, ionic strength, moisture content, and interaction with other food component (Zayas 1997a).

2.7 Protein Gelation

Protein gelation refers to the transformation of the protein from the sol state into a gel-like structure by heat or other agents, in which the individual protein molecules interact with each other to form a three-dimensional network (Damodaran 1989). Gelation is a result of hydrogen bonding, van der waals forces, and covalent disulfide bonding (Katsuta *et al.* 1990).

Protein-protein, protein-solvent interactions, the strength of junctions, and the flexibility of the polypeptide chains determine the gelling properties of

proteins. If protein-protein interactions are extremely strong, the three-dimensional network collapses and water will released from the structure. Electron microscope studies have shown that linear aggregation of protein molecules was more important than their random aggregation (Hillier *et al* 1980). The properties of gels are different for various proteins, depending on the type of protein-protein and protein-water interactions that stabilize the gel (Schmidt *et al.* 1981). High molecular weight proteins and a high percentage of amino acids with hydrophobic groups tend to establish the strong network of the gel system (Zayas 1997a).

2.7.1 Heat-Induced Gelation

Heat-induced gelation is an important property for preparing soy-based products such as tofu and soy-yogurt. The formation of protein networks is considered to be the result of a balance of protein-protein and protein-water interactions and attraction and repulsion forces occurring between adjacent polypeptide chains. Among attraction forces, are hydrophobic interactions (favored at high temperatures), electrostatic forces (calcium bridges), and disulfide bonds (Puppo and Anon 1998). The contribution of each bond type depends on the nature of the stage in the gelation process as well as on pH and ionic strength of the medium (Utsumi *et al.* 1984; Puppo *et al.* 1995). Heat-induced gel formation was suggested to be a two stage process that involves unfolding of the native protein followed by protein-protein and protein-solvent

interactions resulting in a three-dimensional network which forms the gel during heating (Ferry 1948), such that,

$$XP_N \longrightarrow XP_D \longrightarrow (P_D)_X$$

where X is the number of protein molecules, P_N is the native protein, and P_D is the denaturated protein (Smith, 1994). Thus, gelation is a two-stage process involving the initial denaturation of native protein into uncoiled polypeptides, which may associate gradually to from the gel matrix, if attractive forces and thermodynamic conditions are suitable. The overall process requires that the proteins unfold initially and that the aggregation step proceeds more slowly than denaturation, to allow the denatured protein molecules to orient them and interact at specific points resulting in a three-dimensional network. According to the Ferry theory (1948), the final gel state corresponds to aggregates of partly denaturated protein. When aggregation occurs very rapidly, coagulum characterized by high opacity, low elasticity and considerable syneresis is obtained (Morrissey *et al.* 1987).

2.7.1.1 Types of Heat-Induced Gels

There are two basic types of heat induced gel structures depending on the conditions involved: 1) thermo-set or thermo-reversible and 2) thermoplastic or thermo-irreversible gels. In thermo-set gelation, the sol or progel is formed by

heating and is usually accompanied by increased viscosity. Thermo-set gels can be melted by subsequent heating and form a gel upon cooling but may revert to the progel state by subsequent reheating, suggesting that the aggregation is reversible. The process involves the formation of an elastic solid, permanently cross-linked three-dimensional network as exemplified by soy protein, egg-white protein and conalbumin (Morrissey *et al.* 1987, Damodaran 1989). In thermoplastic gels, such as whey protein and haemoglobin gels, melting or reversion to the progel does not occur under normal conditions; these gels may, however, soften or shrink (Morrissey *et al.* 1987).

2.7.1.2 Heat-Induced Gelation of Soy Proteins

Catsimpoolas and Meyer (1970) proposed the following scheme for the gelation of soy proteins (Figure 2.8), according to this scheme, when a soy protein dispersion (sol) is heated above its denaturation temperature, the sol increases in viscosity and undergoes an irreversible change to the progel state. This step involves dissociation, denaturation, and then unfolding of the protein molecules. Progel becomes gel when it is cooled and viscosity increases again; this step is reversible as it involves mainly noncovalent bonds (Oakenfull *et al.* 1997). Heating of the sol at temperatures much higher than the denaturation temperature of soy protein leads to a metasol state which does not set into a gel upon cooling (Catsimpoolas and Meyer 1970).

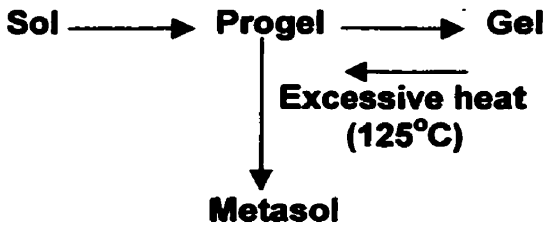


Figure 2.8: Schematic of the heat-induced gelation of soy proteins. Yamauchi *et al.* (1991).

This might be related to B- elimination of disulfide bonds and scission of peptide bonds involving aspartate residue at high temperature (Ahern and Klibanov 1985, Volkin and Klibanov 1987). In addition, soy protein gels melt to the progel state upon heating, suggesting that the progel transition to gel is reversible. This also suggests that the forces responsible for the formation of gel structure, such as hydrophobic and electrostatic interaction and hydrogen bonding are noncovalent (Catsimpoolas and Meyer 1970).

Van-Kleef (1986) proposed that soy protein gelation is irreversible because the native state of the protein is not restored when the solution is cooled. An additional complication is the fact that soy proteins contain several protein fractions. It has been suggested that the glycinin fraction alone accounts for thermoreversibility and that the physical state of this fraction before aggregation determines the extent of reversibility (Utsumi *et al.* 1997).

2.7.1.3 Heat-Induced Gelation of Glycinin

The heat-induced gelation of glycinin (Figure 2.9) was proposed by Mori *et al* (1982). In step 1, glycinin aggregates (MW 8 X 10^6 Da) are formed when glycinin solution is heated at low and high protein concentration. At low protein concentration, on subsequent heating results in disaggregation to acidic and basic polypeptides (step 2'), while at high protein concentration, there is association resulting in gel formation (step 2). Whether the heated protein undergoes disaggregation or gel formation is governed by the concentration of the soluble aggregates. Although the formation of the network structure is completed by step 2, the stabilization of the network structure by formation of non-covalent bonding and disulfide cross-links take place by subsequent heating (step 3) (Utsumi *et al.* 1997).

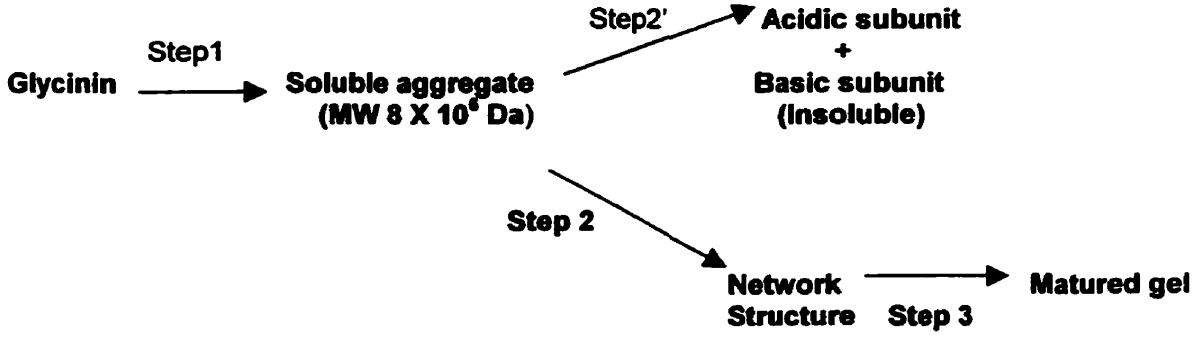


Figure 2.9: Schematic of the heat-induced gelation of glycinin. Utsumi *et al.* (1997).

2.8 Factors Affecting the Gelling Properties of Soy Proteins

2.8.1 Effect of Protein Concentration

Protein concentration is an important factor in determining the type of gel and final gel characteristics. Protein concentration required for gel formation varies depending on the protein properties; gelatin will form a gel at relatively low concentration, while, globular proteins require higher initial concentration for gel formation, therefore, gelation of these proteins will not occur below certain concentration (Zayas 1997a). At low concentration, a protein network is difficult to establish and protein-protein interaction tend to occur within molecules rather than between molecules and gel network can not be obtained. The inter-molecular interactions are possible at increased protein concentration, resulting in firm gels (Jayaprakasha and Brueckner 1999). The minimum protein concentration required for soy isolates to form a heat induced gel is 8 % (Catsimpoolas and Meyer 1970). Mori *et al.* (1982) reported that high concentrations of glycinin led to gel formation, while low concentrations of glycinin favored disaggregation. Iwabuchi and Yamauchi (1984) and Nakamura *et al* (1986) reported that β-conglycinin dissociated and then recombined into soluble aggregates at high protein concentration and ionic strength. The mechanical strength of the gel also depends upon protein concentration, since it is related to the number of crosslinks formed per protein chain (Stanley and Yada 1992). Damodaran (1989) showed a linear relationship between gel strength and protein concentration for a soy isolate and β–conglycinin protein.

2.8.2 Effect of Heating Temperature

One effect of heat on soy proteins is to produce conformational changes involving destruction of the quaternary structure (Stanley and Yada 1992). 11S and 7S proteins registered thermal denaturation temperatures of 80 °C and 67 °C respectively (Babajimopoulos *et al.* 1983); while German *et al.* (1982) reported values of 92 °C and 77 °C; this difference between the two proteins was attributed to the large number of disulfide bonds in the glycinin protein. Generally, heating temperature above the minimum denaturation temperature of the protein is required for gel formation and the heating conditions used to form a gel greatly influence its rheological properties (Paulsson *et al.* 1986). Catsimpoolas and Meyer (1970) reported that heating soy protein isolate at lower temperature requires longer heating time for gelation than does at higher temperature; this also results in the formation of a weaker gel, indicating that at relatively low temperature the soy isolates are not sufficiently unfolded to from the appropriate three-dimensional network for maximum gel strength. Excessive heating of soy protein dispersion at temperatures much higher than the denaturation temperature can lead to a metasol, which does not gel upon further heating (Kinsella *et al.* 1985). Shimada and Cheftel (1988) suggested that soft gels obtained above 130 °C are due to disulfide bond breakdown. Baldwin (1986) reported that heating glycinin at 100 °C caused more unfolding and more exposure of hydrophobic regions on the surface of the glycinin molecule. In this case, hydrophobic interaction contributed predominantly to the formation and

stabilization of the gel network. When glycinin was heated at 80 °C, the lesser extent of unfolding and lesser exposure of hydrophobic regions on the molecular surface suggested that hydrogen bonding was the predominant force in the gel formation at this temperature.

2.8.3 Effect of Ionic Strength

Hermansson *et al.* (1986) reported that gels formed at low ionic strength (0.25M KCl) showed a fine microstructure, while gels formed at high ionic strength (0.6M KCl) showed coarsely aggregated microstructure. Wang (1981) reported that the gel strength of soy protein gels with various ratios of β-conglycinin and glycinin increased with addition of 2 % NaCl. However, a high NaCl concentration (10 %) caused failure of soy protein gel formation. The effects of NaCl on gelation of soy protein at low concentration are attributed to charge neutralization effects. However, soy protein failed to form gel at high concentration of NaCl due to a change in protein conformation, and due to an increase in hydrophobic interaction between proteins (Utsumi and Kinsella 1985).

2.8.4 Effect of pH

Protein denaturation, protein-protein and protein-solvent interactions are affected by pH (Zayas 1997a). pH adjustment may be necessary to achieve the proper balance between the rate of denaturation and aggregation as well as

forces of attraction and repulsion between adjacent protein chains that are necessary to achieve a protein gel (Doi 1993). The maximum gel strengths of soy protein isolates are obtained at neutral pH whereas weaker gels are obtained at high acidic and alkaline pHs, above pH 12 the formation of gel is completely inhibited (Catsimpoolas and Meyer 1970). At high acidic and alkaline pHs, the polypeptides possess high positive and negative charges respectively. Under these conditions the electrostatic repulsive forces may destabilize other favorable protein-protein interactions required for the formation of the gel network and thus decrease the gel strength. At neutral pH, intermolecular interactions between positively charged groups provide additional energy favoring gelation (Kinsella *et al.* 1985). The gels formed at alkaline pH values were elastic, transparent and had greater gel strength than the gels produced at lower pH values (Jayaprakasha and Brueckner 1999). Gels formed at the isoelectric pH of soy proteins are less hydrated and less firm because of the lack of repulsive forces. The type and stability of the gel structure is significantly affected by the net charge of the protein (Hermansson and Lucisano 1982).

2.8.5 Effect of Reducing Agents

Utsumi and Kinsella (1985) reported that glycinin formed a soft gel in the presence of 0.002M 2-mercaptoethanol, while coagulated gels were obtained at concentrations above 0.01M 2-mercaptoethanol. The lack of gel forming ability of glycinin in the presence of low concentration of 2-mercaptoethanol may be

caused by its dissociation into acidic and basic subunits, and/or inhibited the inter-subunit disulfide bonds. Catsimpoolas and Meyer (1970) reported that high concentration of 2-mercaptoethanol (10 %) enhanced soy protein gelation. Circle *et al.* (1964) reported that 0.05 % cysteine inhibited gelation but that 0.5 % cysteine was not effective in inhibiting gelation possibly due to the presence of both intermolecular and intramolecular disulfide bonds. Intermolecular bonds disrupted by low concentrations of 2-mercapotethanol may inhibit gelation whereas at higher concentration of 2-mercaptoethanol the intramolecular disulfide bonds may be reduced, facilitating complete unfolding of the protein, thereby exposing reactive groups and enhancing gel strength.

Nakamura *et al.* (1984) found that the presence of 100 mM N-ethyl maleimide (NEM) inhibited the heat-induced gelation of glycinin, and that heating only produced linear soluble aggregates. This indicated that the disulfide bond formation through intermolecular disulfide exchange reaction may participate in the formation of strands, particularly branched ones, followed by subsequent network formation and gel formation. Mori *et al.* (1982) suggested that hydrophobic interaction contribute to the formation of soluble aggregates of glycinin, because soluble aggregates were found to be disaggregated in the presence of sodium dodecyl sulfate (SDS) which is known to destabilize mainly hydrophobic interactions. Utsumi and Kinsella (1985) reported that the gel strength of glycinin markedly increased in the presence of propylene glycol (PG) at concentrations above 10 %; PG may have diminished the hydrophobic

contributions, but it enhanced the contribution of hydrogen bonds and electrostatic interaction by lowering the dielectric constant.

In the case of β-conglycinin, the gels were found to be more soluble in sodium dodecyl sulfate (SDS) solution suggesting that the formation of gel structure occurs mainly via hydrophobic interactions (Saio *et al.* 1974).

2.9 Cold-Set Gelation

The ability of pre-heated whey protein to form a gel at room temperature has recently been reported by Barbut and Foegeding (1993). By this procedure, a pre-heated whey protein suspension is cooled to room temperature and gelation is induced by salt. This phenomena was previously only employed in some carbohydrate gels (Barbut and Drake 1997) and was called Ca^{+2} –induced gelation (Barbut and Foegeding, 1993) or cold-setting gelation by McClements and Keogh (1995). The gel formed by this method has fine–strand structure, better water holding capacity (WHC), and higher gel strength than that formed by the conventional heat-induced gelation. These unique gel characteristics indicate that the Ca^{+2} –induced or cold-set gelation mechanism is different from the heat-induced gelation (Hongsprabhas and Barbut 1996).

A commercial cold-set gel has been prepared for use in various foods, such as surimi, salad dressing and bakery products (Ju and Kilara 1998). In the case of soy proteins, glycinin forms stable and firm gels at heating temperature between 90-100 °C, this limits its application in many types of meat products

which should be heated only to 70 °C, therefore glycinin does not promote gel formation in meat products (Shiga and Nakamura 1987). Often, it is necessary to improve the functional properties of soy proteins by modification of their structure using chemical, enzymatic, or physical processes (Creamer *et al.* 1989, McClemets and Keogh 1995).

2.9.1 Mechanism of Cold-Set Gelation

The possible mechanisms of cold-set gelation as shown in Figure 2.10 can be separated into two stages: (i) the preparation of a heat-denaturated protein solution and (ii) the induction of gelation by salt at low temperature. (Hongsprabhas and Barbut 1997a, Bryant and McClements 1998).

(i) Preparation of a heat-denaturated suspension

Pre-heating is a necessary step in cold-set gelation; it causes protein unfolding and form linear aggregates, but a gel does not form (Honsprabhas and Barbut 1997a). This requires careful control of the initial solution conditions (pH, mineral content, protein concentration) and heating conditions (temperature and holding time) (Bryant and McClements 1998). The protein suspension must be heated above its denaturation temperature and held for 5 to 60 min to insure the correct degree of protein unfolding and aggregation into the required structure (Barbut and Foegeding 1993, McClements and Keogh 1995).

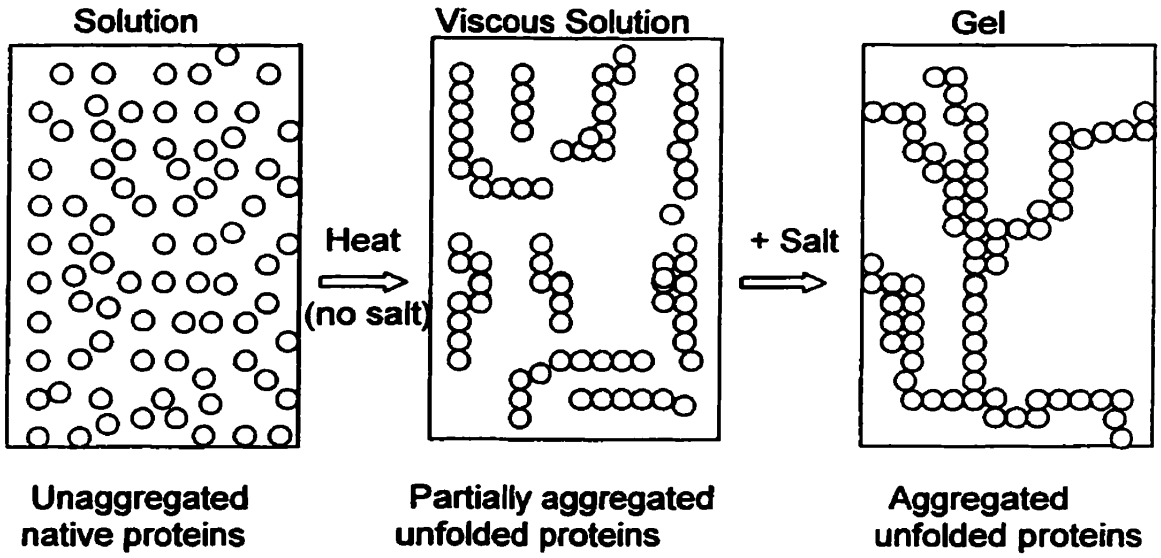


Figure 2.10: Schematic representation of the gelation of cold-setting WPI gels at pH 7. McClements and Keogh (1995).

The pH of the initial solution should be sufficiently different from the isoelectric point of the protein to ensure that they do not immediately aggregate upon heating (Bryant and McClements 1998). In the case of whey protein, most experiments have been done at pH 7 (Ju and Kilara 1998). The protein concentration must be low enough to prevent the molecules from forming a three-dimension network (Barbut and Foegeding 1993). The salt concentration of the initial solution must be low to prevent excessive aggregation of the protein molecules (Bryant and McClements 1998). However, whey protein suspensions ≤ 10 % did not form gels after heating in the absence of additional salt

suggesting that the original concentration of salts in the protein was below the critical level needed to form cold-set gelation (Mc Clements and Keogh 1995).

(ii) Salt induced gelation

Salt can be added to enhance gel formation, after the protein suspension has been heat denatured (Sato *et al.* 1995). However, the precise effects of salts on cold-set gelation is mostly unknown (Hongsprabhas and Barbut 1997b). Three hypotheses have been proposed to explain the role of salt in cold-set gelation: Jeyarajah and Allen (1994) suggested that $CaCl_2$ addition to a pre-heated protein suspension resulted in further denaturation (of the already denatured protein) producing a more open conformation than the native structure and hence, different degrees (and modes) of protein-protein interactions; Sato *et al.* (1995) suggested that the addition of salt caused a decrease of negative charge on the surface of the soluble aggregate, leading to formation of a three-dimensional network through hydrophobic interactions and sulfhydryl / disulfide inter-change reactions between the soluble aggregates; McClements and Keogh (1995) suggested that when a pre-heated protein suspension is mixed with NaCl or $CaCl_2$ the salts will shield the electrostatic repulsion between the soluble protein aggregates causing them to come together and form a gel.

2.10 Factors Affecting Cold-Set Gelation

2.10.1 Effect of Protein Concentration

Heating a 9 % whey protein isolate suspension at 80 °C for 30 min did not form a gel unless a salt was added to the protein suspension (Ju and Kilara 1998); once the protein concentration was increased, the viscosity increased. Above a critical protein concentration, a heated protein solution formed a gel rather than a viscous solution (Bryant and McClements 1998). Whey protein isolates required protein concentration of 12 % to form a self-supporting gel after heating at 80 °C for 30 min (Ju *et al.* 1995).

Hongsprabhas and Barbut (1997b) reported that the addition of $CaCl_2$ to a pre-heated whey protein suspension (10 %) resulted in formation of more transparent gels compared with preheated whey protein suspension with lower protein concentration. This indicated that a finer protein strand network was formed at high protein concentration. Relkin and Launay (1990) suggested that inter-molecular interactions occurring during heating were more predominant at high protein concentrations. Shimada and Cheftal (1989) suggested that both inter-molecular hydrophobic and inter-molecular disulfide bond formation increased as protein concentration increased. However, Morr and Ha (1993) reported that intra-molecular interaction were predominant at low protein concentration. Thus, it is likely that at low protein concentration, inter-molecular forces would have less effect on stabilization of the network than at high protein concentration.

2.10.2 Effect of Heating Temperature

Barbut and Foedeging (1993) reported that a whey protein isolate suspension preheated to > 70 °C was required to achieve enough structural change to make the protein molecule suspectable to salt–induced gelation. The combined effects of heating temperature (70 – 90 °C) and time (0 – 30 min) were demonstrated to affect the final gel characteristics (Barbut and Foedeging 1993). Kinsella (1984) suggested that at a higher degree of inter-molecular bonding can be expected at higher temperature. Overall, pre-heat treatments carried out at different temperatures result in differences in the degree of unfolding, interactions among proteins and a subsequent response to $CaCl_2$. Hongsprabhas and Barbut (1996) reported that $CaCl_2$ did not enhance gel formation of cold-set gelation when whey protein suspensions were heated at ≤ 70 °C. It has been suggested that this may be due to the importance of hydrophobic interactions in determining the initial aggregation of the protein (McClements and Keogh 1995). The hydrophobic force is relatively strong and its magnitude increases as the temperature is raised (Bryant and McClements 1998); consequently, it is believed to play an important role in determining the aggregation rate of the protein. Regardless of this observation, once the gels have formed, their strengths tend to increase as the temperature is decreased (Van-Camp *et al.* 1997) suggesting that hydrophobic interactions play a dominant role in the initial stages of aggregation. Non-hydrophobic interactions

play more important role in determining the final gel strength (Bryant and McClements 1998).

2.10.3 Effect of Ionic Strength

The effect of salt concentration on gel structure depends on whether the salts are mono-valent or di-valent (Kuhn and Foedgeding 1991). Maximum gel strength was achieved with 120 mM $CaCl_2$ (Hongsprabhas and Barbut 1997), compared to 400 mM of NaCl (Barbut and Drake 1997); indicating that gelation caused by mono-valent cations is mainly induced by charge dispersion while di-valent salts induce charge dispersion as well as aggregation because of their ability to act as bridges between the negatively charged carboxylic groups on neighboring protein molecules.

2.10.4 Effect of pH

The fact that electrostatic interactions play a dominant role in determining the aggregation of proteins in heat-denatured protein solutions suggests that the pH should also have a large influence in cold-set gelation, however there is no evidence of previous work which relates pH to cold-set gelation characteristics (Bryant and McClements 1998).

2.11 Water Holding Capacity (WHC)

An important property of gels is their water holding capacity (Stanley and Yada 1992). The term WHC has been defined by Kinsella (1984) as the grams of water held per 100 grams of protein, while Hermansoon (1986) defined WHC as a physical property and is the ability of a gel to prevent water from being released from the three-dimensional structure of the gel.

Some protein gels retain up to 95 % water (w / w); this water is physically immobilized by the protein structure and can not be easily released by pressure. The CO- and NH- groups that are exposed during denaturation become polarized and clustered, creating a water multilayer along the polypeptide chain, which on cooling, interact forming hydrogen bonds, producing the structure required to immobilize free water (Puppo and Anon 1998).

WHC plays a major role in the formation of food texture, especially in comminuted meat products and baked dough (Zayas 1997b).

Soy protein products (soy isolate and concentrate) rank above wheat flour, sodium caseinate, skim milk powder, and potato starch based on WHC (Comer and Dempster 1981). Using soy proteins (flour, concentrate, and isolate) in comminuted meat increased WHC and yield, decreased cook losses and had no detrimental effect on color (Kinsella *et al.* 1985). Also, addition of soy proteins to these products enhanced textural and sensory properties (Gnanasmbandam and Zayas 1992; Leconate *et al.* 1993). Van-Denover (1977) reported that the use of soy proteins in fish fillets increased the WHC of these products.

2.12 Factors Affecting WHC

2.12.1 Effect of Protein Concentration

The amount of water retained by a protein depends upon the amino acid composition (especially the number of polar groups), conformation of proteins, surface hydrophobicity and processing history (Kinsella 1984). Soybean products can have various protein concentrations (Hutton and Campbell 1977). For instance soy flour, soy concentrate and soy isolate had WHCs of 2.6, 2.75 and 6.25 g water / gram solids, respectively (Fleming *et al.* 1974). Lin *et al.* (1974) reported values of 1.3, 2.2 and 4.4 g water / gram solids for soy flour, soy concentrate and soy isolate, respectively. The higher WHC of soy isolate may be related to better swelling capacity and to the capacity to dissociate and expose water binding sites, while in the soy concentrate, carbohydrate and other components may impair the ability to retain water (Zayas 1997b).

2.12.2 Effect of Heating Temperature

The WHC of a gelling protein increases with gel formation. Heating above the gelation temperature results in increased degree of phase separation and poor WHC for certain proteins (e.g whey protein) due to the formation of a coarser structure with larger aggregates and pores (Boye 1995). In the case of glycinin, however, increased heating led to transition from an aggregated disordered gel to an ordered structure consisting of strands (Stanley and Yada 1992). Overall, there has been no reported systematic study of the effects of

heat treatment and of interaction of heat and salt on water holding capacity of soy proteins (Kinsella *et al.* 1985).

2.12.3 Effect of Ionic Strength

Fleming *et al.* (1974) reported that NaCl (5%) enhanced the WHC of soy flour but reduced that for soy concentrate and soy isolate. On the other hand, Lopez de Ogara *et al.* (1978) reported that WHC of soy isolate was not greatly affected by the addition of solutes, NaCl (2 - 15 %), $CaCl_2$ (2.5 - 10 %) and Na_2HPO_4 (2 – 10 %). Hermassom and Akesson (1975) reported that the WHC of soy protein in chopped meat was enhanced by salt.

2.12.4 Effect of Reducing Agents

Addition of reducing agents greatly affected the WHC (Kinsella *et al.* 1985). Ochiai-Yangi *et al.* (1978) indicated that the optimum concentration of urea for maximum water holding was around 0.5 M, indicating the disruption of the structure of native soybean protein by urea treatment. Sodium dodecyl sulfate (0.5 %) increased the WHC of soy concentrate. This could be related to disruption of hydrophobic bonds and an increased negative charge of the protein by sodium dodecyl sulfate treatment (Kinsella *et al.* 1985).

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF A GLYCININ-RICH PROTEIN FROM DEFATTED SOYBEAN.

3.1 MATERIALS AND METHODS

3.1.1 Materials

Commercial defatted soybean flour (50 % protein, 1.2 % fat, 3.5 % fiber, 8 % moisture) was obtained from Daminco Inc (Dorval, Quebec, Canada) and stored in airtight containers at 4 °C.

3.1.2 Preparation of Proteins

3.1.2.1 Sodium Hydroxide and Water Extraction / Acid Precipitation.

Proteins were extracted using the procedure of Fan and Sosulski (1974) as modified by Alli and Baker (1980). Defatted soybean (100g) was mixed with distilled H_2O (1 L) or dilute NaOH (1L, 0.02%, pH 10.7) and allowed to stand for 1 h with intermittent stirring. The mixture was centrifuged (12,000 x g) for 10 min, the extract was filtered through glass wool and the residue was discarded. The pH of the filtrate was adjusted to 4.5 by the dropwise addition of HCl (2N) with continuous stirring. The precipitated proteins were recovered by centrifugation (12,000 x g) for 10 min and then lyophilized. These isolates were

designated as H_2O isoelectric isolate (H_2O-IE) and NaOH isoelectric isolate (NaOH-IE). Samples from the NaOH and H_2O extracts and supernatants were dialyzed against deionized water (Spectra / Pormembrane, molecular weight cut off 8,000 Da) and lyophilized.

3.1.2.2 Citric Acid Extraction / Cryoprecipitation.

The procedures described by Melnychyn (1969) and modified by Alli and Baker (1980) were used for the extraction of proteins with citric acid solution. Defatted soybean (100g) was mixed with citric acid solution (1L, 0.2N, pH 4) and allowed to stand for 1 h with intermittent stirring; the mixture was centrifuged (12,000 x g) for 10 min. The extract was filtered through glass wool and the residue was discarded. The filtrate was refrigerated (4 °C) for 18 h, the proteins, which precipitated, were recovered by centrifugation (12,000 x g) for 10 min followed by lyophilization. This isolate was designated as citric acid cryoprecipitate (CA-CP). Samples from the citric acid extracts and citric acid supernatants were dialyzed against deionized water (Spectra / Pormembrane, molecular weight cut off 8,000 Da) and lyophilized.

3.1.2.3 Sodium Hydroxide Extraction / Cryoprecipitation

Proteins were extracted using the procedure of Davidson *et al.* (1979) with some modifications. The defatted soybean meal (100g) was mixed with dilute NaOH (1L, 0.02 %, pH 10.7) and allowed to stand for 1 h at room temperature

with intermittent stirring. The mixture was centrifuged (12,000 x g) for 10 min. The extract was refrigerated (4 °C) for 18 h and the proteins which precipitated were recovered by centrifugation (12,000 x g) for 10 min followed by lyophilization. This isolate was designated as sodium hydroxide cryoprecipitate (NaOH-CP). Samples from the NaOH extract and NaOH-CP supernatant were dialyzed against deionized water (Spectra / Pormembrane, molecular weight cut off 8,000 Da) and lyophilized.

3.1.3 Protein Contents and Yields of Isolates

The protein contents of the isolates were determined by the micro-Kjeldahl procedure (A.O.A.C., 1980); nitrogen content was converted to protein content using a factor of 6.25. All analyses were performed in triplicate. Protein yield was calculated on the basis of the weight of the isolate obtained and on the protein contents of defatted soybean.

3.1.4 Protein Characterization

3.1.4.1 Electrophoresis on Polyacrylamide Gels (PAGE)

PAGE was performed according to the method of Davis (1964) using a Mini-Protean II Electrophoresis Cell unit (Bio-Rad, Hercules, CA). A 4 % acrylamide stacking gel and a 6 % separation gel were used. Sample solutions (15 μl), prepared from 1 mg of freeze-dried protein extracts, isolates, or

supernatants dissolved in 1 ml sample buffer (0.3M tris-HCl pH 8.8, 1 % glyecrol and 0.05 % bromophenol blue), were applied to each sample well. Electrophoresis was carried out for 3 h at constant current (6 mA / gel) using tris glycine buffer (pH 8.3). Gels were stained with Coomassie Brilliant Blue R-250 (0.1 % w/v) in water / methanol / acetic acid and destained with the same solvent system but without dye.

SDS-PAGE electrophoresis was carried out on slab gels (4 % stacking and 12 % separation gels) using the technique described by Laemmli (1970). The protein samples (15 μl) prepared from 1 mg of extract, isolates or supernatants dissolved in 1 ml sample buffer (3 % SDS, 0.7M 2-mercaptoethanol, 25 mM tris-HCl pH 6.8, 1 % glycerol and 0.05 % bromophenol blue), were heated at 95 °C for 5 min. Electrophoresis was performed at constant current (30 mA / gel) for 1 h. The gels were stained with 0.1 % Coomassie Brilliant Blue R-250 in water / methanol / acetic acid, and destained in the same solvent system but without dye. A SDS-PAGE broad range molecular weight standard, (Bio-Rad Hercules, CA) was subjected to the same procedure as described above.

3.1.4.2 Fractionation of Proteins by Size-Exclusion Chromatography

A glass column (34 cm long, 2.6 cm internal diameter) was packed with Sepharose-CL 6B gel (Pharmacia, Sweden) previously washed with distilled water, followed by a phosphate buffer (0.0325 M K_2HPO_4, 0.0026 M KH_2PO_4, 0.4M NaCl, 0.01 M 2-ME, 0.02 % NaN_3, pH 7.6), and equilibrated (0.8 ml / min)

for three days with the same buffer. A quantity (100 mg) of the lyophilized soy protein extracts, isolates, or supernatants were dissolved in 7 ml of the phosphate buffer and the solution was filtered through a membrane filter (0.45 μm, Millipore). Filtrate (5 ml) was loaded to the column and eluted with the same phosphate buffer used to equilibrate the column, using a constant flow rate of 0.8 ml / min. The eluted protein was collected with an automatic fraction collector. Detection of the protein fraction was done by measurement of UV absorbance at 280 nm. The collected fractions were dialyzed against deionized water (Spectra / Pormembrane, molecular weight cut off 8,000 Da) and lyophilized. Fraction 2 obtained from the protein isolates (Figure 3.3) was subjected to SDS-PAGE using the procedure described above.

3.1.5 Differential Scanning Calorimetry (DSC)

The denaturation characteristics of the soy protein isolates were studied using differential scanning calorimeter (DSC) equipped with TC11 processor (Mettler TA 3000, Mettler Instrument Corporation, Greitensee, Switzerland). For each run, a sealed empty DSC medium pressure pan was used as reference. Protein solutions (50 μl; 12 % w / v, pH 8) were placed in preweighed DSC medium pressure pans which were hermetically sealed and reweighed. The samples were heated from 20 to 180 °C (heating rate was 10 °C / min), cooled to 20 °C, then reheated in the same manner to investigate the reversibility of

protein denaturation. Indium standards were used for DSC calibration. All analyses were performed in duplicate.

3.2 RESULTS AND DISCUSSIONS

3.2.1 Protein Contents and Yield of Isolates

The protein contents of the isolates (Table 3.1) were similar (84 – 86 % protein) except for the NaOH-CP (91 % protein), these values are similar to protein contents of soy protein isolates reported by other researchers (Johnson and Kikuchi 1988; Dilollo *et al.* 1991, Hettiarachchy and Kalapathy 1997). The yield of proteins which were obtained from deffated soybean with the various solutions, are shown in Table 3.1. The highest protein yield (78 %) was obtained for NaOH-IE while the yield of NaOH-CP was 22 %. By extraction soy protein with a salt solution at pH 8, Howard *et al.* (1983) obtained 22.8 % glycinin. The lowest yield (2.5 %) was obtained from CA-CP; Alli and Baker (1980) reported that a limitation of organic acid extraction was its relatively low yield of protein.

3.2.2 Polyacrylamide Gel Electrophoresis

3.2.2.1 Native Conditions

The acid precipitated proteins (NaOH-IE, H_2O-IE) and the citric acid cryoprecipitated (CA-CP) gave two distinct bands designated as I and II (Figure 3.1) whereas the cryoprecipitate NaOH-CP showed a predominance of band I and only trace of band II. Sathe (1991) reported that soy protein contains two

Table 3.1: Protein contents and protein yields of soy protein isolates

Protein Isolate	% Protein[1]	% Protein Yield
NaOH-IE	86.0 ± 1.40	78.0 ± 0.21
H_2O-IE	84.5 ± 1.91	63.0 ± .22
NaOH-CP	91.0 ± 1.84	22.0 ± 0.90
CA-CP	84.2 ± 1.09	2.5 ± 0.98

1. Kjeldahl N X 6.25
Results are means and standard deviation of triplicate determinations.

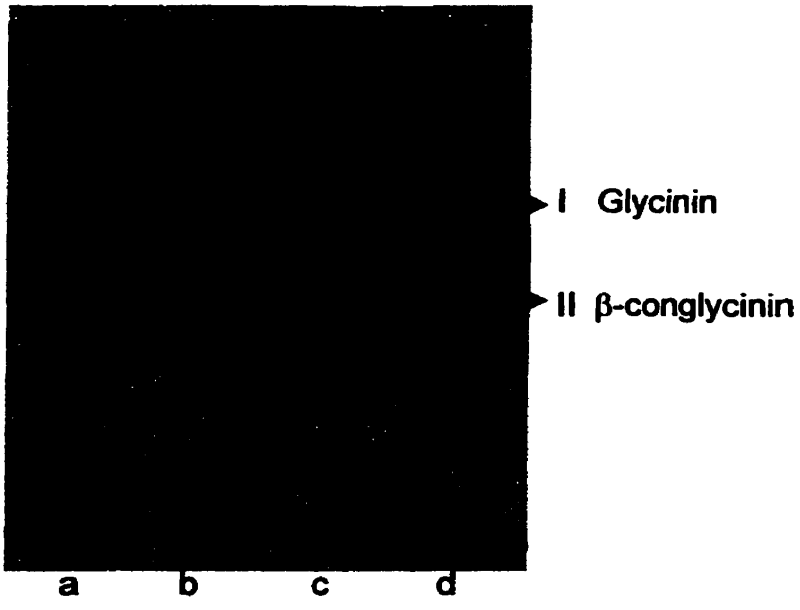


Figure 3.1: Electrophoresis (native PAGE) of
a- NaOH-IE, b- H_2O-IE, c- NaOH-CP, d- CA-CP

major proteins with a molecular weight of 350 kDa for glycinin and 180 kDa for β-conglycinin.

3.2.2.2 SDS-PAGE

The molecular weights of the subunits of the proteins in soybean have been reported by several researchers (Table 3.2). Sathe *et al.* (1987) reported the MW of lipoxygenase was 93330 Da, while β-conglycinin subunits α', α, and β were 82220, 70630 and 48420 Da, respectively and acidic and basic subunits of glycinin were 33570 and 20650 Da respectively. MW similar to these have been reported by Petruccelli and Anon (1995), Sathe (1991), Iwabuchi *et al.* (1987) and Fontes *et al.* (1984). Brooks and Morr (1985) reported the MW of β-conglycinin α' subunits ranged from 83000 - 57000 Da, α subunit ranged from 76000- 57000 Da , and β subunits ranged from 53000 – 42000 Da, these values was confirmed by Arrese *et al.* (1991) who also reported that glycinin acidic subunits and basic subunits showed MW, ranging from 37000 to 42000 Da and 17000 to 20000 Da, respectively. Koshiyama *et al.* (1981) reported that some of the 2-S globulins precipitated between pH 5.8 and pH 4.5 were identical with Kunitz trypsin inhibitor (MW 21500 Da). In the present work, the assignment of bands obtained with SDS-PAGE was based on the published information summarized in (Table 3.2).

In the presence of SDS, the NaOH-IE and the extract from which this isolate was prepared gave at least 15 subunits (Figures 3.2Aa, 3.2Ba). The bands with MW 85, 75 and 50 kDa correspond to β–conglycinin subunits while those of MW 42, 38, 37, 35, 22 and 12 kDa correspond to glycinin subunits (Table 3.2). The supernatant obtained after precipitation of the NaOH-IE showed a marked decreased in the intensity of the bands which represent β–conglycinin and glycinin (Figures 3.2 Ca); this suggests that both β–conglycinin and glycinin were mainly removed from the extract during isoelectric precipitation.

The results from the H_2O extract, H_2O-IE and H_2O supernatant (Figures 3.2Ab, 3.2Bb, 3.2Cb) were similar to those from the NaOH extract, NaOH-IE and NaOH-IE supernatant.

The SDS-PAGE results of extract from the NaOH-CP and the NaOH-IE are identical (Figure 3.2Aa, 3.2Ac); this is expected since the extraction procedures are identical. However, the SDS-PAGE of the NaOH-CP was substantially different from that of NaOH-IE (Table 3.2, Figure 3.2Ba, 3.2Bc). The major bands in NaOH-CP were 42, 38, 22 and 12 kDa representing glycinin subunits; while the bands 85, 75 and 50 kDa which representing β–conglycinin subunits were minor bands. This is consistent with the results from the native PAGE which suggested that glycinin was the major component in the NaOH-CP. β-

Table 3.2: The molecular weight (Da) of subunits of soybean proteins

Protein / Subunits	MW reported by previous researchers (Da)		MW obtained in present work Isolate			MW obtained in present work F_2 from proteins isolates		
			NaOH-IE	NaOHCP	CA-CP	NaOH-IE	NaOH-CP	CA-CP
Aggregates	100000[a]		98000	2	2			
7S								
Lipoxygenase	93330[b]	91000[c]	92000	ND	2			
β–conglycinin α'	82200[b]		85000	2	1	85000	ND	1
α	70630[b]	68000[d]	75000	2	1	75000	ND	1
β	52000[e]	48420[c]	50000	2	1	50000	2	1
Agglutinin or H_I and H_{II} basic 7S globulin	30000[a, f]		31000	2	2	30000	2	1
L_I and L_{II} basic 7S	18000[l]		17000	2	2			
11S (glycinin)								
A_3	42000[g]	40740[b]	42000	1	2	42000	2	ND
	40000[b]							
$A_{1a}A_{1b}A_2A_4$	38000[h]	37000[b]	38000, 37000	1	2	38000	1	ND
	34000[h]	33570[b]	35000	2	2			
$B_{1a}B_{1b}B_2B_4$	20650[b]	20000[g]	25000, 22000	1	2	22000	1	2
A_5	10000[b]	11750[k]	12000	1	2	12000	1	2
2S								
Kunitz trypsin inhibitor	18000[l]		17000	2	2			

ND Not detected, 1 Major band, 2 Minor band

a. Petruccelli and Anon 1995, b.Sathe *et al* 1987, c. Wolf *et al* 1992, d. Dieckert and Dieckert 1985, e. Nielsen 1985b, f. Iwabuchi and Yamauchi 1987, g. Moreia *et al* 1979, h.Utsumi *et al* 1985, I. Sathe *et al* 1989, k. Fonte *et al* 1984.

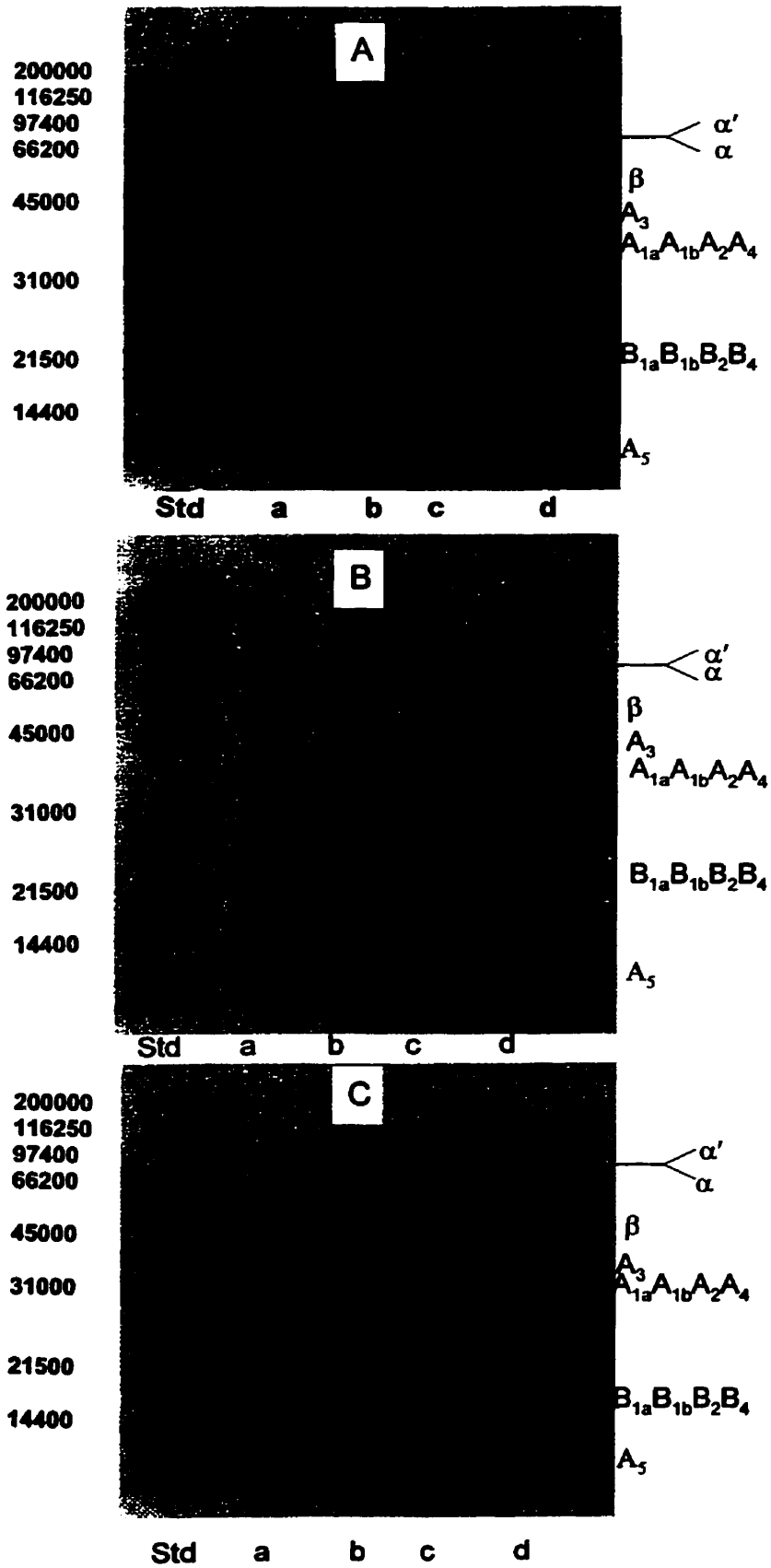


Figure 3.2: Electrophoresis (presence of SDS) of extract (A), isolate (B) and supernatant (C). a- NaO-HIE, b- H_2O-IE, c- NaOH-CP, d- CA-CP

conglycinin subunits, (85, 75 and 50 kDa) which were minor bands in the gel of NaOH-CP, were major bands in of the supernatant remaining after precipitation of this NaOH cryoprecipitate (Figure 3.2Cc). This confirmed that there was minimal precipitation of β-conglycinin during cryoprecipitation of the NaOH-CP. The bands representing glycinin subunits were also found as minor components in the supernatant suggesting that the glycinin in the extract did not precipitate completely during cryoprecipitation.

The bands detected in the citric acid extract were comparable to those bands in the NaOH extract (Figures 3.2Ad, 3.2Aa); however, the bands obtained from the citric acid extract are less intense. The CA-CP showed bands with MW from 97 to 12 kDa (Figure 3.2Bd). Bands with MW of 85, 75 and 50 kDa, which represent β–conglycinin subunits, were the major bands in the CA-CP while glycinin subunits 42, 38, 22 and 12 kDa (Table 3.2) were minor bands. This suggests that the major component in the CA-CP was the β–conglycinin, with glycinin as a minor component.

3.2.3 Fractionation by Size-Exclusion Chromatography

SE chromatograms (SEC) from the NaOH extract, the NaOH-IE and the supernatant remaining after precipitation of NaOH-IE are shown in Figure 3.3. The NaOH extract and the NaOH-IE contained four fractions (F_1, F_2, F_3, and F_4) with F_2 representing the major protein fraction. The F_2 was absent from the SEC

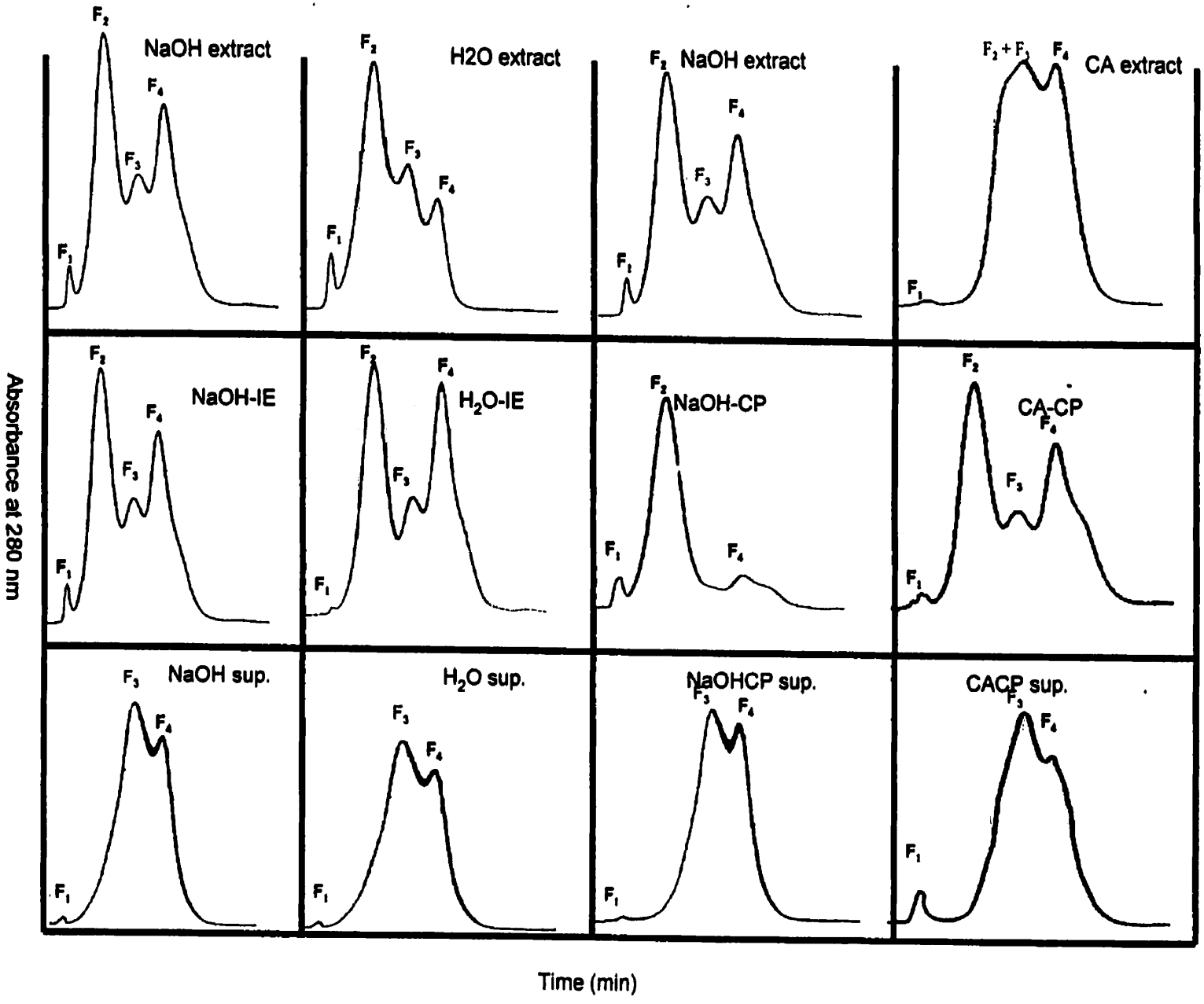


Figure 3.3: SEC of soy protein extracts, isolates and supernatants

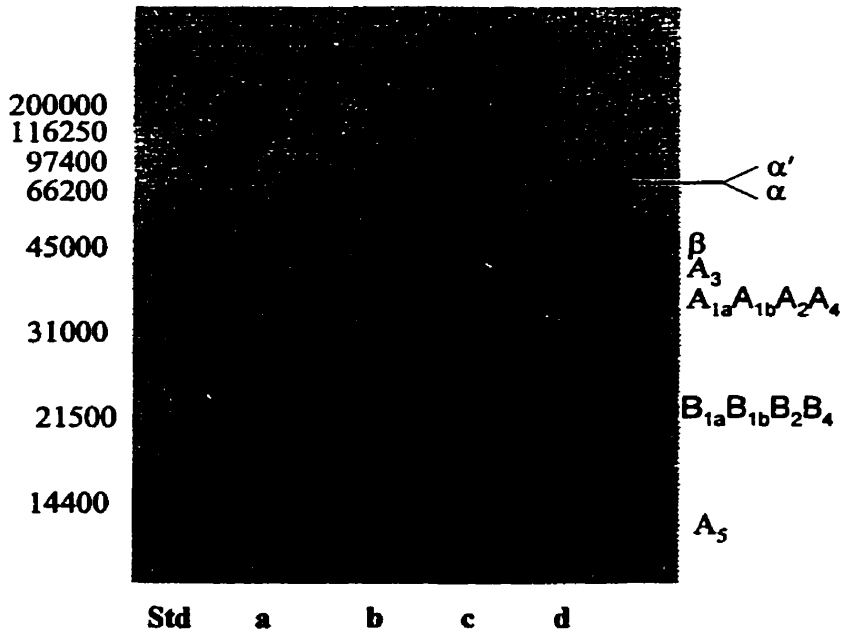


Figure 3.4: Electrophoresis (presence of SDS) of F_2 from SEC.
a- F_2 (NaOH-IE), b- F_2 (H_2O-IE), c- F_2 (NaOH-CP), d- F_2 (CA-CP)

of the supernatant, suggesting that during isoelectric precipitation of the NaOH-IE primarily, F_2 was recovered from the extract. SDS-PAGE of F_2 from the NaOH-IE (Figure 3.4a) gave bands with MW 85, 75 and 50 kDa representing subunits of β–conglycinin and bands with MW 42, 38, 22 and 12 kDa representing subunits of glycinin. The SEC results indicate that F_2 from NaOH-IE is a mixture of glycinin and β-conglycinin. Koshiyama (1969) also reported that SEC did not separate glycinin from β–conglycinin.

The results from the H_2O extract, the H_2O-IE and the supernatant remaining after precipitation of H_2O-IE were similar to those from NaOH extract, NaOH-IE, and the NaOH-IE supernatant.

Three major fractions (F_1, F_2, F_4) were separated from NaOH-CP by SEC, while four fractions (F_1, F_2, F_3, F_4) were found in the extract. Fraction F_3 (in the extract) was absent for NaOH-CP whereas F_1 and F_4 were relatively minor fractions. SEC suggested that the NaOH-CP was a relatively homogenous protein. SDS-PAGE of F_2 obtained from NaOH-CP showed three major bands with MW of 38, 22 and 12 kDa and four minor bands with MW of 50, 42, 30 and 17 kDa (Figure 3.5c). These results suggest that F_2 of NaOH-CP contained subunits similar to those identified in the NaOH-CP, the MW of the major subunits are similar to those of glycinin (Table 3.2).

Figure (3.4) shows the SEC of the citric acid extract, CA-CP and the supernatant obtained after the cryoprecipitation. The citric acid extract and the CA-CP contained four fractions (F_1, F_2, F_3, and F_4) with F_2 representing the

major protein fraction CA-CP. SDS-PAGE of F_2 of CA-CP (Figure.3.5d) gave major bands with MW of 85, 75, 50 and 30 kDa corresponding to 7S (β–conglycinin) and also two minor bands with MW of 22 and 12 kDa corresponding to 11S (glycinin) can be detected in the gel.

3.2.4 Differential Scanning Calorimetry (DSC)

DSC thermograms of NaOH-IE, CA-CP and NaOH-CP are shown in Figure 3.5a. Two thermal transition peaks at 72.8 and 86.4 °C were obtained with the NaOH-IE corresponding to the denaturation temperature (T_d) of β–conglycinin (lower endothermic temperature 72.8 °C) and the thermal denaturation temperature (T_d) of glycinin (higher endothermic temperature 86.4 °C). These values are similar to those reported by other workers (Sheard *et al.* 1986; Damodaran 1988; Arrese *et al.* 1991; Wagner *et al.* 1996).

The NaOH-CP exhibited only one endothermic transition peak at 85.3°C, while the CA-CP exhibited one endothermic transition peak at 75.3°C; these results suggest that the NaOH-CP and CA-CP which are obtained by the cryoprecipitation procedures are composed mainly of glycinin and β–conglycinin, respectively. A β–conglycinin isolate obtained by the procedure described by Thanh and Shibasaki (1976) showed two transition peaks representing β–conglycinin and glycinin, presumably because of presence of glycinin as a contaminant in the β–conglycinin isolate (Damodaran 1988).

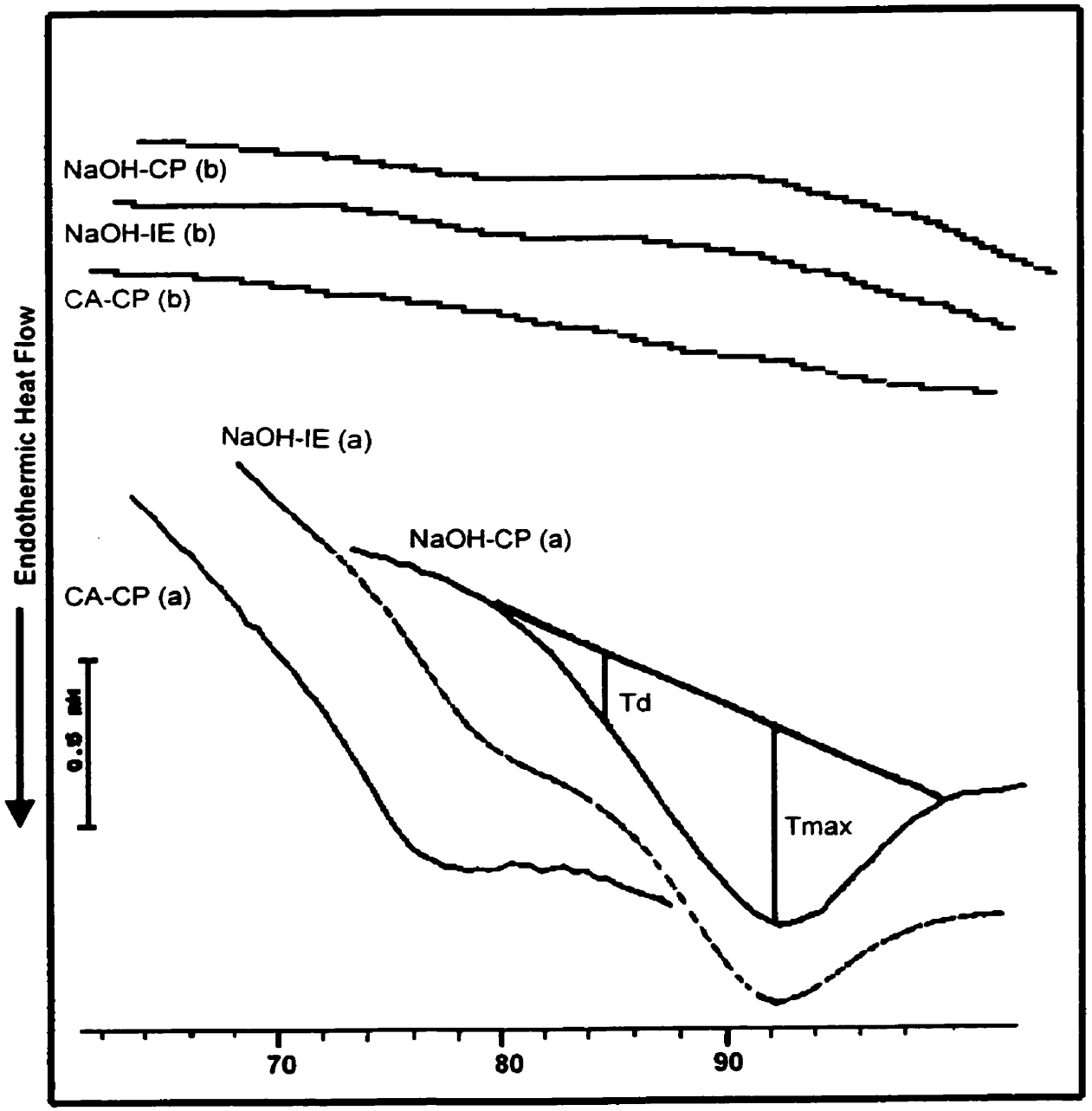


Figure 5.3: DSC thermograms of soy protein isolates.
a) First heating cycle, b) Second heating cycle

The much higher thermal stability of the NaOH-CP compared the β–conglycinin-rich protein CA-CP can be attributed to the difference in the conformational structures of the two proteins. Glycinin contains 21 disulfide bonds, of which six are intersubunit bonds and fifteen are intrasubunits bonds. On the other hand, β–conglycinin does not have any inter or intrasubunits disulfide linkages which makes it less a stable structure when subjected to thermal treatment (Badlely *et al.* 1975; Lin 1991). The extensive intra- and intersubunit disulfide bonds in glycinin provide greater stability against thermal denaturation, which is reflected in its higher Td and enthalpy of denaturation (Damodaran 1988).

Enthalpy (ΔH) of NaOH-CP was 17.5 J/g and of CA-CP was 2.1J/g. Damodaran (1988) reported that the ΔH of glycinin and β-conglycinin were 18.9 J/g and 12.3 J/g respectively. The value of ΔH (2.1 J/g) obtained from CA-CP, suggests that this protein may have been already denaturated due to the extraction procedure. Sorgentini *et al.* (1991) reported that ΔH for 10 commercial soy isolates ranged from 0.0 to 6.3 J/g.

No transition peaks were obtained (Figure 3.5b) when the proteins were cooled after the first DSC run and then subjected to a second thermal analysis. This suggested that both glycinin and β–conglycinin showed irreversible denaturation, regardless of the isolation in which they were present, while some globular proteins such as α–lactalbumin showed reversible thermal denaturation (Boye 1995).

CHAPTER 4

INVESTIGATION OF MECHANISMS OF PROTEIN CRYOPRECIPITATION

4.1 MATERIALS AND METHODS

4.1.1 Materials

Commercial defatted soybean flour (50 % protein, 1.2 % fat, 3.5 % fiber, 8 % moisture) was obtained from Daminco Inc (Dorval, Quebec, Canada) and stored in airtight containers at 4 °C.

4.1.2 Mechanism of Cryoprecipitation

To study the mechanism of protein cryoprecipitation, the extraction was carried out using the following three different treatments shown in Figure 4.2. 1) Defatted soybean flour was extracted using dilute NaOH (0.02 %, pH 10.7) as described before in Section 3.1.2.3. The extract was divided into 2 parts (A, B); part A was stored at 4 °C and part B was stored at ambient temperature (21 °C) for 18 h. Proteins were recovered from the stored solutions as described in Section 3.1.2.3 Chapter 3. 2) The extraction was carried out using the same procedure, but in the presence of sodium azide (NaN_3, 0.1 %) as an antimicrobial agent and the extract was then divided into two parts (C, D), part C was stored at 4 °C for 18 h and part D was stored at ambient temperature (21 °C) for 18 h.

3) The defatted soybean was extracted with sodium carbonate buffer (pH 10.8), instead of dilute NaOH solution and the extract store at 4 °C for 18 h.

4.1.3 Factors Affecting Cryoprecipitation Yield

4.1.3.1 Effect of Extraction Temperature on Cryoprecipitation Yield

Defatted soybean (30 g) was mixed with 300 ml of dilute NaOH solution (0.02 %, pH 10.7). The mixture was allowed to stand with intermittent stirring for 1 h at (20°, 40°, 50°, and 60 °C). The insoluble residues were removed by centrifugation (12,000 x g) for 10 min and the extract was filtered through fine glass wool. Cryoprecipitation of proteins was carried out at 4 °C for 18 h. The protein precipitates were recovered by centrifugation (12,000 x g) for 10 min and then lyophilized. The protein yield was calculated on the basis of the weight of the cryoprecipitated protein and the protein content of the defatted soybean.

4.1.3.2 Effect of Cooling Time on Cryoprecipitation Yield

The extraction procedure described above was carried out at ambient temperature. The extracts were cooled at 4 °C for 3, 6, 18 and 24 h for cryoprecipitation of proteins and then centrifuged at 12,000 x g for 10 min. The cryoprecipitated proteins were washed with distilled water to remove soluble residue and then lyophilized. The protein yield was calculated on the basis of the weight of the cryoprecipitated protein and the protein content of the defatted soybean.

4.1.3.3 Effect of Recycling of Extraction Solvent

Commercial, ground, defatted soybean flour (30 g) was extracted with 300 ml of dilute NaOH solution (0.02 %, pH 10.7). An initial extraction (first extraction cycle) was carried out at room temperature for 1 h. The mixture was then centrifuged (12,000 x g) for 10 min to separate the protein solution from the residue. From this solution, a protein fraction (NaOH-CP) was cryoprecipitated by storing at 4 °C for 18 h and the protein was recovered by centrifugation (12,000 x g) for 10 min and lyophilized. The supernatant was adjusted to pH 4.5 with dilute HCl to further precipitate proteins (NaOH-IE) according to their isoelectric properties. This second protein fraction was recovered by a second centrifugation (12,000 x g) for 10 min and lyophilized. In order to re-utilize (recycle) the solvent used in the first extraction, the supernatant from the isoelectric protein precipitation was adjusted to 300 ml and pH 10.7 with NaOH solution and used for second extraction (second extraction cycle) of anther 30 g quantity of defatted soybean flour, exactly as described above. The supernatant remaining after the isoelectric precipitation of protein from the second extraction cycle of defatted soybean was adjusted to 300 ml and pH 10.7 with dilute NaOH solution and re-used (recycled) for a third extraction (third extraction cycle) of anther 30 g quantity of defatted soybean flour. Figure 4.1 shows a diagram of the cyclic extraction / protein precipitation process for the three consecutive quantities of defatted soybean flour.

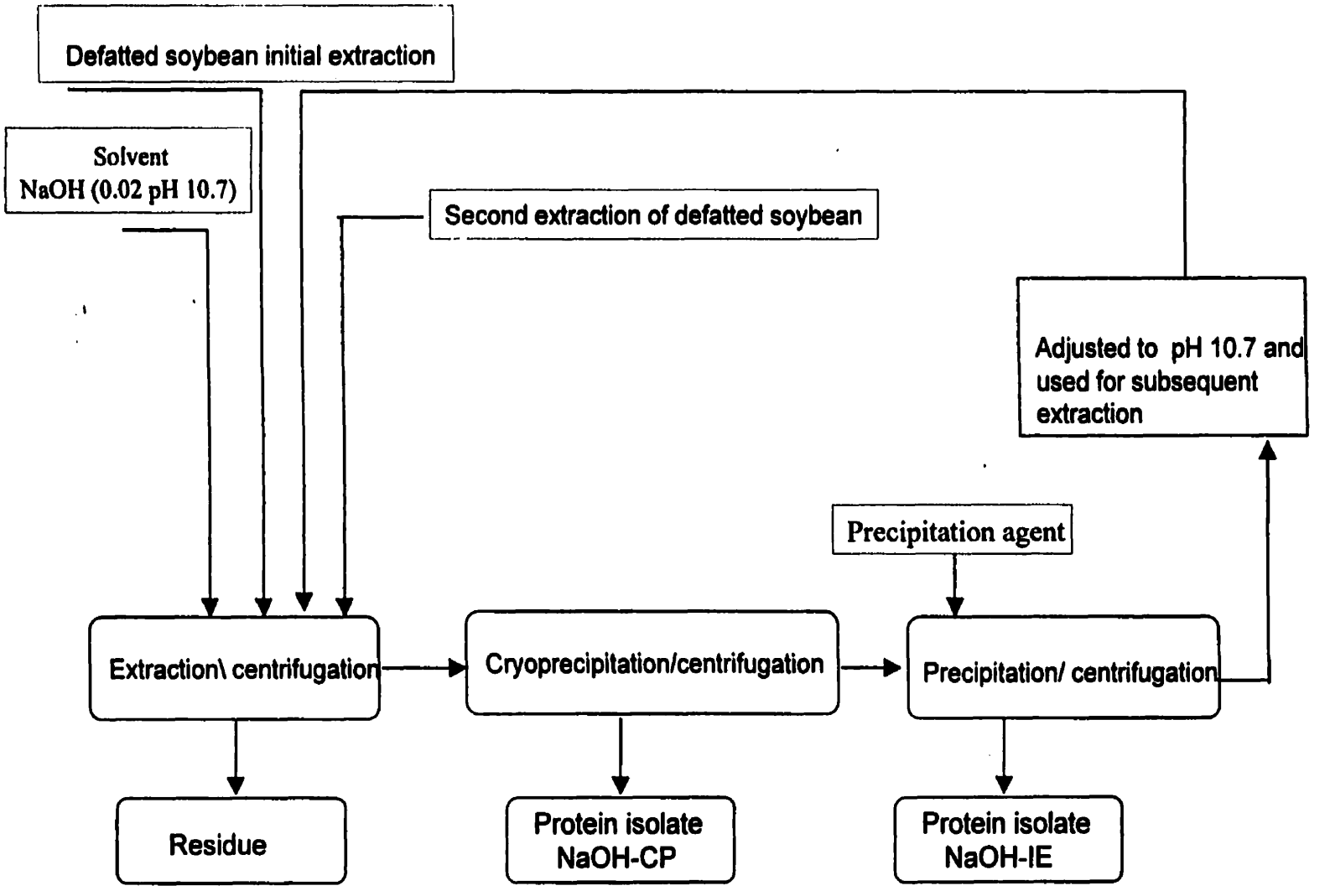


Figure 4.1 Outline of process for recycling extraction

4.1.3.3.1 PAGE and SDS-PAGE

NaOH-CP and NaOH-IE proteins obtained from the three consecutive extraction was subjected to PAGE and SDS-PAGE using the procedure described before in Section 3.1.4.1 of Chapter 3.

4.1.4 Statistical Analysis

A complete random design (CRD) was used. Data were analyzed by the Statistical Analysis System (SAS Institute, Cary, NC, Version 6.12), using General Linear Model (GLM) for analysis of variance and significant differences among means were determined by the LSD test; the level of statistical significance was 0.05.

4.2 RESULTS AND DISCUSSIONS

4.2.1 Mechanism of Cryoprecipitation

Figure 4.2 shows the treatments which were investigated in order to study the mechanism of cryoprecipitation. After 1 h of extraction the pH of the extract obtained using dilute NaOH solution (0.02 %) was 7.0. Storage of part A of the extract at 4 °C for 18 h resulted in cryoprecipitation of the NaOH-CP and a supernatant with of pH 6.8. Storage of part B of the same extract was stored at ambient temperature (21 °C) for 18 h, the pH of supernatant was 4.5, resulting in isoelectric precipitation of soybean proteins (NaOH-IE). The pH 6.8 of the supernatant from the part A extract stored at 4 °C, is identical to the isoeletric precipitation point of glycinin (Liu 1997) but much higher than the isoelectric precipitation point of β–conglycinin (pH 4.5) (Thanh and Shibasaki 1976). This suggests that the NaOH-CP is formed as a result of the loss of solubility of glycinin at its isoelectric precipitation point at 4 °C. The pH 4.5 of the supernatant from the extract of part B stored at ambient temperature (21 °C) for 18 h is similar to the isoelectric precipitation point of β–conglycinin; this suggests that the precipitate obtained from part B was formed by the loss of solubility of both β–conglycinin and glycinin. Since the decrease from pH 10.7 in the extracting solution to pH 4.5 in the supernatant of part B extract at ambient temperature (21 °C) occurred without the addition of acid, it was considered that this pH decrease might have resulted from acid-producing microorganisms.

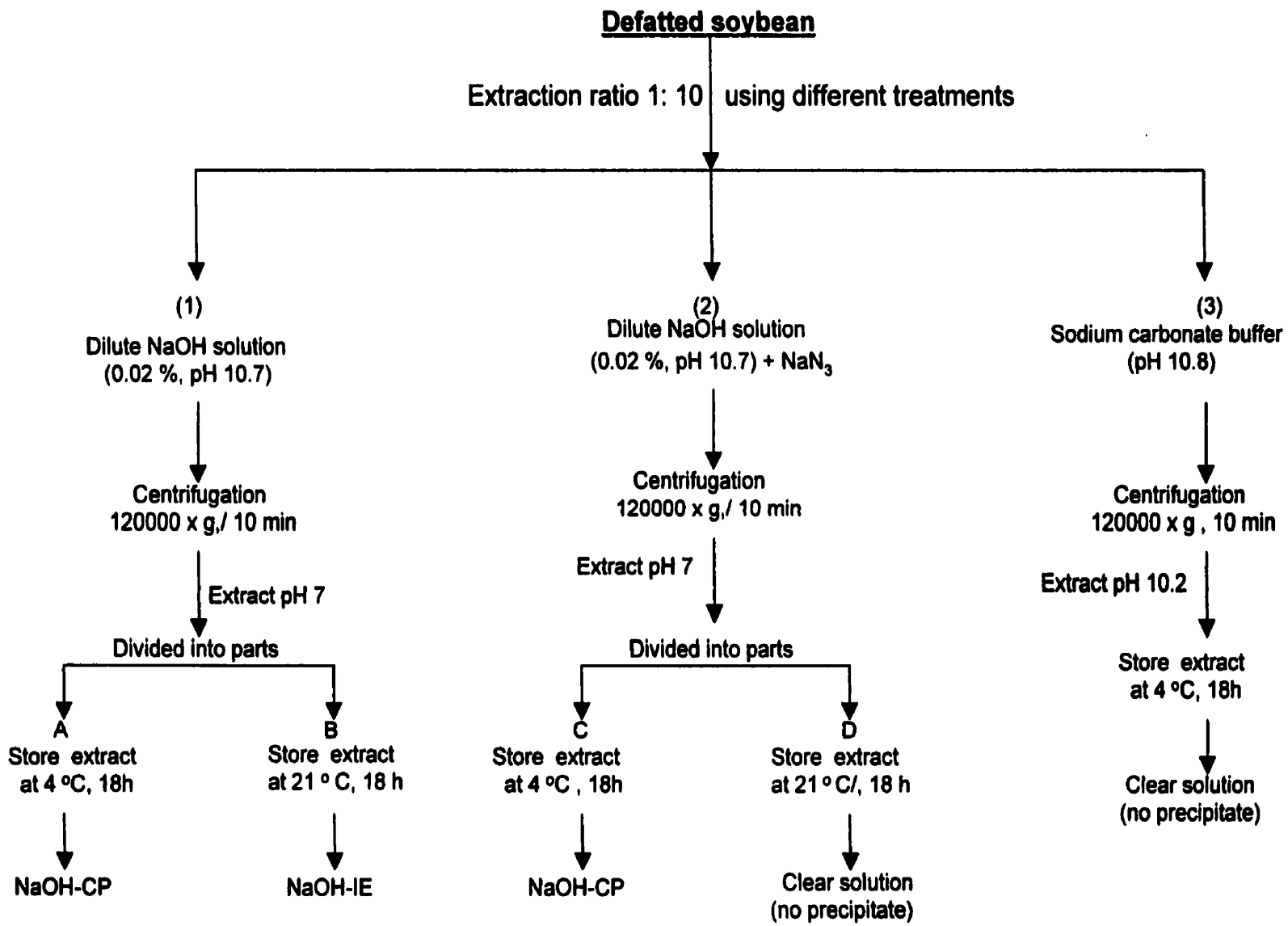


Figure 4.2: Summary of extraction and precipitation of proteins using different conditions

Microbial analysis revealed that lactic acid bacteria was found in the stored extract at ambient temperature for 18 h, suggesting that the decrease to pH 4.5 in the extract resulted in acid production by lactic acid bacteria.

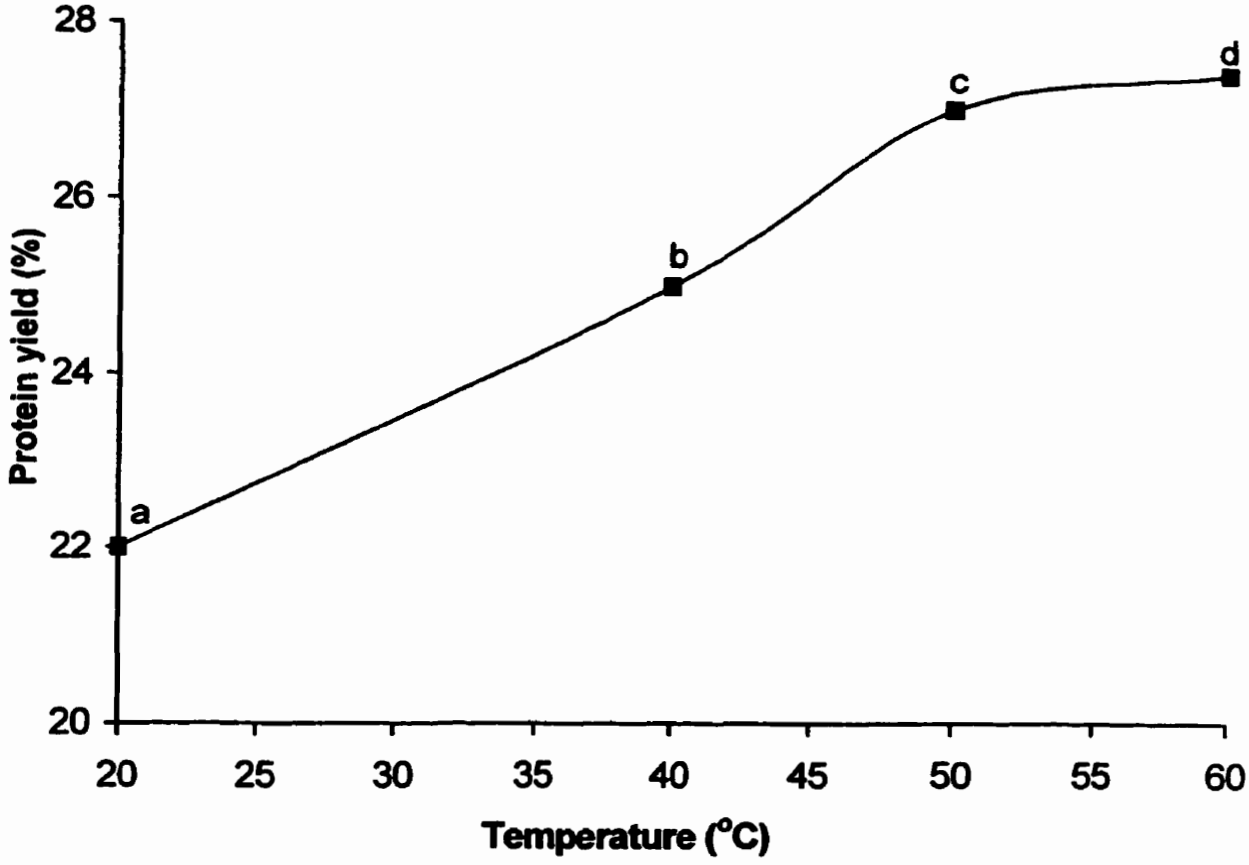
The part D of the extract was clear (no cryoprecipitation) when it was stored at ambient temperature (21 °C) for 18 h. in the presence of 0.1 % NaN_3 which was used as antimicrobial agent to prevent the growth of lactic acid bacteria. On the other hand, the precipitation of NaOH-CP was observed in the presence of NaN_3, when the part C of the extract was stored at 4 °C for 18 h, suggesting that temperature is a major factor affecting cryoprecipitation mechanism.

No precipitate was obtained from the extract obtained from the pH 10.8 buffered sodium carbonate extract. The buffering action of the extracting solution prevented the decrease in pH to 6.8 (the isoelectric point precipitation of NaOH-CP) and therefore prevented the loss of solubility of protein from the extract.

4.2.2 Factors Affecting Cryoprecipitation Yield

4.2.2.1 Effect of Extraction Temperature on Cryoprecipitation Yield

The effect of extraction temperature on yield of NaOH-CP is shown in Figure 4.3. The results indicate that the yield of protein increased when the extraction temperature was increased from 20 to 60 °C. Alli (1977) found that



Line values followed by the same letter are not significantly different (P > 0.05)

Figure 4.3: Effect of extraction temperature on protein yield

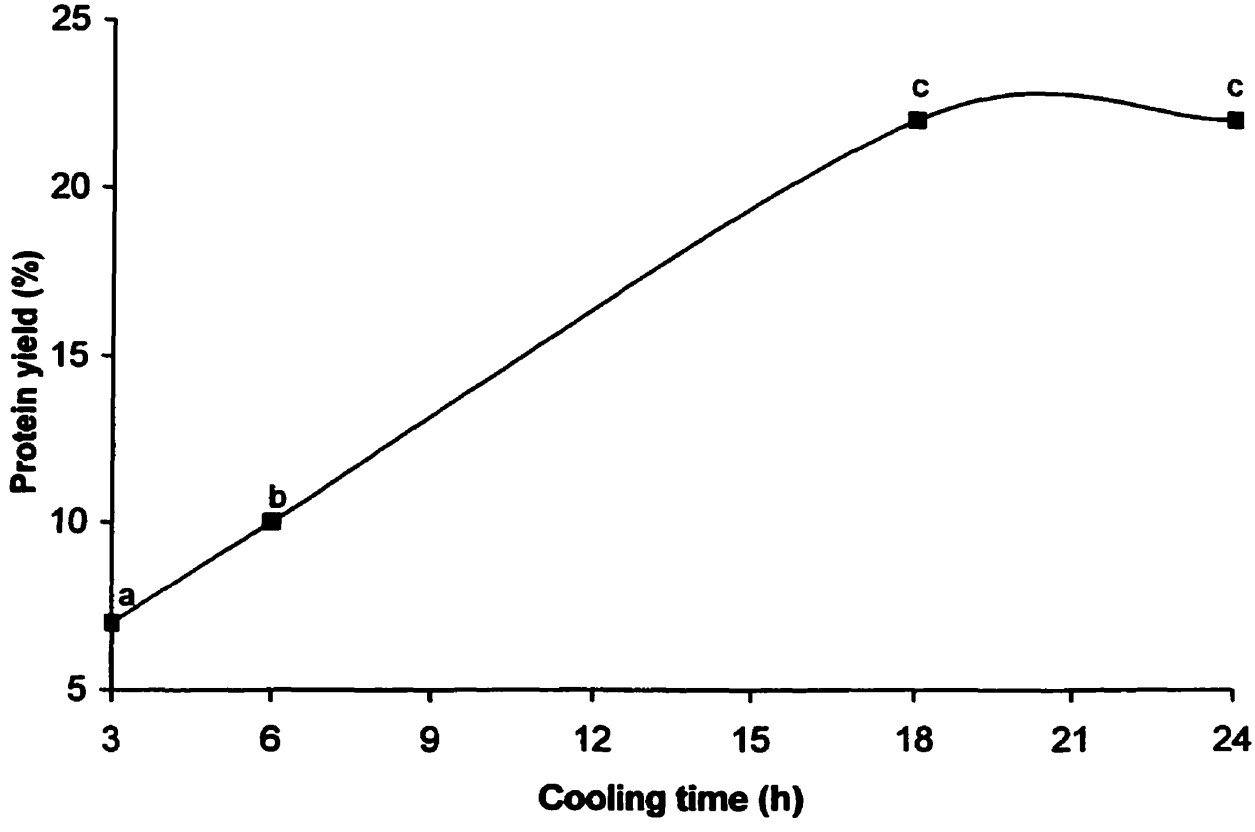
increasing extraction temperature from room temperature to 45 °C increased the yield of cryoprecipitated protein from white kidney bean; Davidson *et al.* (1979) also showed that elevating the extraction temperature was associated with increase the protein yield from soybean.

4.2.2.2 Effect of Cooling Time on Cryoprecipitation Yield

Figure 4.4 shows the effect of cooling time on the protein yield from aqueous defatted soybean. The results indicate that the yield of protein increased from 7% to 22% as the cooling time increased from 3 h to 18 h and remained unchanged when the cooling time increased to 24 h. The results of the present work are in agreement with those obtained by Wolf and Sly (1967) who reported an increase in protein yield from soybean meal as the cooling time increased from 1 h to 20 h.

4.2.2.3 Effect of Recycling of Extraction Solvent

For the first extraction cycle (Section 4.1.3.3), the yield of NaOH-CP protein was 22% of the total protein content of the defatted soybean, the yield of the isoelectric precipitate NaOH-IE was 61% of the total protein. In the second extraction cycle, the yield of the NaOH-CP protein was 32 %; and the yield of acid precipitated NaOH-IE protein was 40 %. In the third extraction cycle, the yields of NaOH-CP and NaOH-IE were 24 % and 44 %, respectively. After the



Line values followed by the same letter are not significantly different (P > 0.05)

Figure 4.4: Effect of cooling time on protein yield

third extraction cycle, the recycled extraction solvent did not yield any NaOH-CP protein, possibly due to accumulation of oligosaccharides in the recycled extract. Ghetie and Buzila (1964) reported that cryoprecipitaion can be inhibited by using sugar concentrations from 0.1 to 0.5 M, while Wolf and Sly (1967) reported that sugar concentration of 0.6 M or higher completely inhibited cryoprecipitation. The negative effect of sugar on cryprecipitation might be due to the possibility of sugar and glycinin interaction, which prevents glycinin from precipitation when the extraction temperature cools down (Ghetie and Buzila 1964).

Electrophoresis of NaOH-CP and NaOH-IE (Figures 4.5A, 4.5B and 4.5C) confirm that the composition of the NaOH-CP protein from the three extraction cycles were identical; this was also the case for NaOH-IE from the three extraction cycles.

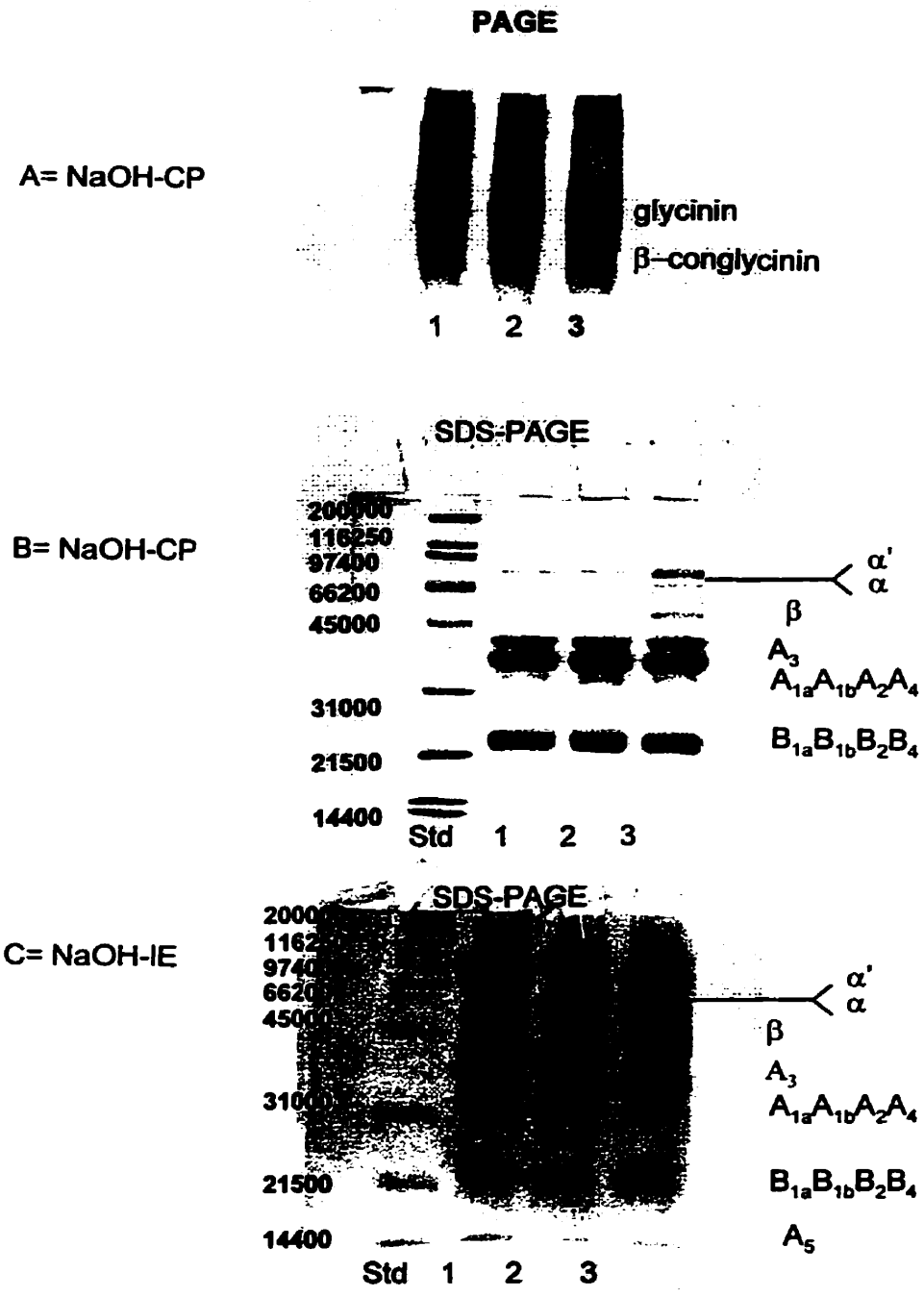


Figure 4.5: PAGE and SDS-PAGE of NaOH-CP and Isoelectric precipitation from 3 consecutive extractions

CHAPTER 5

GELATION CHARACTERISTICS OF A CRYOPRECIPITATED SOY PROTEIN

5.1 MATERIALS AND METHODS

5.1.1 Materials

Commercial defatted soybean flour (50 % protein, 1.2 % fat, 3.5 % fiber, 8 % moisture) was obtained from Daminco Inc (Dorval, Quebec, Canada) and stored in airtight containers at 4 °C.

5.1.2 Preparation of Cryoprecipitated Protein

Proteins were extracted using the procedure of Davidson *et al.* (1979) with some modifications. The defatted soybean meal (100 g) was mixed with dilute NaOH (1L, 0.02 %, pH 10.7) and allowed to stand for 1 h at room temperature with intermittent stirring. The mixture was centrifuged (12,000 x g) for 10 min. The extract was refrigerated (4 °C, 18 h) and the proteins which precipitated were recovered by centrifugation (12,000 x g) for 10 min followed by lyophilization. This isolate was designated as NaOH cryoprecipitate (NaOH-CP).

5.1.3 Preparation of Heat-Induced Gels

Heat-induced protein gels at desired protein concentrations (Section 5.1.3.1) were prepared in beakers (10 ml). Aqueous dispersions (5 ml) of NaOH-CP were prepared in distilled water and mixed to obtain a uniform suspension. The suspensions were adjusted to pH 8 by dropwise addition of 1M NaOH. The beakers were covered with aluminium foil to prevent evaporation during heating and gelation was carried out by heating in a water bath (95 °C, 60 min). Gels were kept at 4 °C for 24 h before removal the gel from the beakers for determination of rheological properties.

The effects of the following factors on gelation properties were studied:

5.1.3.1 Protein Concentration

NaOH-CP protein dispersions of 6, 8, 10, 12, 14, and 16 % (w / v) were prepared and the pHs were adjusted to pH 8 with 1M NaOH; the dispersions were heated (95 °C, 60 min) for gelation studies.

5.1.3.2 pH

The pH of NaOH-CP dispersions (14 % w / v) were adjusted to pH values 3, 4, 5, 6, 7, 8, 9, 10, and 11 using 1 M NaOH or 1N HCl as required. 5ml of each dispersion was heated (95 °C, 60 min) for gelation studies.

5.1.3.3 Heating Temperature

NaOH-CP dispersions (14 % w /v, pH 8) were heated at 40 °, 50 °, 60 °, 70 °, 80 °, 85 °, 90 °, and 95 °C for 60 min for gelation studies.

5.1.3.4 Heating Time

NaOH-CP dispersions (14 % w /v, pH 8) were heated at 95 °C for 10, 20, 30, 40, 50, and 60 min for gelation studies.

5.1.3.5 Ionic Strength

NaOH-CP dispersions (14 % w /v, pH 8) in the presence of NaCl concentrations of 0.5, 1.0, 1.5, and 2.0M were heated (95 °C, 30 min) for gelation studies.

5.1.3.6 Reducing Agents

NaOH-CP dispersions (14 % w / v, pH 8) were heated (95 °C, 30 min) in the presence of 50, 100, 150 mM of SDS or 0.1, 0.5, 1.0. % 2-mercaptoethanol for gelation studies.

5.1.4 Preparation of Cold-Set Gels

Cold-set protein gels at desired concentrations (Section 5.1.4.1) were prepared in beakers (10 ml). Aqueous dispersions (5 ml) of NaOH-CP were

prepared in distilled water and adjusted to pH 8 by dropwise addition of 1M NaOH. The beakers were covered with aluminium foil (to prevent evaporation during heating), heated in a water bath (95 °C, 60 min) and cooled to ambient temperature before addition of $CaCl_2$. The suspensions were kept at 4 °C for 24 h before they were removed from the beakers for determination of rheological properties.

The effect of the following factors on cold-set gelation were studied:

5.1.4.1 Effect of $CaCl_2$ Concentration on Cold-Set Gelation

NaOH-CP dispersions (6 % w/v) were prepared in distilled water, the pH adjusted to pH 8.0 with 1M NaOH, then heated (95 °C, 60 min) and cooled to ambient temperature. After cooling, 10, 20, 30, 50, 70, and 100 mM of $CaCl_2$ were added to form cold-set gels; the gels were kept at 4 °C for 24 h.

5.1.4.2 Effect of Protein Concentration on Cold-Set Gelation

NaOH-CP dispersions (1, 2, 3, 4, 5, and 6 % w / v) were prepared in distilled water, the pHs were adjusted to pH 8.0 with 1 M NaOH then heated (95 °C, 60 min) and cooled. After cooling $CaCl_2$ (50 and 100 mM) were added to form cold-set gels; the gels were kept at 4 °C for 24 h.

The above procedure was also used to prepare cold-set gels from 8 and 10 % of NaOH-CP dispersions heated at 95 °C for 12 min instead of 60 min.

5.1.4.3 Effect of pH on Cold-Set Gelation

The pH of NaOH-CP dispersion (6 % w / v) were adjusted to pH values of 3, 4, 5, 6, 7, 8, 9, and 10 using 1 M NaOH or 1N HCl as required; the procedure described in Section 5.1.4 was used to form cold-set gels.

5.1.5 Measurement of Gel Strength

Samples of thermally-induced gels and cold-set gels of NaOH-CP were compressed to 50% deformation using the Universal Instron Testing Machine (Instron Canada, Burlington, Ontario). Gel strength (N) was calculated as the force required to break the gels (load at yield) (Boye 1995). All measurements were done in duplicate.

5.1.6 Water Holding Capacity (WHC)

WHC was determined using the centrifugation technique of Shiga and Nakamura (1987). Duplicate samples (0.7 g) of NaOH-CP were placed in centrifuge tubes, 5 ml of water added, the pH was adjusted to pH 8 using 1M NaOH and heated (95 °C, 60 min) to form gels. The gels were kept at 4 °C for 24 h and centrifuged at 12000 x g for 10 min and the value of the supernatant layer that separated was measured. The WHC was expressed as:

WHC (%) = water content of sample – separated water x 100 / water content of sample.

5.1.7 Electrophoresis Gels (PAGE) and (SDS-PAGE)

PAGE and SDS-PAGE of NaOH-CP (14 % w / v, pH 8) which heated at 40 °, 50 °, 60 °, 70°, 80 °, 85 °, and 95 °C for 60 min was performed as described in Section 3.1.4.1 of Chapter 3.

5.1.8 Gel Microstructure

Samples of thermally-induced gel (section 5.1.3) and cold-set gel (section 5.1.4) prepared from NaOH-CP dispersions (6, 8 and10, % w / v) were fixed in glutaraldehyde (2 %) Na cacodylate buffer (0.1M, pH 7.3) for 2 h at room temperature, rinsed several times over a period of 1 h in buffer, dehydrated in a graded ethanol series (30, 50, 70, 100 %), critical point dried, mounted and covered with 8 nm of gold. Observations were done on a scanning electron microscope (Hitachi Model S-3000) using a vacuum of 15 kV

5.1.9 Statistical Analysis

A complete random design (CRD) was used. Data were analyzed by the Statistical Analysis System (SAS Institute, Cary, NC, Version 6.12), using General Linear Model (GLM) available in SAS for analysis of variance and significant differences among means were determined by LSD test. The level of statistical significance was 0.05.

5.2 RESULTS AND DISCUSSIONS

5.2.1 Thermally-Induced Gels

5.2.1.1 Effect of Protein Concentration

Figure 5.1 shows the gel strengths of thermally induced gels of NaOH-CP at various protein concentrations. The gel strength increased from 0.4 at 8 % protein concentration to 7.8 N at 16 % protein concentration. The gel strength of heat-induced gels of globular proteins is known to be affected by the protein concentration (Hillier *et al.* 1980; Hegg, 1982). Shimada and Matsushita (1980) reported that the minimum protein concentrations of glycinin and β-conglycinin prepared by isoelectric precipitation using the procedure of Thanh and Shibasaki (1976) required to form gels, were 12 % and 10 % respectively, while Damodaran (1988) using the same method reported that the minimum protein concentration of glycinin required to form a gel was 9 %. Mori *et al.* (1982) found that whether the heated glycinin undergoes disaggregation or gel formation depends on protein concentration; low levels of glycinin concentration favored disaggregation while high levels favored gel formation. A linear relationship between protein concentration of both soy isolate and β-conglycinin and gel strength was reported by Damodaran (1989).

Figure 5.1 shows the relationship between protein concentration and WHC of NaOH-CP gels. The WHC of 8 % gel was 93 %; this increased slightly to 98%

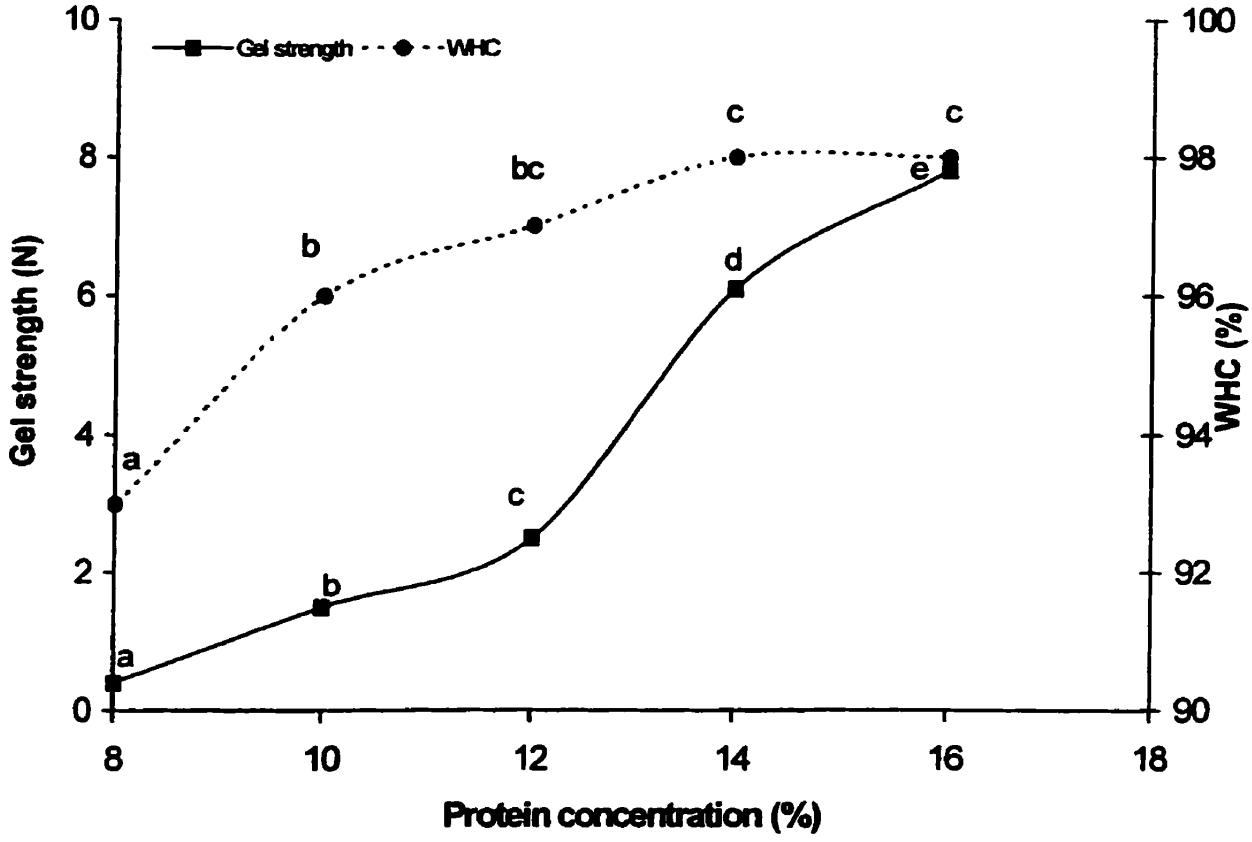


Figure 5.1: Effect of protein concentration on gel strength and WHC of thermally-induced NaOH-CP gels. (line values by the same letter are not significantly different, P>0.05).

at protein concentration of 16 %. There is little published information on WHC of glycinin gels. However several researchers (Saio *et al.* 1974; Saio and Watanabe 1978; Peng *et al.* 1984) reported that tofu made from glycinin hold more water than tofu made from β-conglycinin. Smith *et al.* (1960) found that soy isolate gels showed higher WHC than those from milk protein gels.

5.2.1.2 Effect of pH

Figure 5.2 shows the effect of pH on the gel strength of 14 % NaOH-CP gels. Maximum gel strength (7.0 N) was observed at pH 7, while at pH 11 or pH 3 weaker gels were observed. This may be due to intermolecular repulsion forces at the high net charge so that protein-solvent interactions are favored rather than protein-protein interaction (Hermansson 1978). Between pH 4 and 6, firm gels were not formed, rather white, opaque, coagulums were obtained. This may be attributed to the lack of repulsive forces which lead to less expansion, less hydration and therefore formation of weaker gels (Cheftel and Cug.1983). Standing and Hermansson (1991) studied the effect of pH on gelation of β–lactoglobulin (12 % protein) and found that aggregated, opaque gels were formed at pH 4 to 6 and that transparent gels were formed at pH values below or above this range. Boye *et al.* (1995) reported that at pH values between pH 4 to 6, whey protein (15 %) formed opaque, coarse, coagulum gels

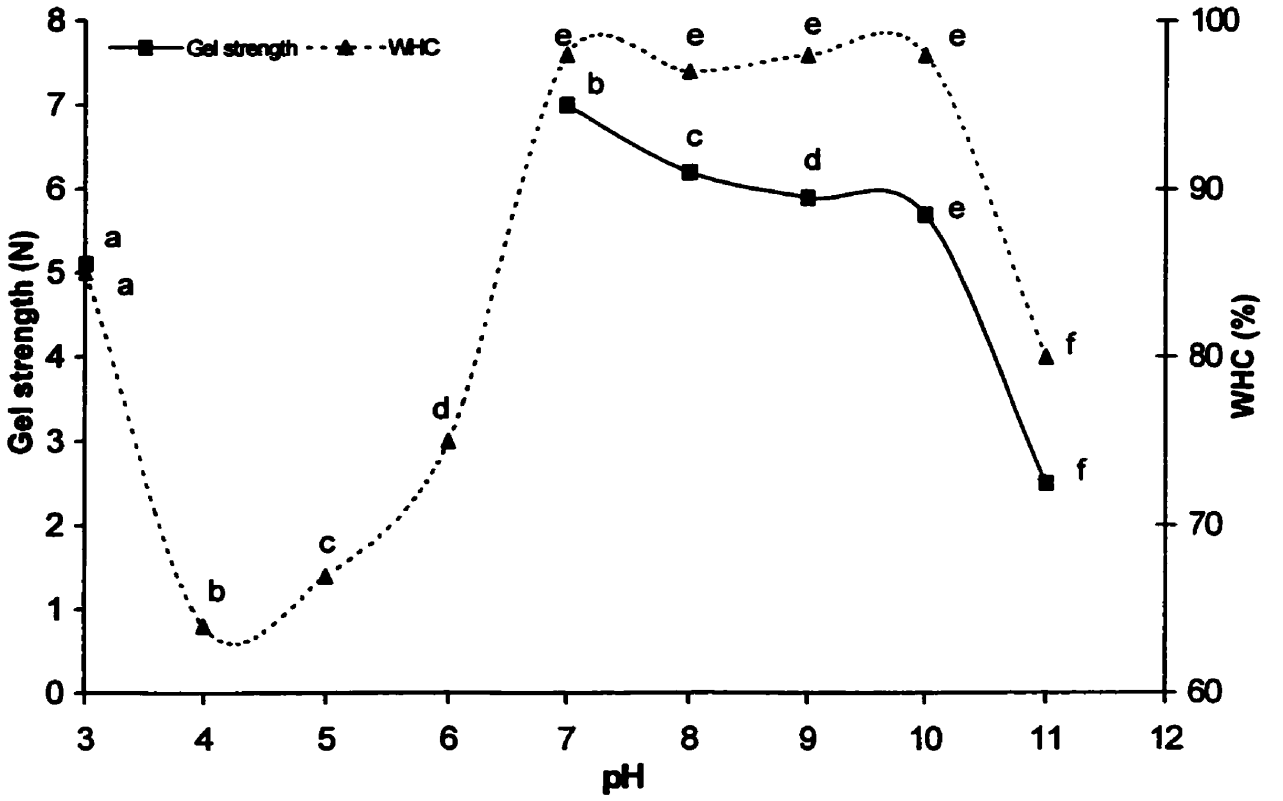


Figure 5.2: Effect of pH on gel strength and WHC of thermally-induced NaOH-CP gels (line values by the same letter are not significantly different, $P>0.05$).

while firm gels were obtained in the alkaline region (pH 8 to 10) compared to soft gels obtained in the acidic region (pH 2 to 3); similar results on whey protein concentrate gels at different pH were reported by Katsuta *et al.* (1990).

Figure 5.2 shows the effect of pH on WHC; WHC decreased from 85 % to 64 % when the pH was increased from pH 3 to pH 4. Between pH 4 and 6 lower WHC was obtained. Above pH 6, WHC increased from 75 % at pH 6 to 98 % at pH 7, remained constant between pH 7 and 10, then decreased. Hutton and Campbell (1977) reported that the WHC of soy protein concentrate is minimum at isoelectric pH and increases with increasing pH, being maximum at pH 7, they reported 241 and 349 g of water per 100 g of protein at pH 5 and pH 7, respectively.

5.2.1.3 Effect of Heating Temperature

Changes in gel strength of NaOH-CP gels formed by heating at 85 °, 90 °, and 95 °C for 60 min are shown in Figure 5.3. No gels were obtained below 85 °C. This supports the theory that protein denaturation is a prerequisite for heat-induced gelation of globular proteins (Clark and Lee-Tuffnell, 1986; Utsumi *et al.*1997). Kang *et al.* (1991) reported that for soybean protein isolates, heating above 60 °C is necessary to induce dissociation of quaternary structure of globulins and to cause unfolding of the protein subunits.

Gel strength increased with increasing temperature from 85 °C to up 95 °C. These results are in a good agreement with the results of Furukawa *et al.* (1979)

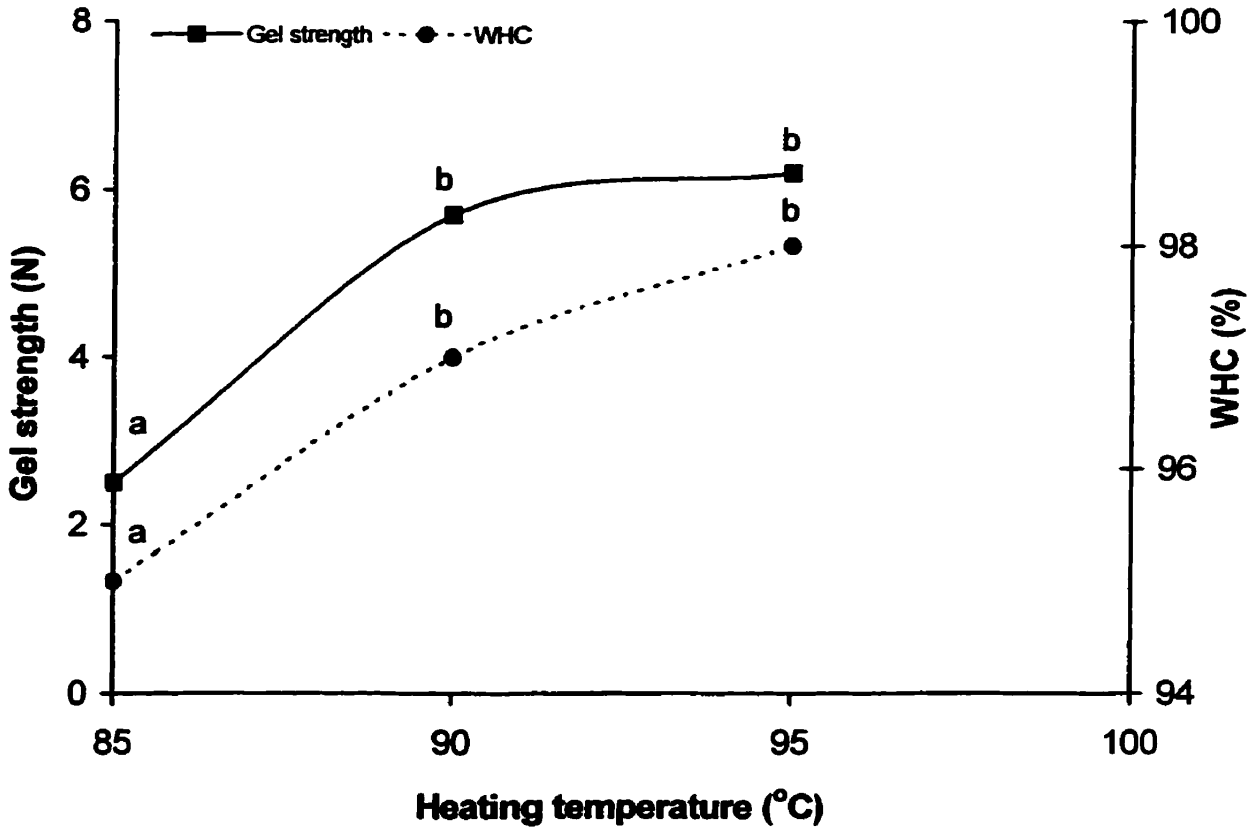


Figure 5.3: Effect of heating temperature on gel strength and WHC of thermally-induced NaOH-CP gels (line values by the same letter are not significantly different, P>0.05).

who reported an increase in gel strength with increased temperature of soy isolate protein paste. Schmidt *et al.* (1979) and Boye *et al.* (1995) reported that an increase in temperature from 75 ° to 100 °C caused an increase in gel strength of whey protein concentrate gels, the marked increase in gel strength at 95 °C suggests that the formation of the three-dimensional network occurred at this temperature. The increase in gel strength with increase heating temperature may be attributed to conformational changes followed by aggregation of glycinin molecules (Utsumi *et al.* 1997).

The WHC of gels (14 % w / v, pH 8) prepared at 85 °C was 95 % and this value increased significantly to 98 % for gel prepared at 95 °C (Figure 5.3). The increase in WHC at 95 °C may be attributed to the fact that glycinin possess a complex quaternary structure; with increasing temperature this may lead to a transition from an aggregated disordered gel to an ordered structure consisting of strands. In the case of whey protein, heating above the gelation temperature produced phase separation and reduced WHC (Stanley and Yada 1992).

Figure 5.4 shows PAGE of NaOH-CP (14 % w/v) heated at 40 °, 60 °, 70 °, 85 °, 90 °, and 95 °C. Heating at 40 ° to 70 °C did not result in a gel; the electrophoretic patterns of these samples were similar to that of the control (unheated sample) (Figure 5.4). The gels obtained by heating at 85 °, 90 °, and 95 °C did not show the band of glycinin. This can be attributed to the aggregation of glycinin to give larger molecular wieght aggregates that did not

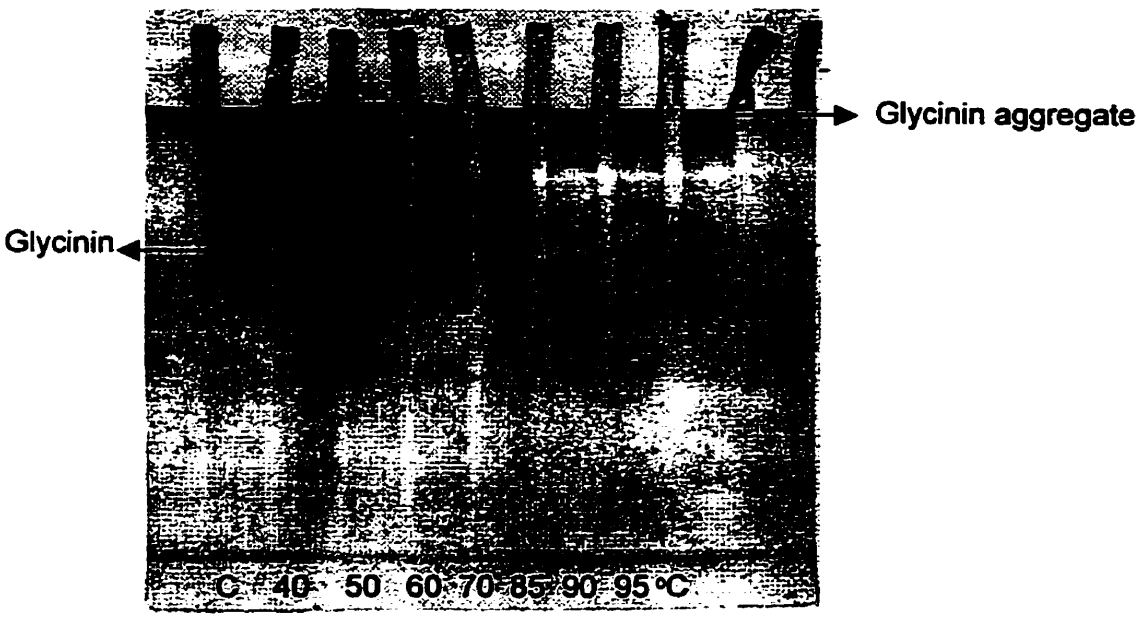


Figure 5.4: PAGE electrophoretic patterns of NaOH-CP heated for 60 min at different temperatures : C- control (unheated sample).

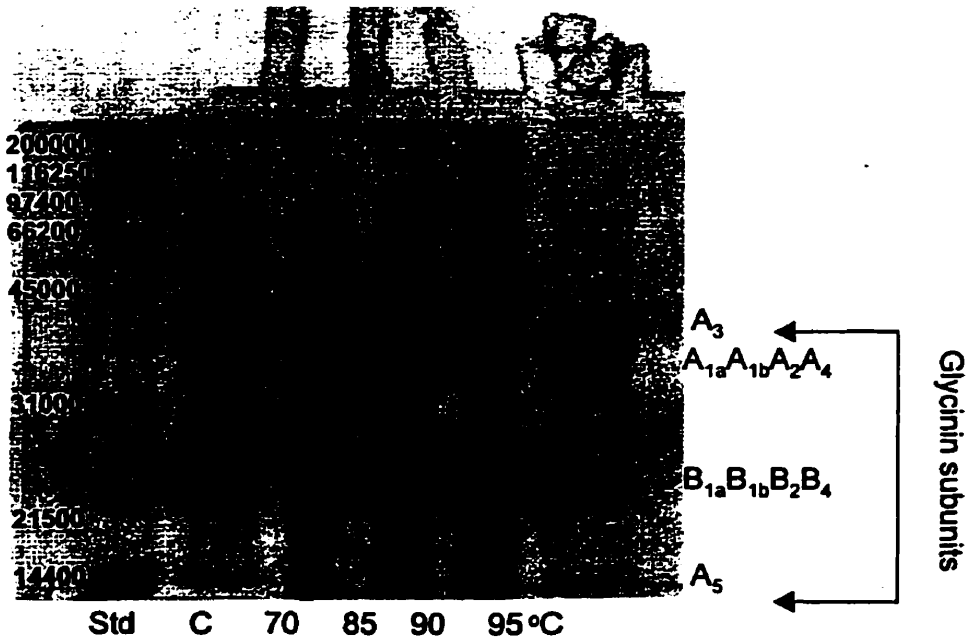


Figure 5.5: SDS-PAGE electrophoretic patterns of NaOH-CP heated for 60 min at different temperatures : Std- standard, C- control (unheated sample).

enter the separation gel but remained at the top of the of the separation gel (Figure 5.4). Mori *et al.* (1982) reported that glycinin aggregates (MW $8x10^6$ Da) are formed when glycinin solutions were heated at 100 °C.

Figure 5.5 shows the SDS-PAGE electrophoretic patterns of the heated dispersions of NaOH-CP. The acidic and basic subunits of glycinin were observed regardless of the temperature of heating and regardless of whether or not gelation took place suggesting that (a) the acidic and basic subunits of glycinin and (b) the aggregation reactions remained intact through noncovalent bonds (such as disulfide and hydrophobic bonding) which take place during gelation are reversible in the presence of SDS / 2-mercaptoethanol.

5.2.1.4 Effect of Heating Time

The effect of heating time on gel strength is shown in Figure 5.6, gel strength increased with increasing heating time (at 95 °C), from 1.6 N for 10 min of heating to 6.1 N for 60 min of heating; however the effect of heating time was limited to the first 40 min of heating. Catsimpoolas and Mayer (1970) reported that heating at lower temperature requires longer heating times for gelation than at higher temperatures and resulted in the weaker gels. This suggest that glycinin must be sufficiently unfolded and must develop the appropriate three-dimensional network, as a result of the heating period, in order for a gel to form (Kinsella *et al.* 1985; Zayas 1997a).

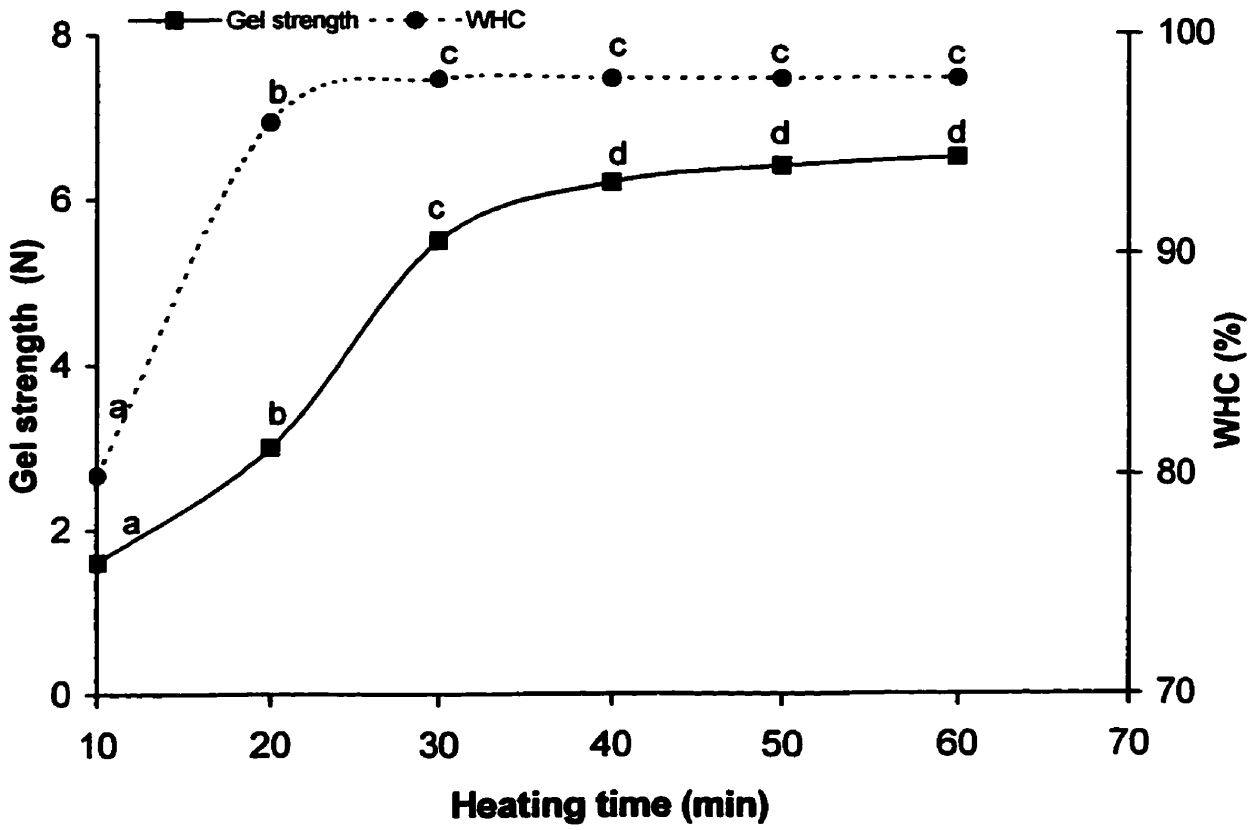


Figure 5.6: Effect of heating time at 95 °C on gel strength and WHC of thermally-induced NaOH-CP gels (line values by the same letter are not significantly different, P>0.05).

The WHC of the gels heated (at 95 °C) for 10 min was 80 %; after 30 min of heating the WHC remained constant at 98 % (Figure 5.6). This suggested that the increase in WHC was limited to the initial stage of gel formation. These results agree with those reported by Boye (1995) for whey protein concentrate.

5.2.1.5 Effect of Ionic Strength

No gels were obtained in the presence of 0.5, 1.0, 1.5, and 2.0 M NaCl. Hermansson (1979) showed that aggregation was increased by NaCl up to 0.2 M, but at higher levels was suppressed. Wang and Damodaran (1991) reported that glycinin prepared by the isoelectric precipitation procedure of Thanh and Shibasaki (1976) did not form gels in the presence of 0.5 M or higher concentration of NaCl, while Utsumi and Kinsella (1985) reported that glycinin formed soft gel in the of 0.05 M NaCl, but did not form gels above 0.1 M NaCl.

5.2.1.6 Effect of Reducing Agents

No gel was obtained in the presence of SDS in the concentration range of 50 to 150 mM of SDS. This suggests that hydrophobic bonds are responsible for the initial step of glycinin gelation.

Soft gels were obtained in the presence of 0.1 % 2-mercaptoethanol, while over the range of 0.5 % to 1.0 % of 2-mercaptoethanol only coagulum type gels were obtained. This suggests that disulfide bonds are not essential for gel formation from glycinin but that it is important in forming a strong elastic gel

(Utsumi and Kinsella 1985). Phillips *et al.* (1994) also reported that the role of intermolecular disulfide bonds in protein gelation might be related to their ability to increase the chain length of the polypeptides rather than as initial network former / stabilizer.

5.2.2 Cold-Set Gelation

The preheating step in which the NaOH-CP was heated at 95 °C for 30 min is essential, for cold-set gelation; when 6 % dispersion of NaOH-CP (pH 8) was heated at 80 °C for 30 min and $CaCl_2$ (30 mM-100 mM) was added, gelation was not observed. This suggests that preheating treatment at 95 °C for 30 min cause more protein unfolding, and addition of $CaCl_2$ after heating enhanced gel formation through increased protein-protein interactions. Ju and Kilara (1998) proposed that for cold set gelation, there are two groups of bonds in the gels: intra-aggregation bonds and inter-aggregation bonds, for cold-set gelation, preheating facilitated the formation of intra-aggregation bonds and addition of $CaCl_2$ promoted the formation of the inter-aggregate bonds.

The minimum protein concentration required to form thermally-induced gel of NaOH-CP was 8 %. Ju and Kilara (1998) suggested that the maximum protein concentration required for cold-set gelation of WPI should be below that required to obtain heat-induced gels.

5.2.2.1 Effect of $CaCl_2$ Concentration

The minimum $CaCl_2$ concentration required to form cold-set gelation was 30 mM. Addition of 10 mM of $CaCl_2$ did not cause cold-set gelation while a very soft gel was obtained with 20 mM $CaCl_2$. The gel obtained at 30 mM $CaCl_2$ was a clear white gel and as $CaCl_2$ concentration increased, gel clarity increased. Figure 5.7 shows the effect of $CaCl_2$ concentration on the gel strength of a cold-set gel; increasing $CaCl_2$ concentration resulted in an increase in the gel strength. Hongsprahas and Barbut (1997a) reported that the gel strength of a cold-set whey protein isolate gel increased with increasing $CaCl_2$ concentration from 10 to 120 mM. Ju and Kilara (1998) also reported that increasing $CaCl_2$ concentration resulted in increased gel hardness.

The effect of $CaCl_2$ concentration on the WHC of cold-set gels is shown in Figure 5.7. The WHC was lowest (48 %) at 30 mM of $CaCl_2$ and increased to a maximum (56 %) at 100 mM $CaCl_2$ Figure 5.7. Hongsprabhas and Barbut (1997b) reported that increasing $CaCl_2$ concentrations from 5 to 150 mM slightly affected the WHC of cold-set WPI gels.

5.2.2.2 Effect of Protein Concentration

Figure 5.8 shows the gel strength of cold-set gels at various protein concentrations. Gel strength increased from 1.3 N at 4 % protein concentration to 2.6 N at 6 % protein concentration.

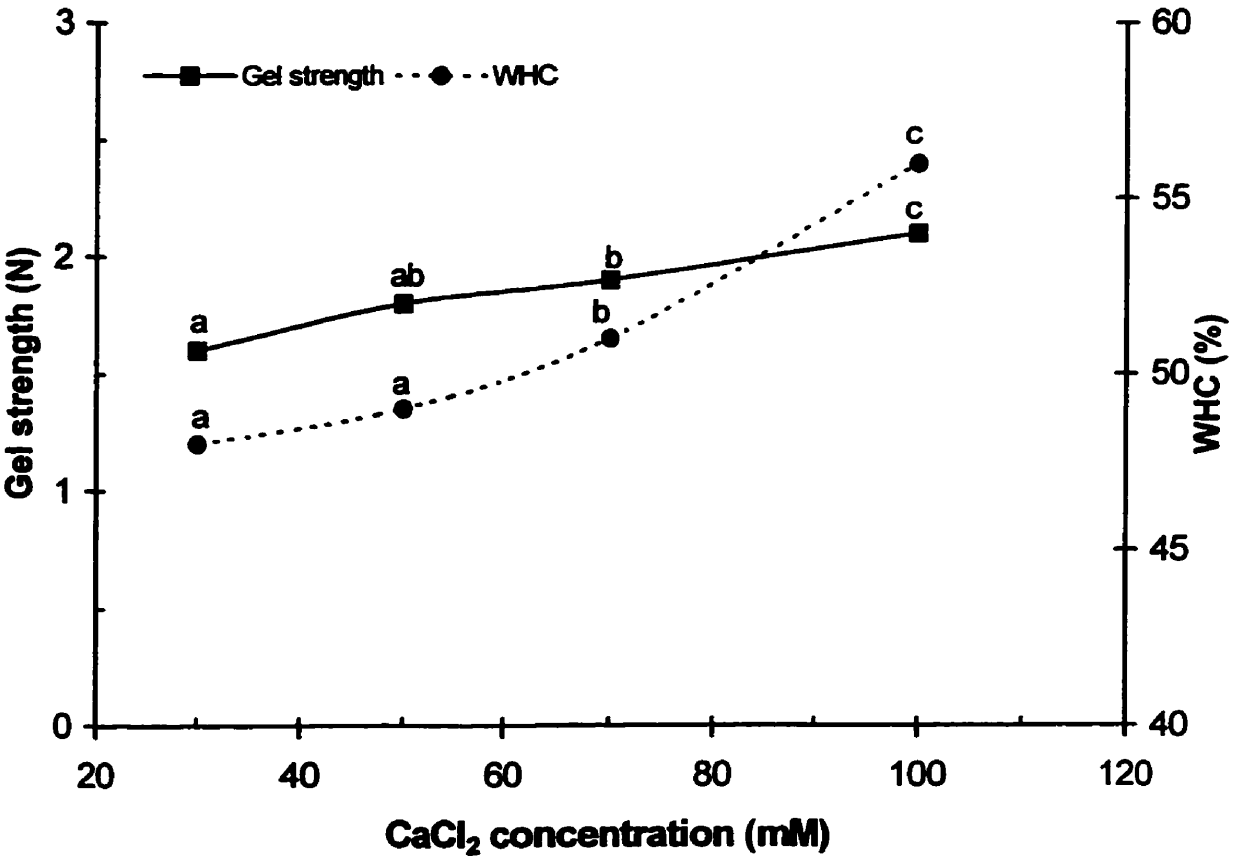


Figure 5.7: Effect of $CaCl_2$ concentration on gel strength and WHC of cold-set gelation of NaOH-CP (line values by the same letter are not significantly different, P>0.05).

Hongsprabhas and Barbut (1997b) have reported 10 % cold-set gels of WPI were more transparent gels than 6 % cold-set gels of WPI. Ju and Kilara (1998) reported that gel hardness increased slowly at lower protein concentration and more rapidly at higher protein concentrations (4 to 8 %).

Figure 5.8 shows the relationship between protein concentration and WHC. The WHC of gels with 4 % protein concentration was 38 % and increased rapidly to 56 % for gels with 6 % protein concentration. These results are similar to the results of Hongsprabhas and Barbut (1997b) who reported that increasing the whey protein concentration from 6 to 10 % protein concentration increased WHC from 25 to 45 %, respectively. At 8 % and 10 % NaOH-CP protein concentrations cold-set gels were formed by heating at 95 °C for only 12 min (below the heating time required to obtain thermally-induced gel) followed by addition 100 mM of $CaCl_2$ at ambient temperature. Figure 5.8 shows that the gel strength from the 10 % cold-set gel was 7.4 N which is substantially higher than 2.6 N gel strength obtained for 6 % protein concentrations, the gel strength value (7.4 N) obtained using 10 % cold-set gel was similar to that (7.8 N) obtained for 16 % thermally induced gels.

5.2.2.3 Effect of pH

Figure 5.9 shows the effect of pH on the gel strength of cold-set NaOH-CP gels. The gel strength was increased from 1.8 to 2.6 N when the pH was

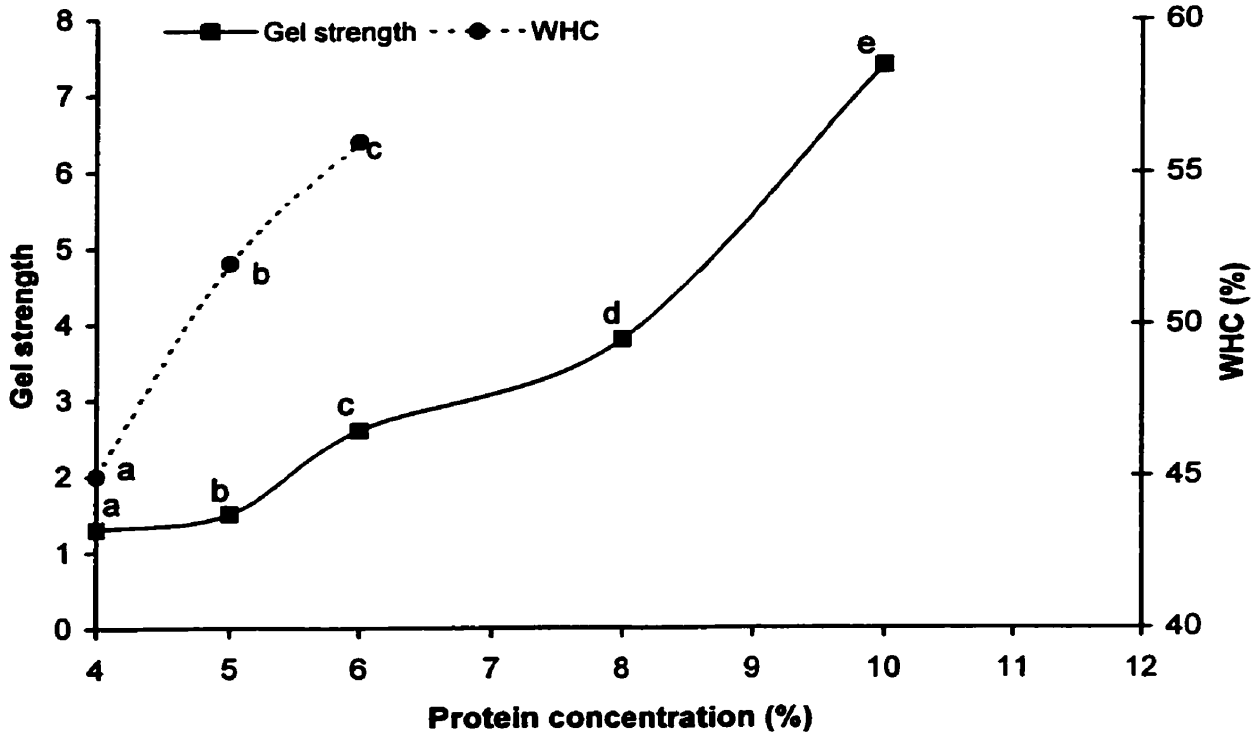


Figure 5.8: Effect of protein concentration on gel strength and WHC of cold-set gelation of NaOH-CP (line values by the same letter are not significantly different, P>0.05).

increased from pH 7 to 9; no significant difference in gel strength was found when the pH increased from pH 9 to 10. These results suggest that cold-set gelation is favored at alkaline pHs because the protein at these pHs will carry more negative charges which can interact with Ca^{+2}. No cold-set gelation was observed at pH 3. Between pH 4 and 6 opaque, coagulums were obtained as in the case of thermally induced gels. Puppo and Anon (1998) reported that at acidic pH, the $Ca+^{2}$ ion competes with the H^{+} for the same binding sites, so that $Ca+^{2}$ would not establish bridges with the protein as at alkaline pH. $Ca+^{2}$ could interact with water, thus modifying the aqueous surroundings of the protein, increasing protein aggregation and decreasing WHC.

Figure 5.10 shows the effect of pH on WHC. WHC increased from 46 % to 60 % when the pH was increased from pH 7 to 9, there was a slight increase in WHC when the pH was increased from pH 9 to 10.

5.2.3 Gel Microstructure of Thermally-induced and Cold-Set Gels

Figure 5.10 shows SEM micrographs of thermally-induced gels and cold-set gels of NaOH-CP. Cold set gels showed a microstructure which consists of homogenous network structure compared to the unhomogeneous network structure shown by the thermally-induced gels. The heat-induced gel shows a particulate microstructure composed of bead-like particles attached to each other (Figure 5.10a), similar to that shown for thermally-induced soy

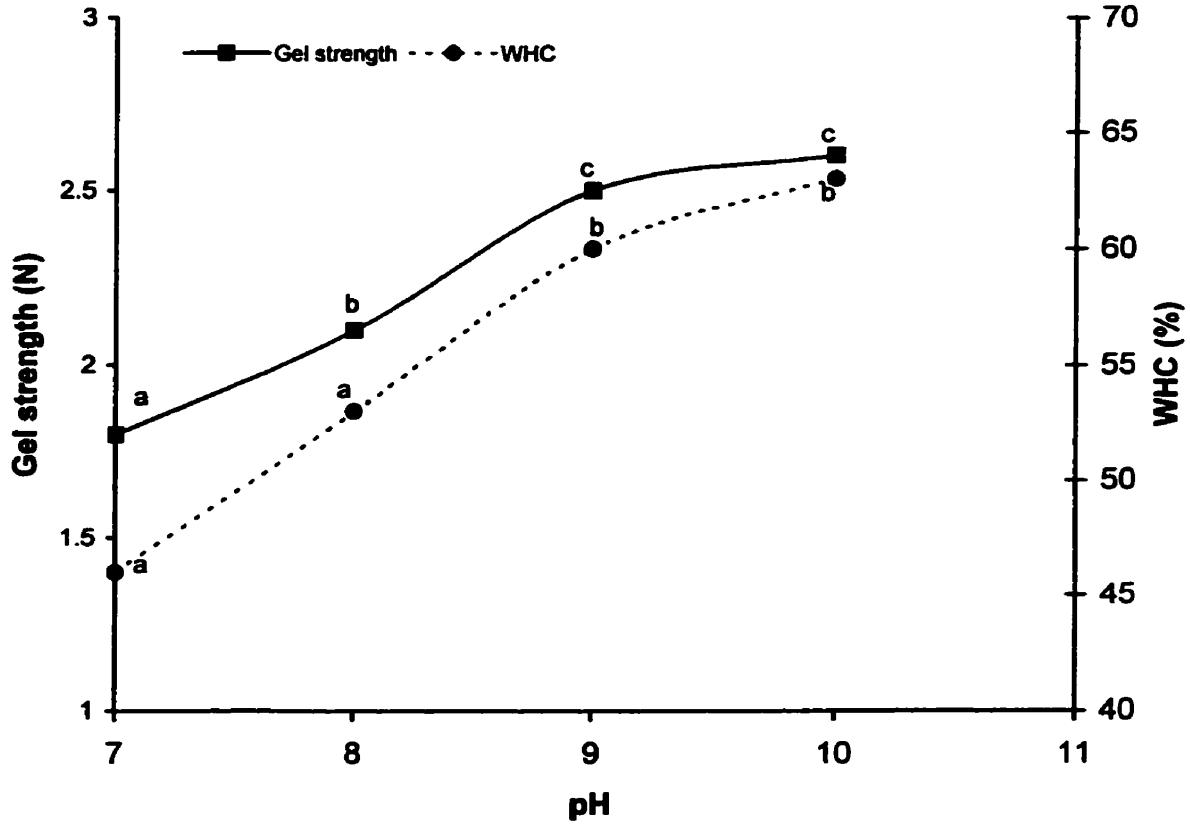


Figure 5.9: Effect of pH on gel strength and WHC of cold-set gelation of NaOH-CP (line values by the same letter are not significantly different, P>0.05).

protein gels (Puppo and Anon 1998), thermally-induced WPI (Barbut and Foegeding 1993), and thermally induced WPC (Beveridge *et al.* 1983). The microstructure of the cold-set gel shows a fine-stranded matrix (Figures 510b, c and d). Similar structure has been reported for cold-set WPI gels (Barbut and Foegebing 1993). The microstructure of cold-set gel was also affected by the protein concentration used. Increasing the protein concentration to 10% (Figure 5.10d) resulted in the formation of thicker protein strands than at 6 % protein concentration (Figure 5.10b), the thicker strands formed from the 10% cold-set gel had higher gel strength compared to 6% cold-set gel (Figure 5.8), indicating that the thicker protein strands formed more rigid network structure.

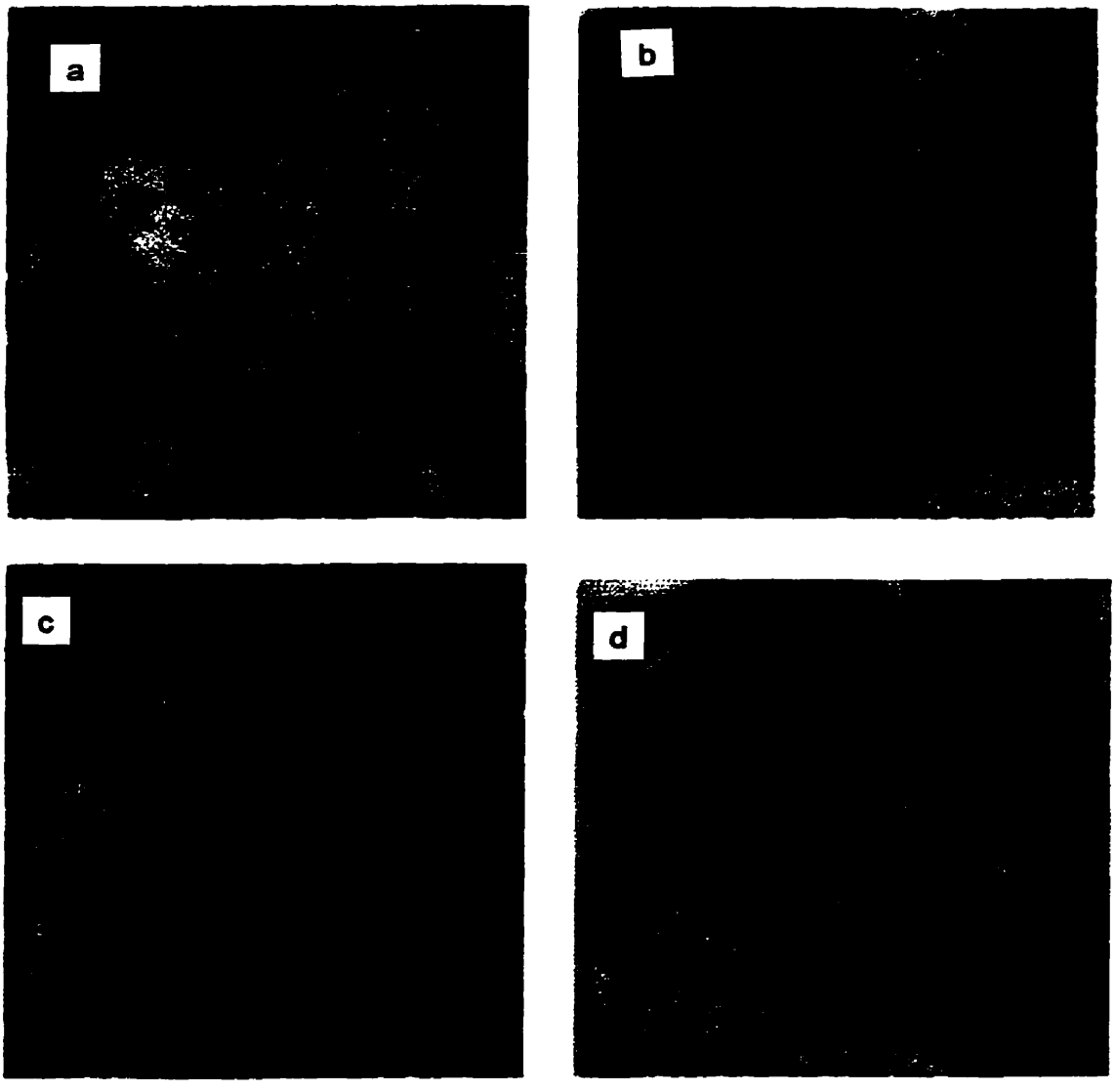


Figure 5.10: Scanning electron micrographs of NaOH-CP. a- thermally-induced gel (14 % w / v) b- cold-set gel (6 %, 50 mM $CaCl_2$), c- cold-set gel (8 %, 50 mM $CaCl_2$), d- cold-set gel (10 %, 50 mM $CaCl_2$),

CHAPTER 6

FTIR AND DSC ANALYSIS OF CRYOPRECIPITATED HIGH-GLYCININ PROTEIN

6.1 MATERIALS AND METHODS:

6.1.1 Materials

The commercial defatted soy flour used in Section 3.1.1 of Chapter 3 was used.

6.1.2 Preparation of NaOH-CP Protein

NaOH-CP protein was prepared using the procedures described in Section 3.1.2.3 of Chapter 3.

6.1.3 Preparation of Protein Samples for FTIR Analysis

FTIR analysis was used for determination of NaOH-CP protein secondary structure. NaOH-CP (12 % w / v) was prepared by dissolving the protein in deuterium oxide (D_2O) or H_2O. To study the effect of ionic strength, the protein was dissolved in D_2O solutions containing 0.5 and 1.0 M NaCl. To study the effect of denaturing agents, NaOH-CP (12 % w/v) were prepared using 50 and 100 mM SDS and 0.1and 0.5 % 2-mercaptoethanol

Infrared spectra were recorded with a 8210E Nicolet Fourier transform infrared spectrometer FTIR equipped with a deuterated triglycine sulfate

detector. The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA, 018350723, USA). Samples (7 µl) were held in an IR cell with a 25µm pathlength (for samples prepared in D_2O) and 12µm (for samples prepared in H_2O) between two CaF_2 windows. The IR cell was place in a temperature-controlled cell holder. The temperature was increased in 5 °C increments and the cell allowed to equilibrate for 10 min before recording the spectrum. For the cooling cycle, the temperature was decreased in 10 °C increments and the cell was allowed to equilibrate for 10 min before recording the spectrum. The second derivative spectrum was obtained as described by Cameron and Moffatt (1987).

6.1.4 Preparation of Protein Samples for DSC Analysis

DSC was used to study the thermal properties of NaOH-CP. NaOH-CP (12 % w / v) was prepared by dissolving the protein in H_2O. To study the effect of ionic strength on the thermal properties, NaOH-CP (12 % w / v) was dissolved in solutions containing 0.5, 1.0, 1.5, and 2 M NaCl and 0.5, 1.0, 1.5, and 2.M $CaCl_2$. To determine the effect of reducing agents, NaOH-CP was dissolved in solutions containing 50, 100, and 150 mM SDS, 0.1, 0.5, and 1.0 % 2-mercaptoethanol, 50,100, and 150 mM cysteine and 1, 2, 3, and 4 M urea using the DSC and the procedure described in Section3.15 Chapter 3. All analyses were performed in duplicate.

6.2 RESULTS AND DISCUSSIONS

6.2.1 FTIR of NaOH-CP

Figure 6.1a and b shows plots of stacked second derivative spectra of the amide I band in the infrared spectra of NaOH-CP dissolved in D_2O and H_2O, respectively. The second derivative spectra were generated from the spectra of NaOH-CP after subtraction of the absorption due to water from each spectrum. At 25 °C, the amide I band in the infrared spectrum of NaOH-CP in D_2O (Figure 6.1a) showed four peaks at 1633, 1650, 1667, and 1689 cm^{-1}. In H_2O, similar peaks were observed, except that the frequency of the band at 1654 cm^{-1} in H_2O shifted to 1650 cm^{-1} in D_2O, this shift can be attributed to hydrogen-deuterium exchange (Surewicz and Mantsch 1988). The band at (a) 1633 cm^{-1} has been assigned to β-sheet structure (Susi and Byler 1988, Dong *et al.* 1990, Herld and Smith 1992); at (b) 1650 cm^{-1} to α-helix structure (Susi and Byler 1988, Mitchell *et al.* 1988, Holloway and Mantsch 1989, Dong *et al.* 1990); at (c) 1667 cm^{-1} to the turn structure (Herald and Smith 1992); at (d) 1689 cm^{-1} to β-turn structure (Arrondo *et al.* 1988; Surewicz and Mantsch 1988; Susi and Byler 1988) or to the high frequency component band of an antiparallel β-sheet (Surewicz and Mantsch 1988). Abbott *et al.* (1996) reported that based on FTIR spectroscopy, the secondary structure of glycinin was 33% β–sheet, 25% α–helix, 31% turns and 12 % unordered structure while Dev *et al.* (1988) had previously reported

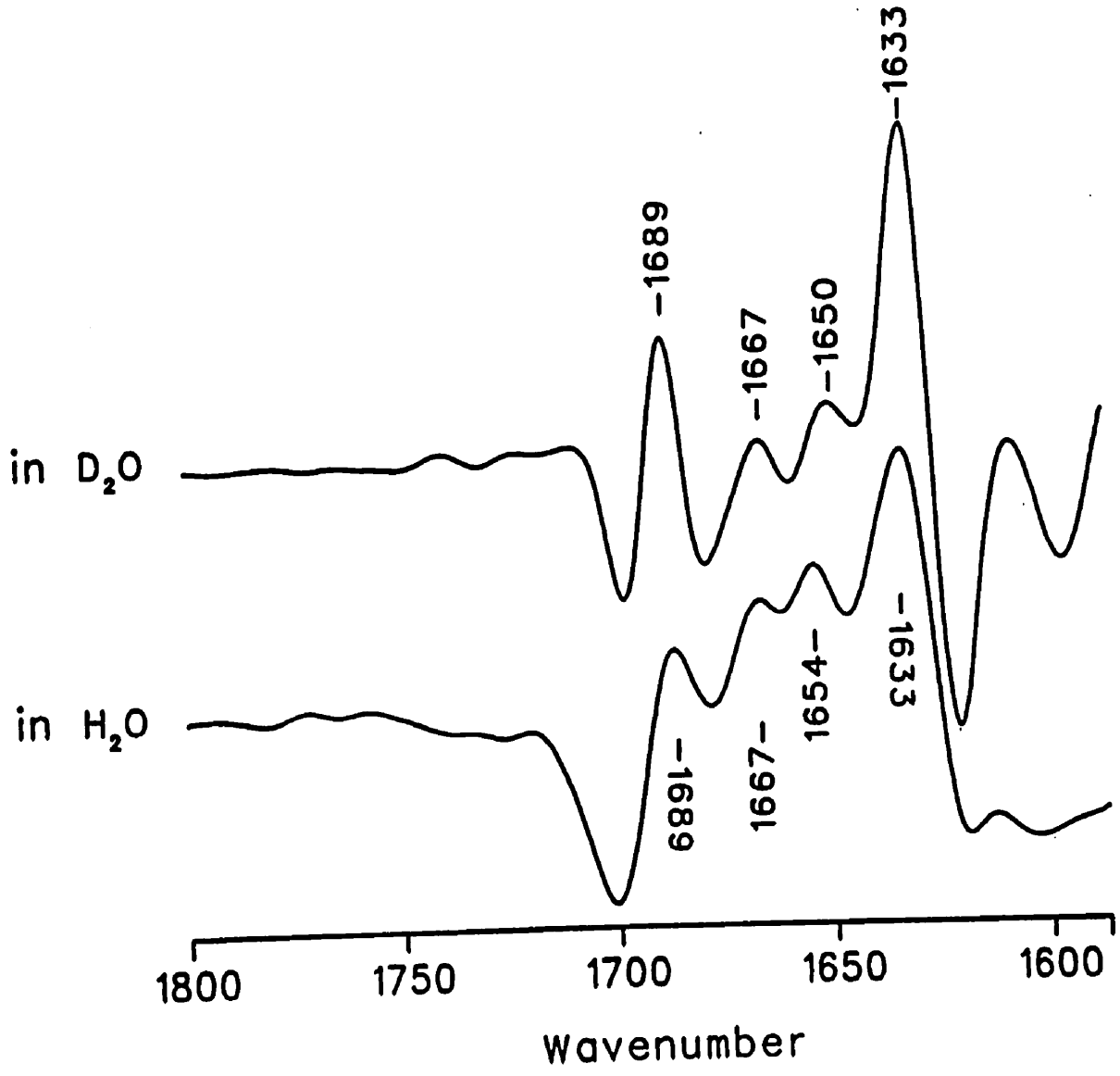


Figure 6.1: Stacked plot of the second dervative infrared spectra of NaOH-CP, a: D_2O, b: H_2O

that the secondary structure of glycinin was mainly of β–sheet structure, β–turns and unordered structure with very little α–helix, since both studies were carried out in H_2O, it was difficult to employ resolution enhancement techniques to ascertain the position of the amide I bands precisely, leading to possible miss-assignment of the unordered and α-helical structures. It is evident from the secondary structure of NaOH-CP in D_2O or H_2O (Figure 6.1a and b) that the major secondary component is β-sheet with smaller amounts of α-helix and turn structures.

6.2.2 Effect of Temperature on Secondary Structure

Heating the NaOH-CP solution (12 % w / v in D_2O) from 25-70 °C had no significant effect on the secondary structure (Figure 6.2). Above 70 °C the 1650 cm^{-1} band attributed to α-helix decreased. Heating the protein above 85 °C (temperature associated with gelation) resulted in a substantial decrease in the intensities of the 1633 1689 and 1667 cm^{-1} bands and an increase in the intensity of a shoulder at 1644 cm^{-1} attributed to random coil (Nagano *et al.* 1994), formed as a result of protein unfolding. The appearance of the 1644 cm^{-1} band coincides with the appearance of two additional bands (at 1685 and 1616 cm^{-1}) attributed to intramolecular β-sheet formed as a result of re-association of unfolded peptide segments which led to formation of a gel and aggregate structures (Clark *et al.* 1981, Ismail *et al.* 1992 and De Las Rivas 1997).

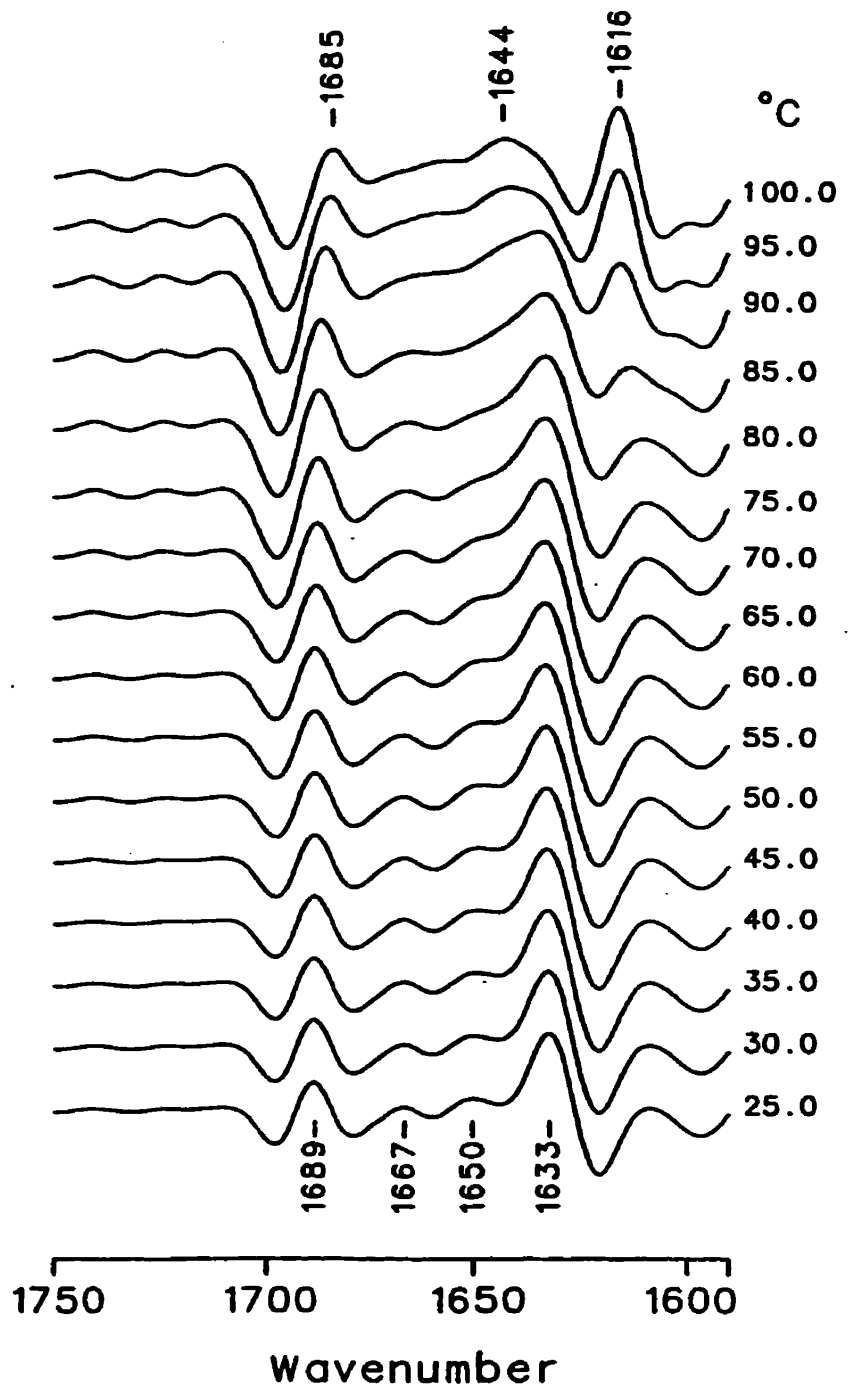


Figure 6.2: Stacked plot of the second derivative infrared spectra of NaOH-CP in D_2O as function of increasing temperature

These results are in contrast to those reported by Chen *et al.* (1990), who observed using FTIR that the amount of α-helix of glycinin was increased from 7.8 to 28 % as the temperature of the protein solution increased from 25 to 90 °C. Also, Wang and Damodaran (1991) reported that at high protein concentration (10 % protein) thermal treatment resulted in extensive denaturation of the secondary structure in soybean 11S protein (glycinin); they suggested that at high concentration, the propensity of intermolecular interactions apparently leads to aggregation and network formation and this prevents refolding and regaining of secondary structure. Nagano *et al.* (1994) investigated the secondary structure changes of glycinin during heat-induced gelation employing FTIR spectroscopy; they reported that bands at 1618 and 1680 cm^{-1} attributed to β-sheet increased with the formation of heat–induced glycinin gel; they also attributed the bands at 1618 and 1680 cm^{-1} to intermolecular hydrogen-bonded antiparallel β-sheet structure resulting from protein aggregation. The transition temperature of NaOH-CP was determined by DSC to be 91 °C (Figure 6.9) and found to be in agreement with the transition obtained by plotting the integrated intensity of the 1616 cm^{-1} band as a function of increasing temperature (Figure 6.3).

The cooling cycle of the heated solution of NaOH-CP showed no changes in the secondary structure of the heated protein (Figure 6.4). This suggests that the

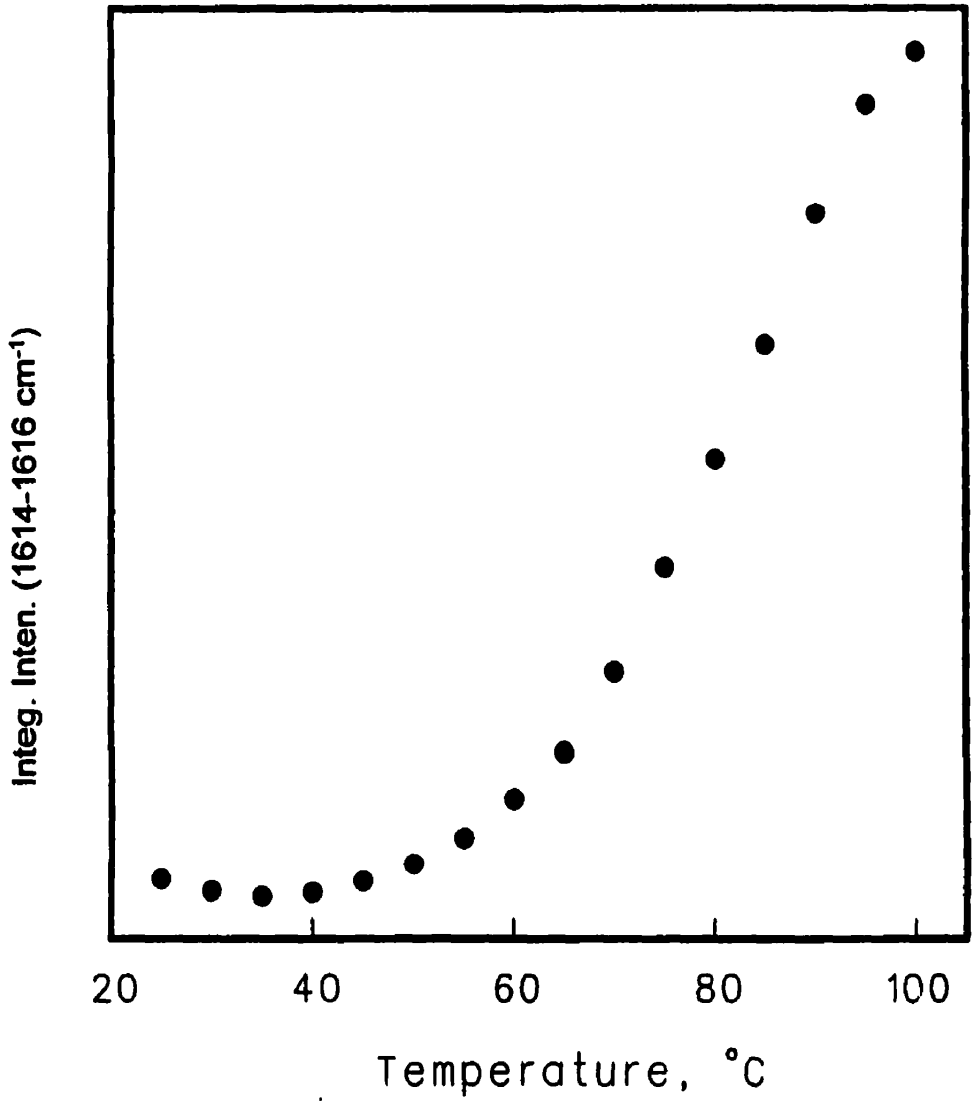


Figure 6.3: Integration intensity of the 1614-1616 cm^{-1} regions in the amide I band of NaOH-CP in D_2O

denaturation temperature of NaOH-CP is irreversible. These findings were similar to that observed in Section 3.2.4 Chapter 3.for NaOH-CP (second heating cycle using DSC).

6.2.3 Effect of Ionic Strength on Secondary Structure

The FTIR spectra in the amide I absorption region of NaOH-CP in the presence of 0.5 and 1.0 M NaCl solutions, recorded as a function of increasing temperature between 25-100 °C, are shown in Figure 6.5a and Figure 6.5b, respectively. No major changes were observed in the amide I bands upon heating of the solutions up to 100 °C in the presence of 0.5 M or 1.0 M NaCl; a notable exception is the small drop in the relative intensity of the band 1650 cm^{-1} (α–helix) to the 1633 cm^{-1} band. This suggests that the α-helix component of NaOH-CP is thermally labile. Elevation the heating temperature of NaOH-CP solution from 25 to 100 °C in the presence of 0.5 M and 1.0 M NaCl did not cause the appearance of the two bands at 1616 and 1685 cm^{-1} which are attributed to protein aggregation. These results indicate that NaCl appeared to stabilize the protein structure aggregate formation possibly through non-specific ion effects of NaCl on electrostatic interaction between charged groups on the protein (Lèger and Arntfield 1993).

Using FTIR, Boye (1995) reported that increasing NaCl concentration up to 2 M led to an increase in the thermal stability of β–lactoglobulin.

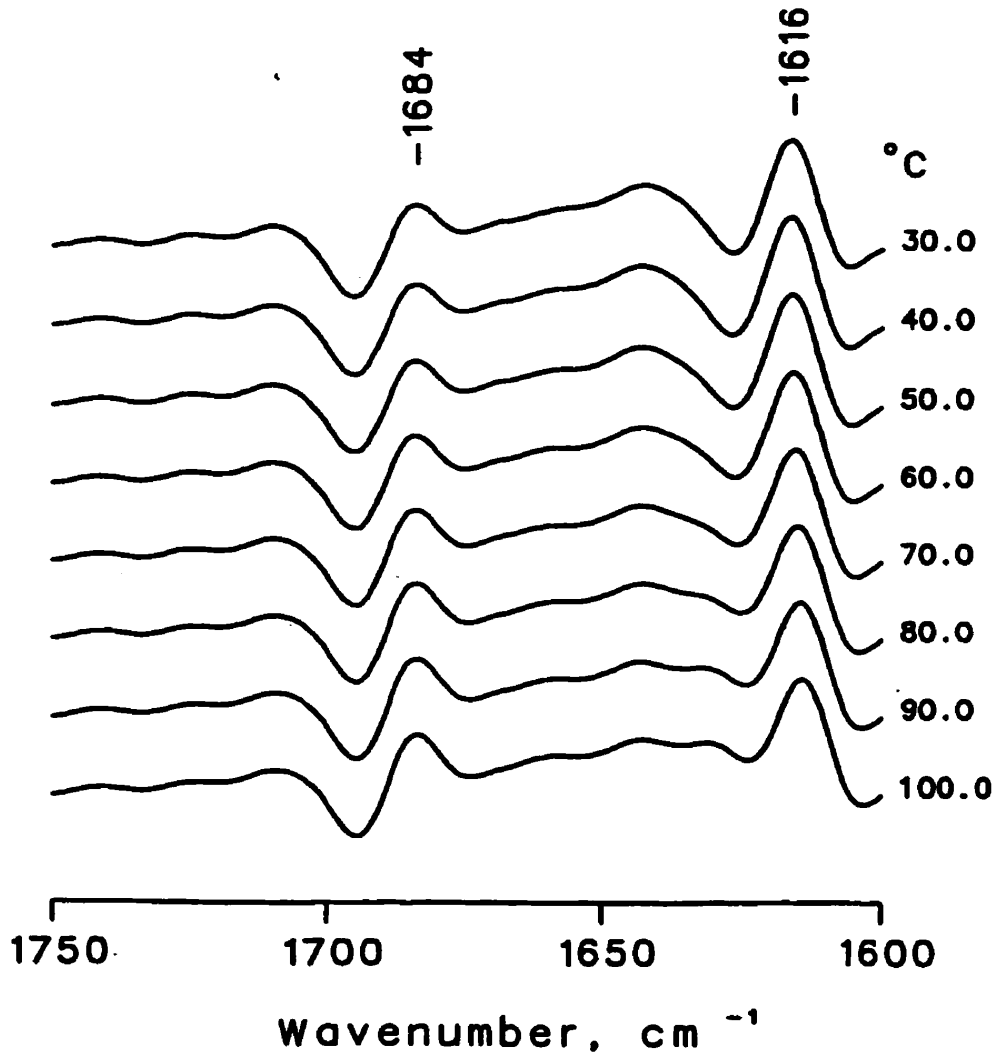


Figure 6.4: Cooling cycle of NaOH-CP in D_2O

Cyclic cooling of NaOH-CP (Figure 6.6) in presence of 1 M NaCl showed the same secondary structure as that of the heating cycle of this protein which indicates that higher heating temperature were required to disrupt the native protein structure at higher ionic strength.

6.2.4 Effect of Reducing Agents on Secondary Structure

Examination of the FTIR spectra of NaOH-CP, in the presence of SDS as a function of increasing temperature, reveals that a 50 mM concentration of SDS stabilized the secondary structure of NaOH-CP against aggregation Figure 6.7a. It is of interest to note that the β-sheet structure (1633 cm^{-1}) remains intact while the turn (1667 cm^{-1}) and helical structures (1650 cm^{-1}) are lost between 80-95 °C. This suggests that SDS at low concentration could form a bridge between cationic groups on the protein and ionic groups on SDS, which seem to solubilize the denatured protein. The aggregation bands at 1616 and 1685 cm^{-1} did not appear with increasing temperature or upon cooling in the presence of 50 mM SDS, indicating the absence of antiparallel β–sheet formation. These results are in agreement with those of Boye *et al.* (1996) who reported no aggregation bands were observed with bovine serum albumin in the presence of 50 mM of SDS.

In the presence of 100 mM SDS (Figure 6.7b), there were major changes in the amide I band above 50 °C, a decrease in the intensity of the 1633 cm^{-1} was

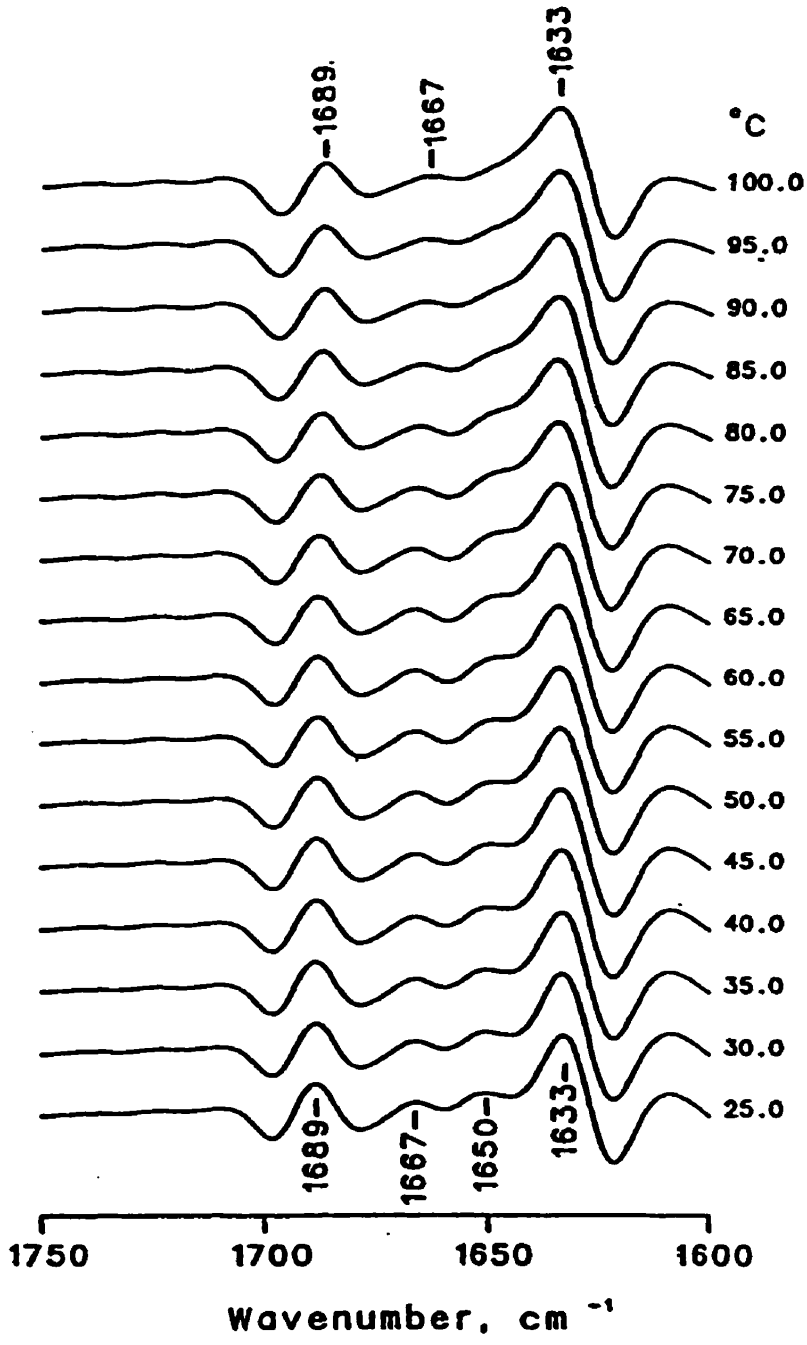


Figure 6.5a: Stacked plot of the second derivative infrared spectra of NaOH-CP in D_2O as a function of increasing temperature in the presence of 0.5 M NaCl

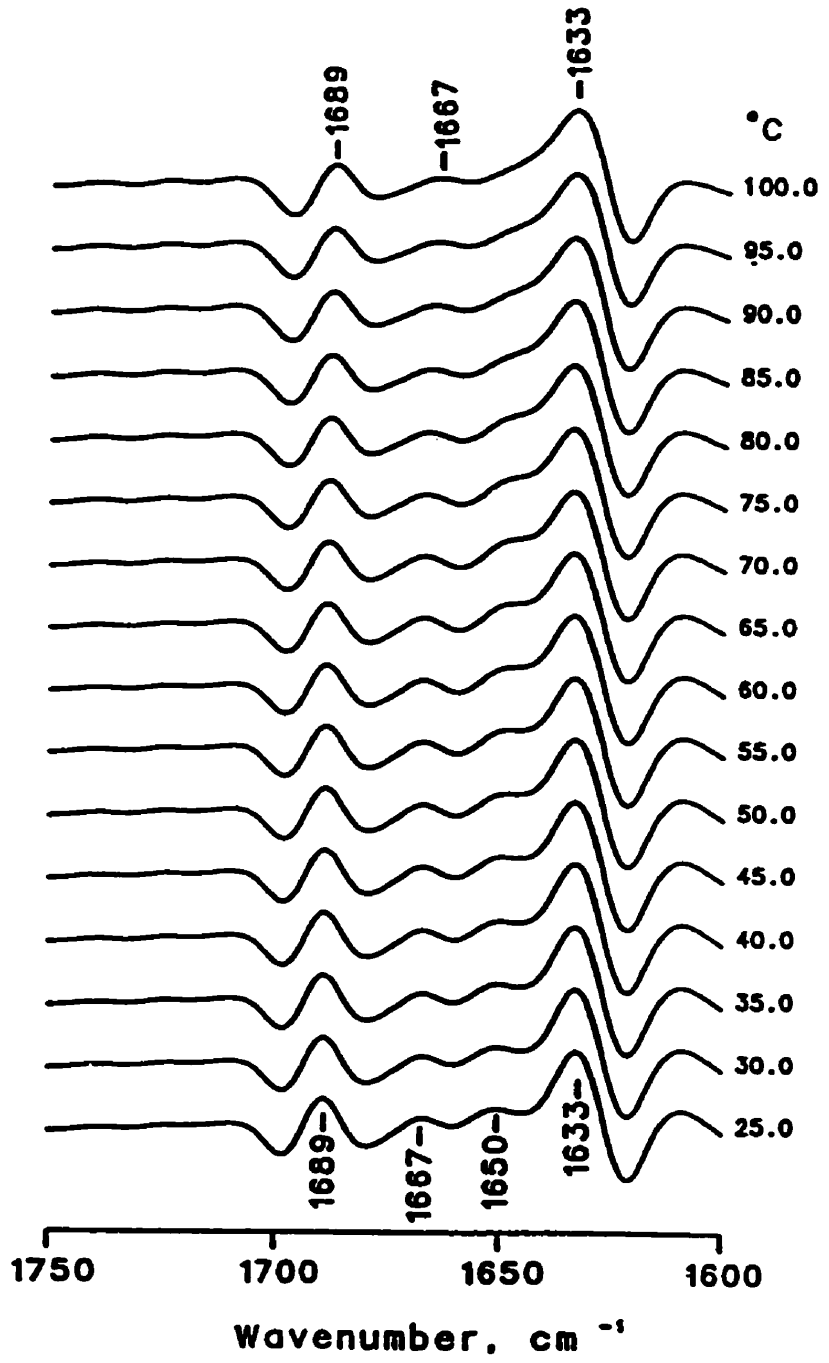


Figure 6.5b: Stacked plot of the second derivative infrared spectra of NaOH-CP in D_2O as a function of increasing temperature in the presence of 1.0 M NaCl

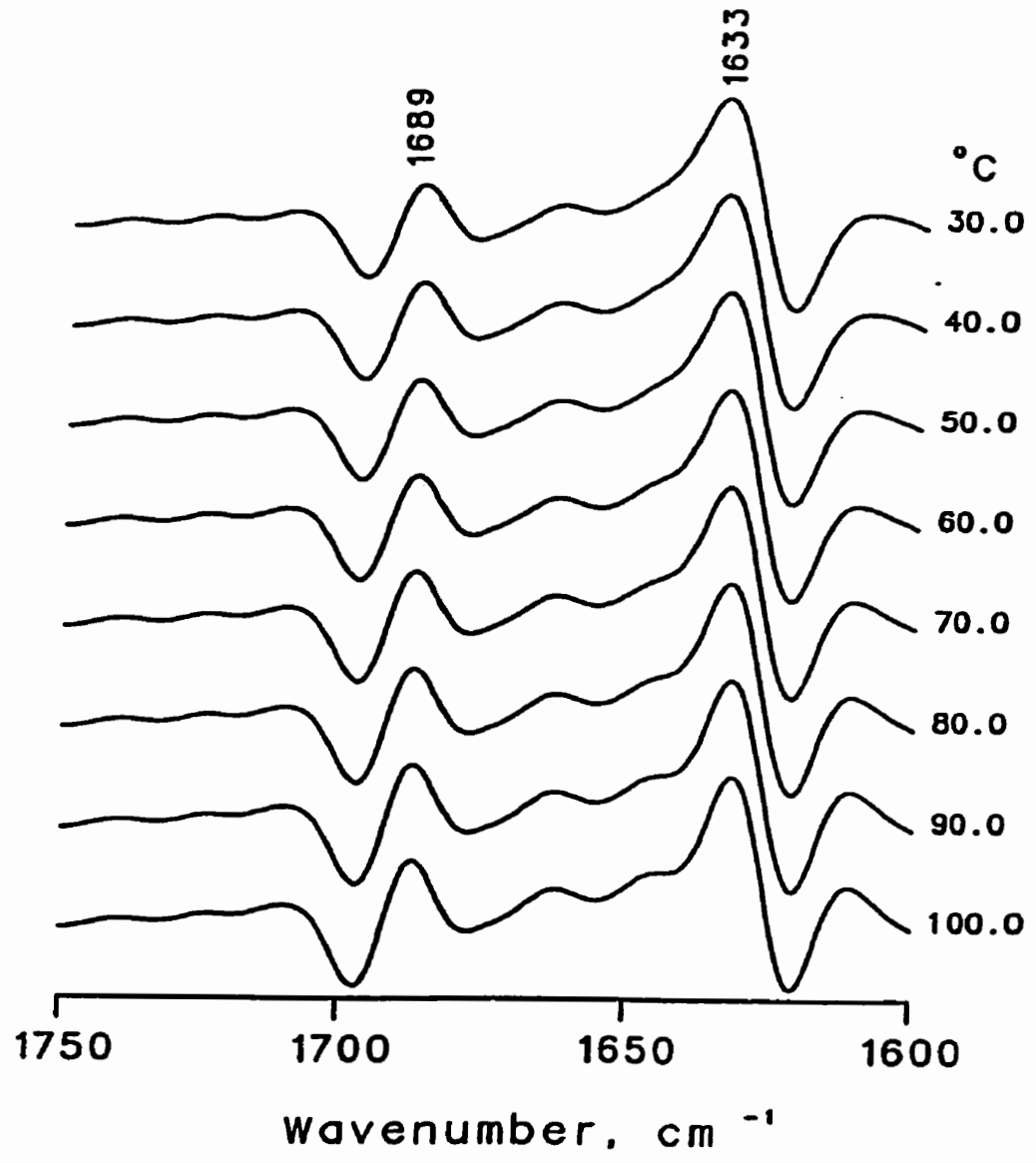


Figure 6.6: Cooling cycle of NaOH-CP in the presence of 1 M NaCl

observed. The bandwidth of amide I bands increased above 70 °C along with the appearance of a weak band at 1616 cm^{-1} attributed to aggregate formation. A broad band centered at approximately 1644 cm^{-1} (unordered structure) is also observed above 70 °C, these results demonstrate that at 100 mM SDS concentration the unfolding of the protein is favored rather than aggregate formation. Steinhardt (1975) reported that high concentrations of SDS favored a decrease in the enthalpy due to the non-specific binding of SDS detergent to the protein, facilitating its denaturation.

The FTIR spectra of NaOH-CP heated from 25-100 °C in the presence of different concentration of 2-mercaptoethanol are show in Figure 6.8a and Figure 6.8b. Major changes were observed in the bands 1633 cm^{-1}, 1650 cm^{-1}, 1667 cm^{-1}, 1689 cm^{-1}, at 75 and 80 °C in the presence of 0.1 and 0.5 % of 2-mercaptoethanol, these changes represented a loss of secondary structure suggesting that increasing 2-mercaptoethanol concentration associated with decreasing the thermal stability behavior of NaOH-CP. The appearance of two new bands at 1616 cm^{-1} and 1685 cm^{-1} which is the result of formation of anti-parallel β–sheet structure (Ismail *et al.* 1992) associated with aggregation, was observed at 80 °C in the presence of 0.1% 2-mercaptoethanol and at 75°C in the presence of 0.5 % 2-mercaptoethanol (Figure 6.8a and b). A broad band representing unordered structure at 1644 cm^{-1} was also observed between the two aggregation bands (1616 cm^{-1} and 1685 cm^{-1}).

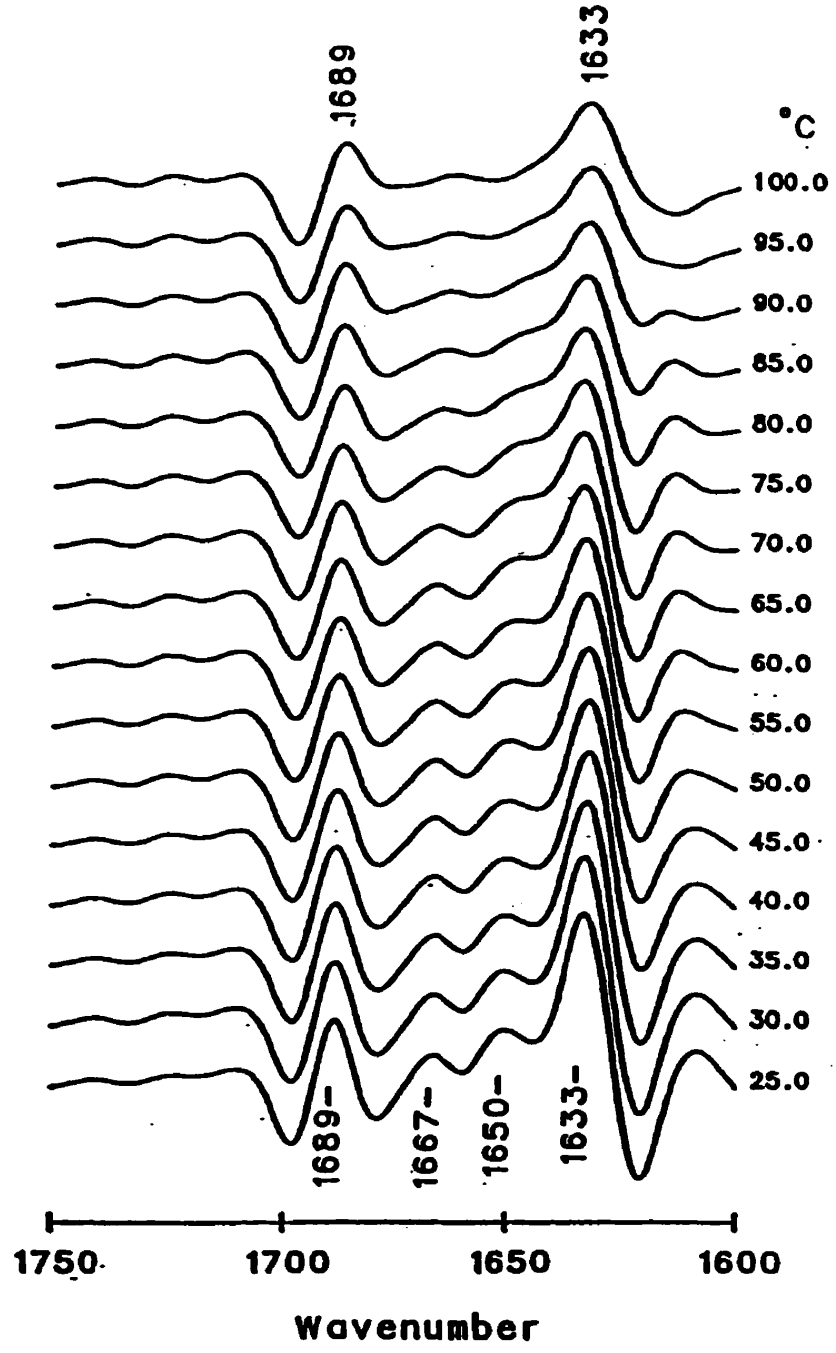


Figure 6.7a: Stacked plot of the second derivative infrared spectra of NaOH-CP in D_2O as a function of increasing temperature in the presence of 50 mM SDS

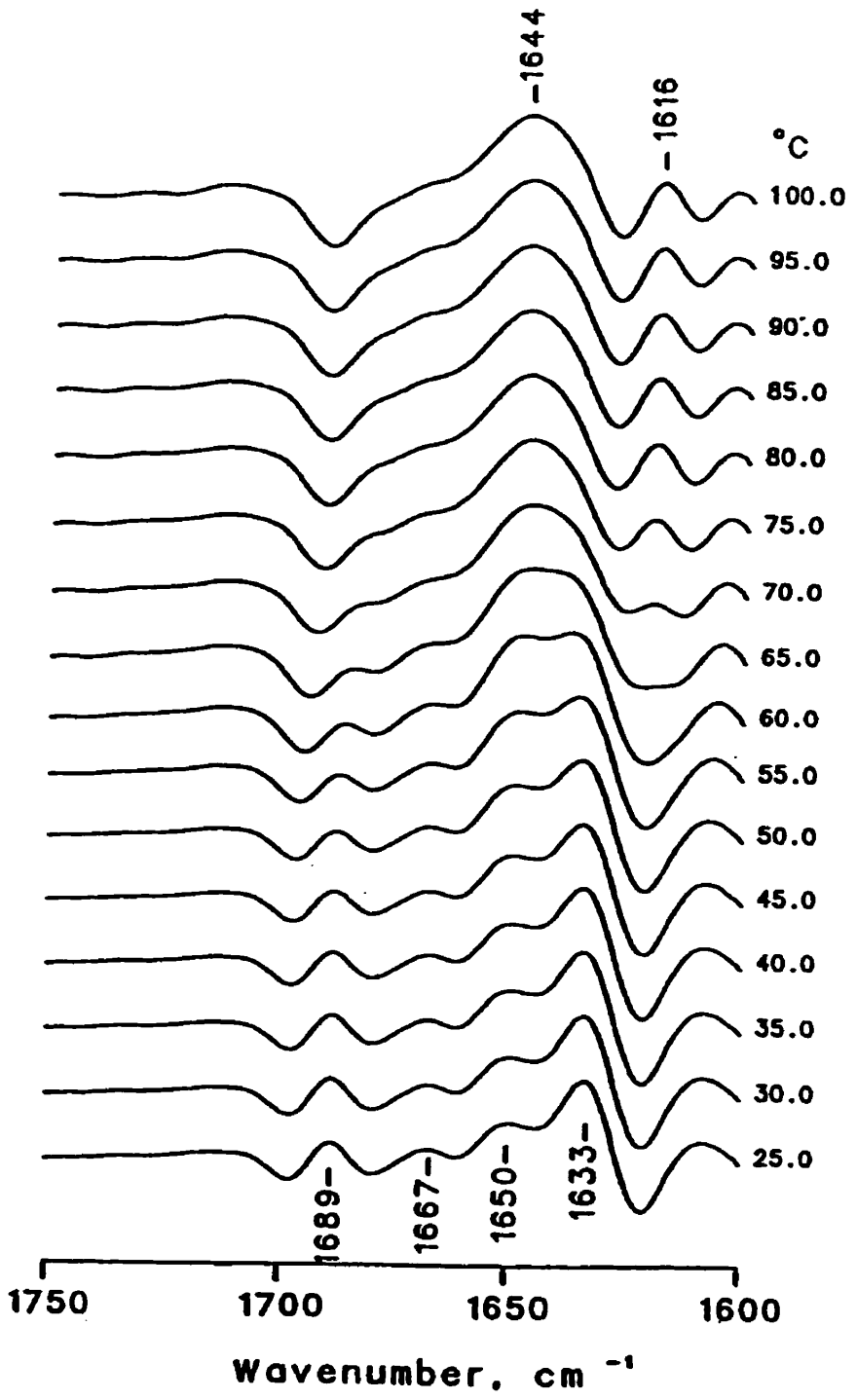


Figure 6.7b: Stacked plot of the second derivative infrared spectra of NaOH-CP in D_2O as a function of increasing temperature in the presence of 100 mM SDS

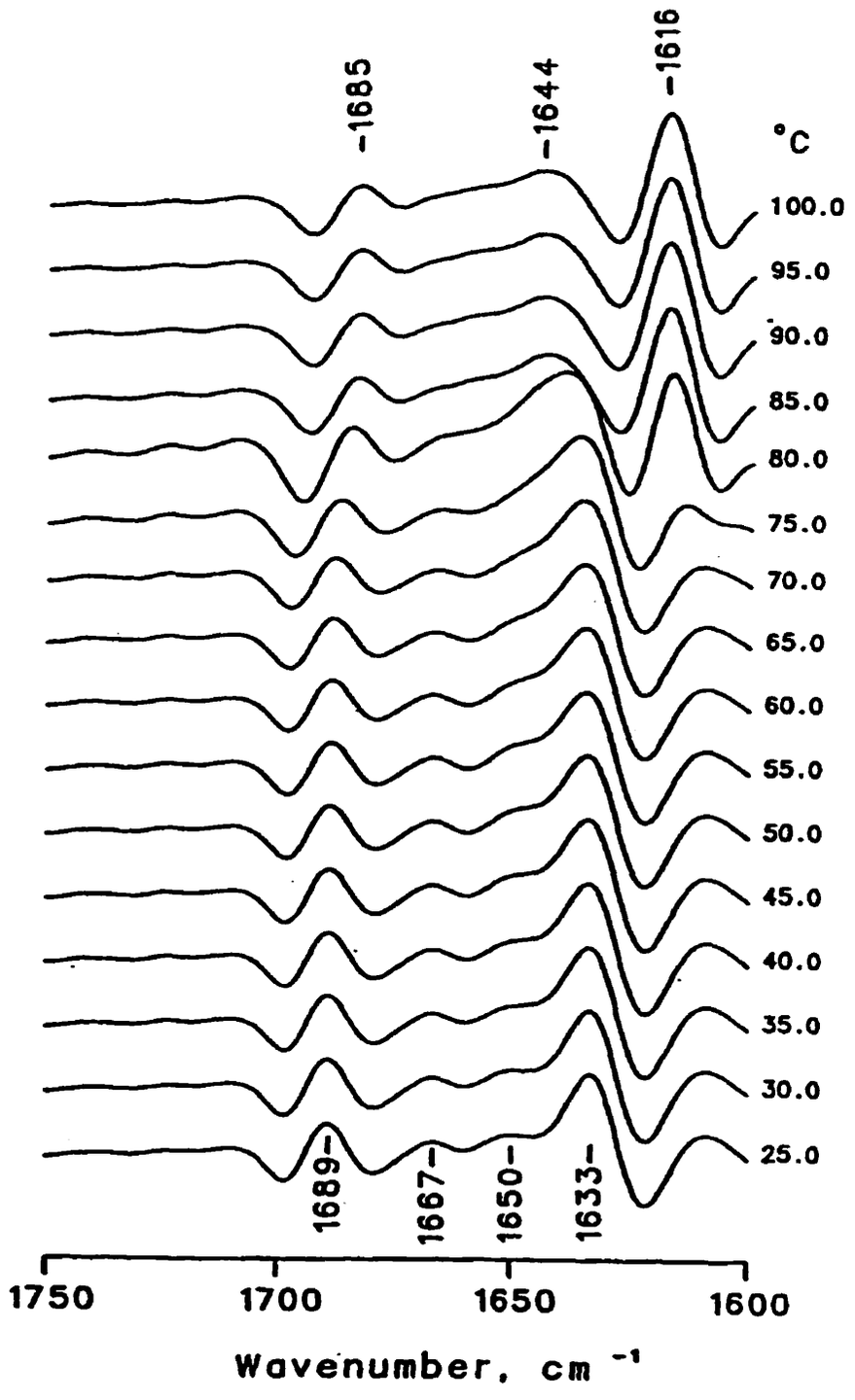


Figure 6.8a: Stacked plot of the second derivative infrared spectra of NaOH-CP in D_2O as a function of increasing temperature in the presence of 0.1 % 2-MeSH

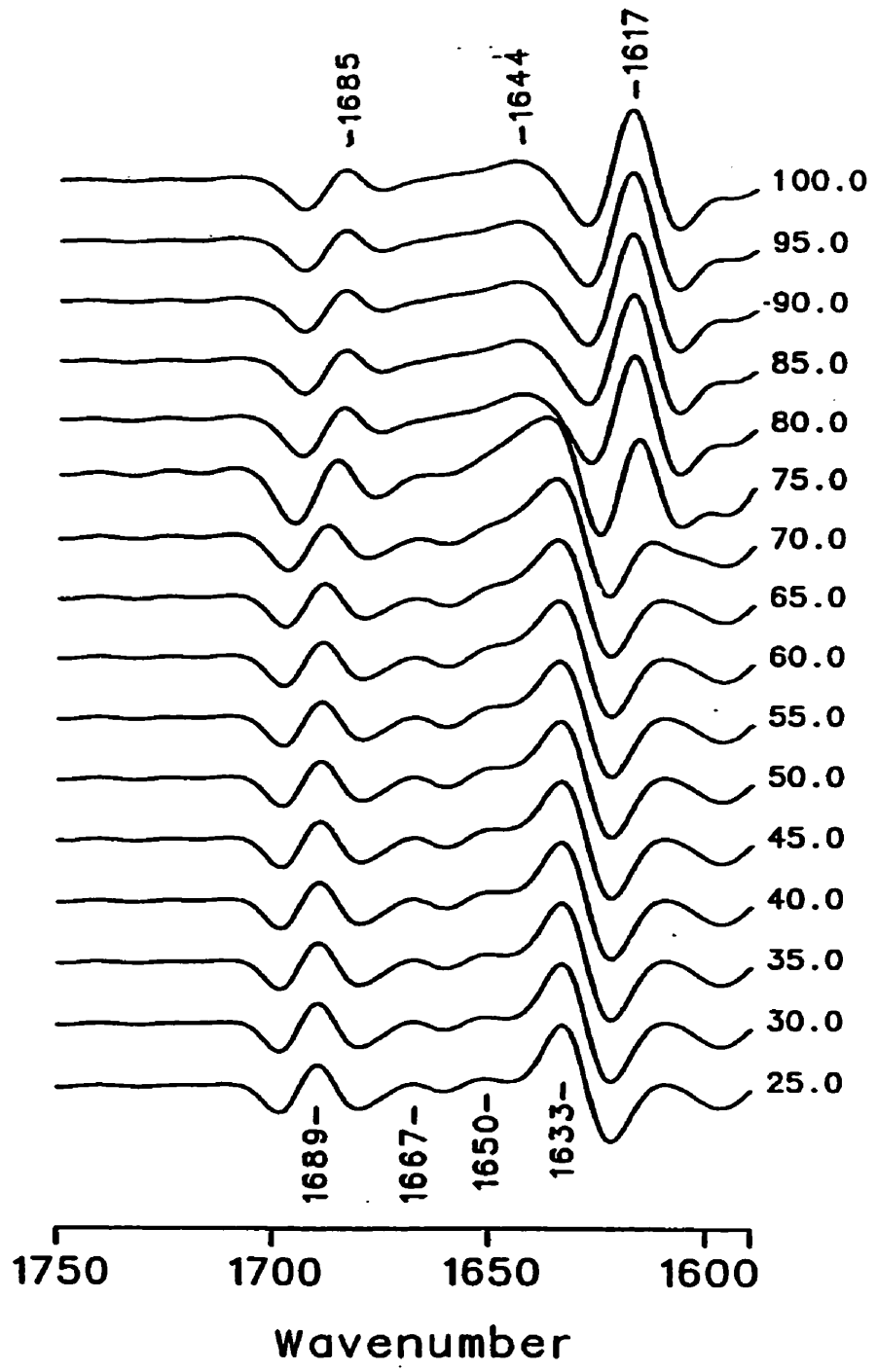


Figure 6.8b: Stacked plot of the second derivative infrared spectra of NaOH-CP in D_2O as a function of increasing temperature in the presence of 0.5 % 2-MeSH

6.2.5 Effect of Ionic Strength on Thermal Properties

Figure 6.9 shows the effect of NaCl concentration on the thermal stability of NaOH-CP; the denaturation temperature (T_d) was increased by approximately 25 °C (91.1° to 115.3 °C) when the NaCl concentration increased from 0.0 to 2.0 M. This could explain why NaOH-CP did not give a gel when heated (at 95 °C, 30 min) in the presence of 0.5 M to 2.0 M and is due to stabilization of the quaternary structure of NaOH-CP against thermal denaturation through nonspecific ionic effects of NaCl on electrostatic interaction between charged groups on the protein (Section 5.2.5, Chapter 5). Increased Td with increasing NaCl concentration has also been reported in oat globulin (Harwalkar and Ma 1987) and legumin (Zheng *et al.* 1993). Enthalpy (ΔH) increased from 17.5 to 23.5 J/g when the NaCl concentration changed from 0.0 to 0.5 M, then ΔH increased to 24.8 J/g in the presence of 1.5 M of NaCl and then decreased to 20.7 J/g at 2 M NaCl as shown in Figure (6.10). Damodoran (1988) proposed two explanations for this type of behavior: first, although a higher thermal denaturation temperature reflect increased resistance to thermal denaturation, the tertiary and quaternary structure of the stabilized protein may not be the same as that of the native molecule and consequently, a lower ΔH can be obtained; secondly, the observed lower ΔH value at 2 M NaCl may be a result of aggregation of denaturated protein so that the exothermic heat effect due to aggregation may partly offset the endothermic heat flow.

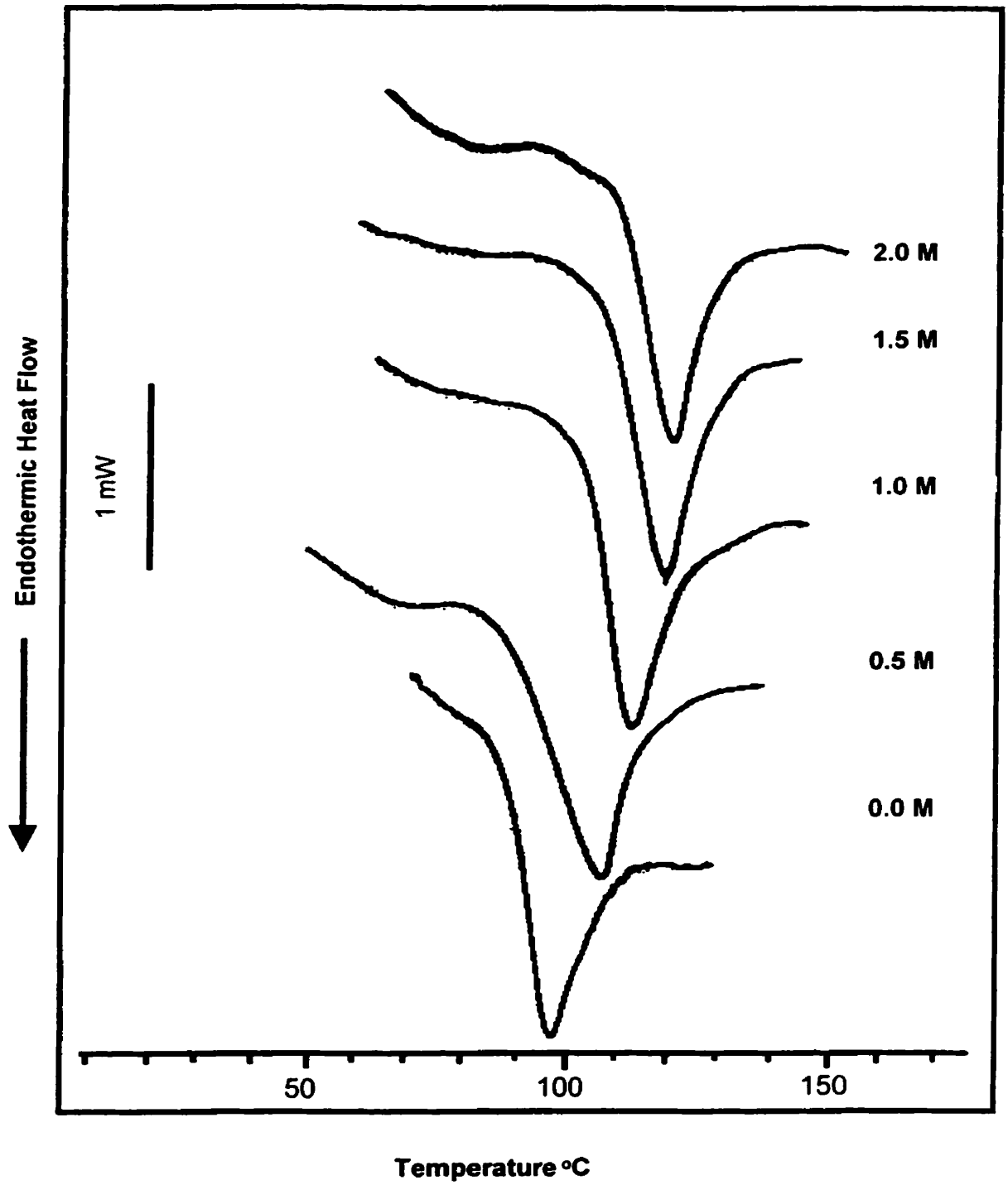


Figure 6.9: Effect of NaCl concentration on T_d of NaOH-CP

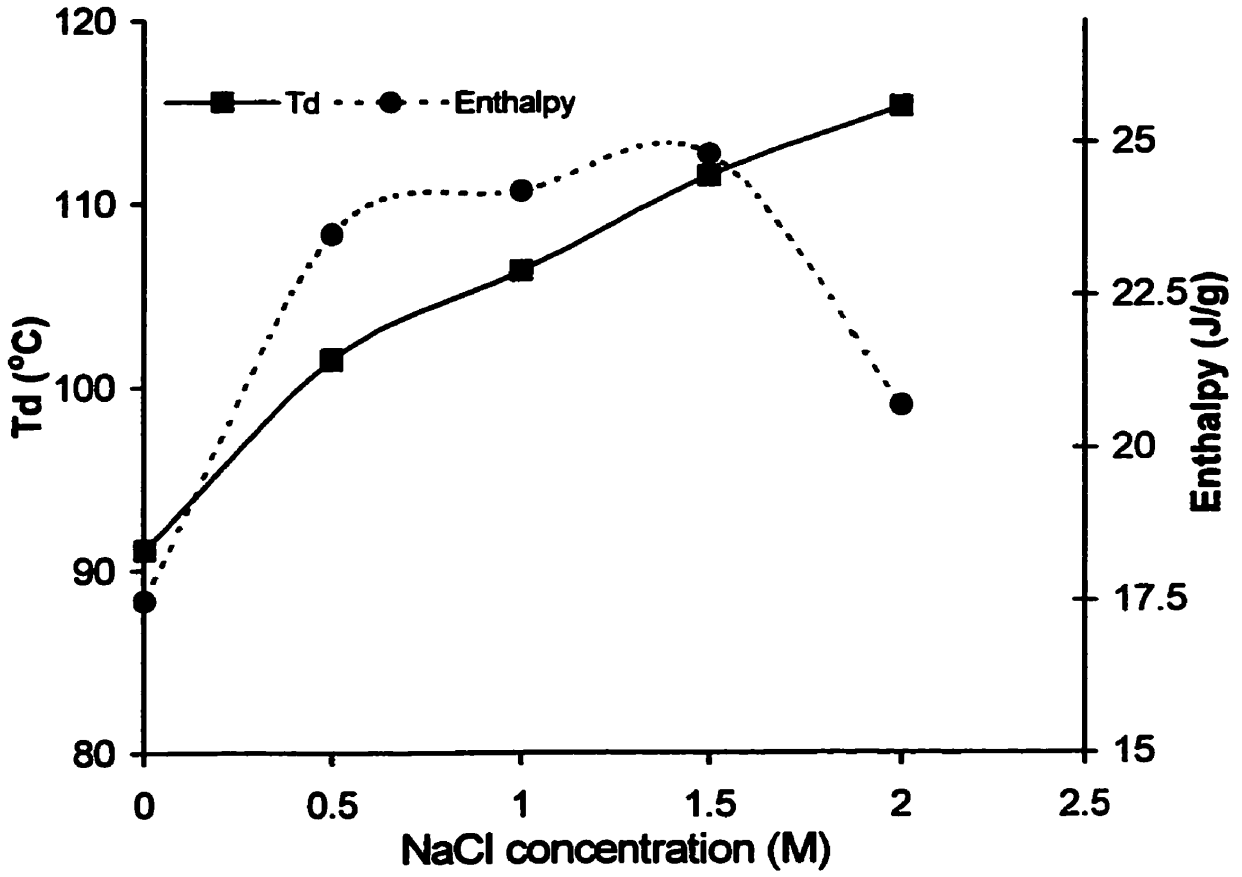


Figure 6.10: Effect of NaCl concentration on T_d and ΔH of NaOH-CP

Figure 6.11 shows the thermograms of NaOH-CP in the presence of different concentrations of $CaCl_2$. The T_d of NaOH-CP increased from 91.1 °C (no $CaCl_2$) to 100.8 °C in the presence of 0.5 M $CaCl_2$. T_d was relatively unchanged from 0.5 to 1.5 M $CaCl_2$ then T_d decreased to 99.2 °C at 2.0 M $CaCl_2$. ΔH decreased with increasing $CaCl_2$ concentration from 22.7 J / g at 0.5 M $CaCl_2$ to 17.9 J / g at 2.0 M $CaCl_2$ (Figure 6.12). Harwalker and Ma (1989) reported that $CaCl_2$ concentration from 0.1 to 0.5 M increased T_d of β–lactoglobulin from 83.5 ° to 85 °C while 1 M $CaCl_2$ concentration decreased the T_d to 84.6 °C; they attributed that decrease at 1 M $CaCl_2$ concentration to a reduction in energy required to transfer apolar groups to water, thereby weakening intramolecular hydrophobic interactions and enhancing protein unfolding which resulted in decreases in both T_d and ΔH. The balance between polar and non-polar residues affect the thermal stability of the protein; the higher proportion of non-polar residues, the higher is the stability to heat (Bigilow 1967, Damodaran 1988).

6.2.6 Effect of Reducing Agents on Thermal Properties

6.2.6.1 Effect of SDS

T_d was decreased from 91.4 °C to 86 °C when the SDS concentration was increased from 50 to 150 mM (Figure 6.13), while ΔH was increased

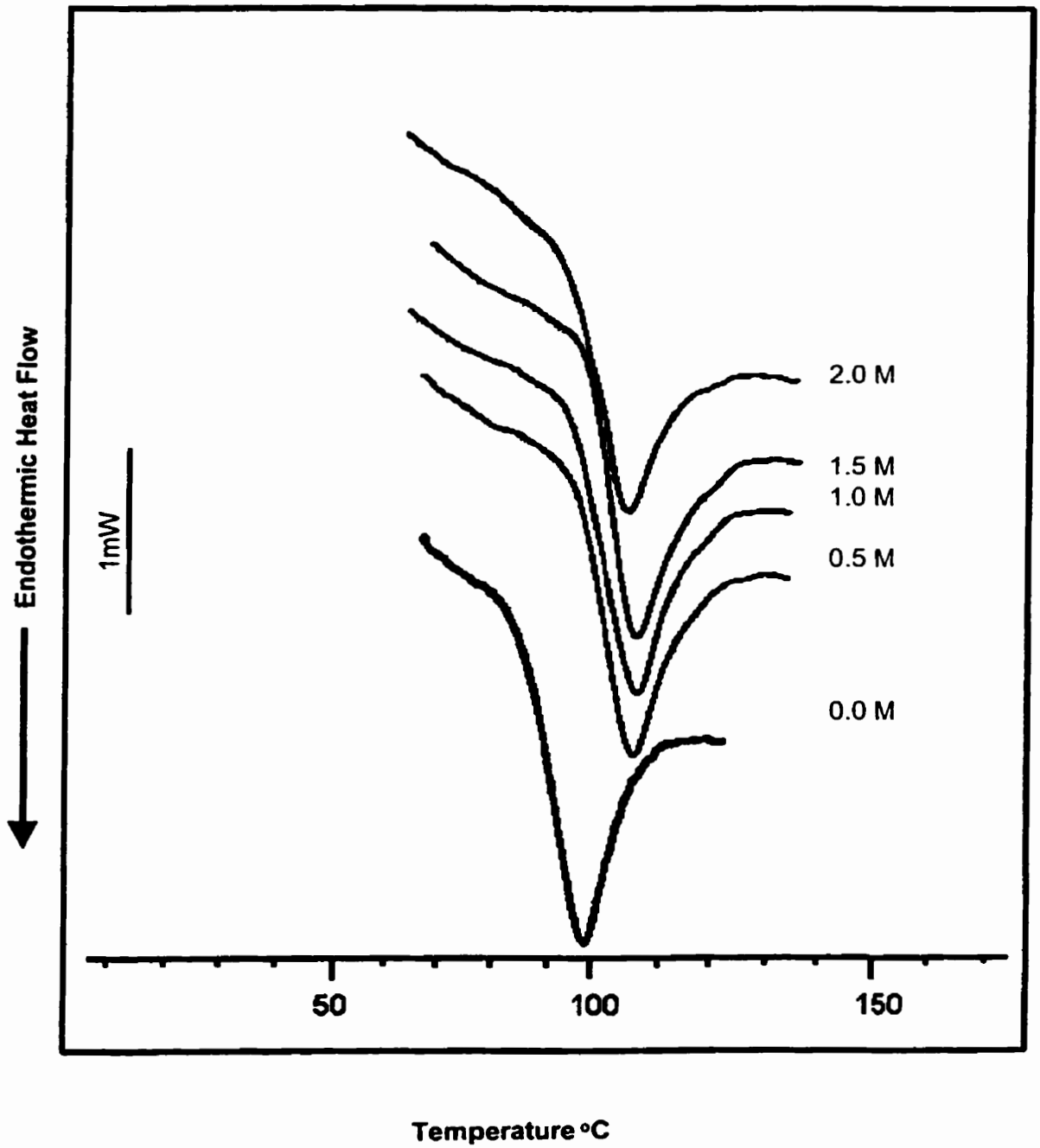


Figure 6.11: Effect of $CaCl_2$ concentration on T_d of NaOH-CP

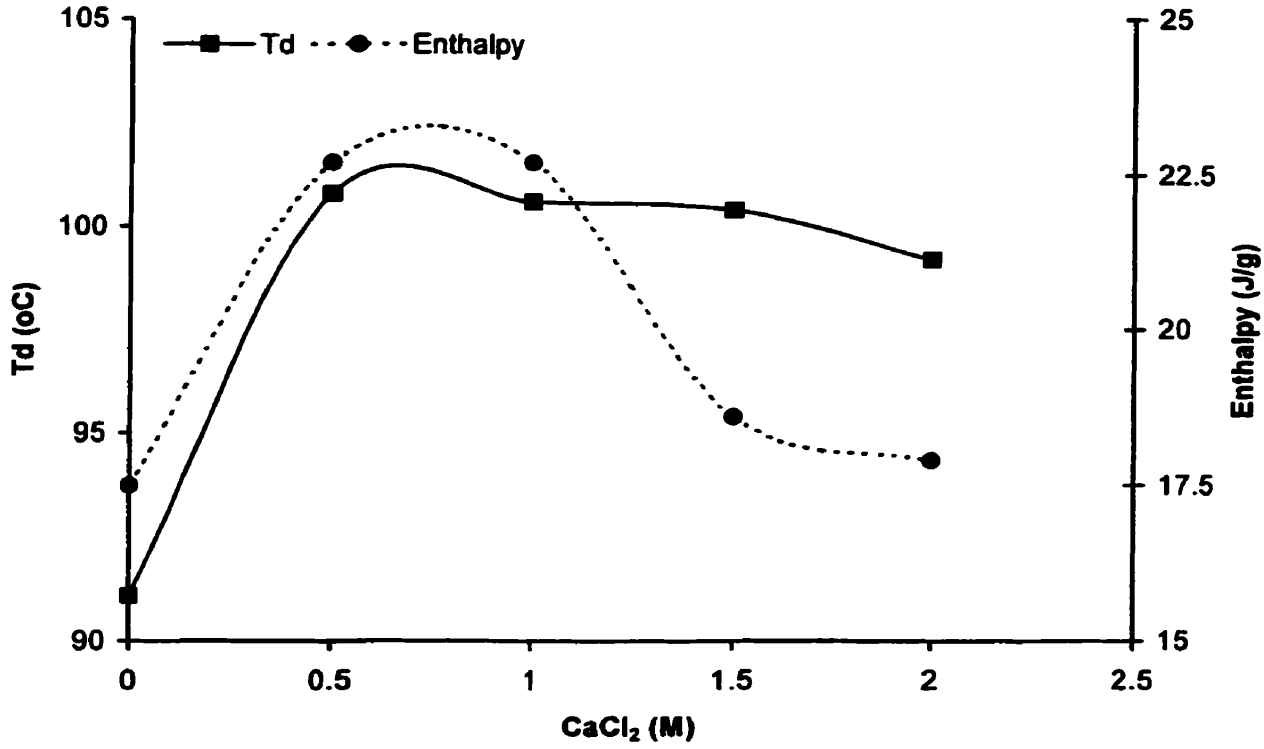


Figure 6.12: Effect of $CaCl_2$ concentration on T_d and ΔH of NaOH-CP

from 17.5 J/g to 21.8 J/g in the presence of 50 mM SDS (Figure 6.14). Harwalkar and Ma (1987) observed similar effects of SDS on oat globulin dispersion (10%), they suggested that SDS, at low concentration can form a bridge between the cationic groups of protein and the ionic groups of SDS, which stabilize the protein structure against denaturation. The SDS hydrophobic chains can be transferred to the interior of the protein molecules leading to formation of additional hydrogen bonds and consequently increased ΔH (Tanford, 1970; Hegg and Lofqvist 1974; Harwalker and Ma 1987). Increasing SDS concentration from 50 mM to 150 mM resulted in a decrease of ΔH from 21.8 J/g to 7.7 J/g (Figure 6.14). The decrease in ΔH at higher concentrations of SDS, indicates weaker hydrophobic interactions in the protein prior to thermal denaturation, aggregation and disruption of hydrophobic interactions which are both exothermic reactions (Myers 1990). Steinhardt (1975) reported that the decrease of ΔH at high concentration of SDS is due to non-specific binding of the detergent to the protein which is reflected in protein denaturation.

6.2.6.2 Effect of 2-mercaptoethanol

In the presence of 0.1 % of 2-mercaptoethanol, an endothermic peak with denaturation temperature 87.3 ^{o}C was observed, along with a small shoulder at

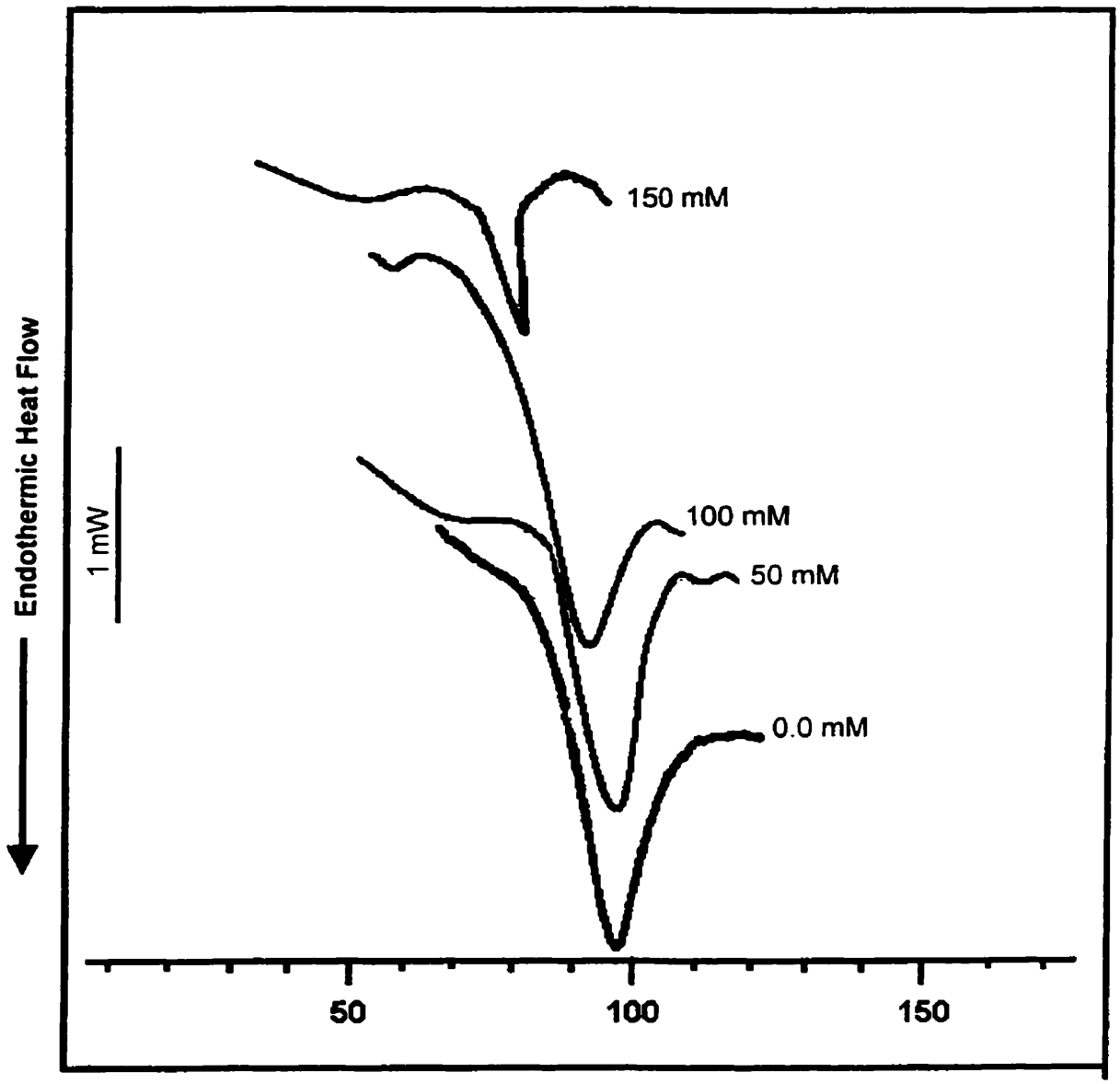


Figure 6.13: Effect of SDS concentration on T_d of NaOH-CP

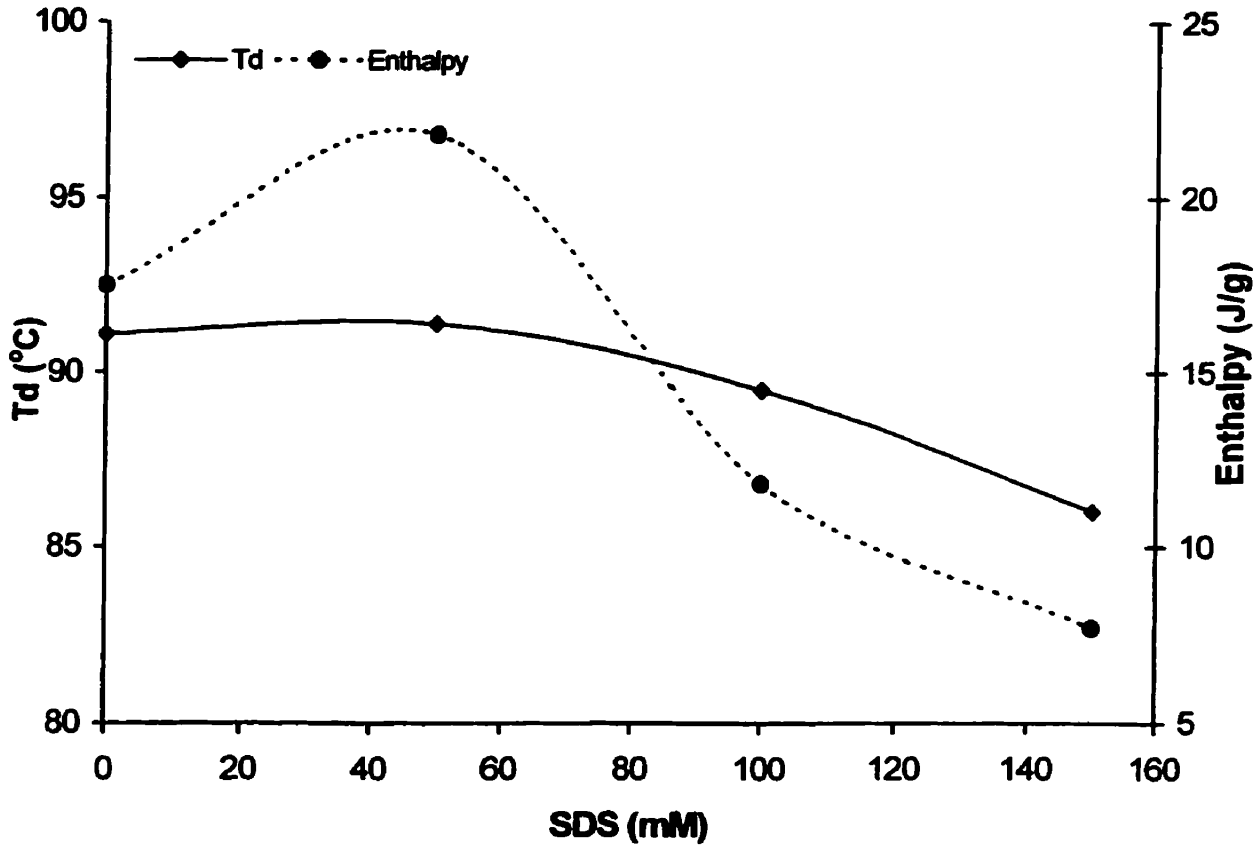


Figure 6.14: Effect of SDS concentration on T_d and ΔH of NaOH-CP

63.2 °C (Figure 6.15); this shoulder increased to a well defined peak when the concentration of 2-mercaptoethanol was increased to 0.5 %. At 1.0 % 2-mercaptoethanol concentration, only one endothermic peak was observed. The changes in thermal properties of NaOH-CP at different concentration of 2-mercaptoethanol might be attributed to the two types of disulfide bonds in glycinin. Catsimpoolas and Meyer (1970) reported that low concentrations of 2-mercaptoethanol reduced only intersubuint disulfide bonds, but at high concentrations of 2-mercaptoethanol both inter and intermolecular disulfide bonds may be reduced, which would facilitate complete unfolding of the protein. Utsumi and Kinsella (1985) reported that at low concentration of 2-mercaptoethanol, glycinin dissociated into basic (insoluble) subunits and acidic (soluble) subunits. SDS-PAGE showed that glycinin heated at 80 °C in the presence of low concentration of 2-mercaptoethanol, revealed that it contained only the basic subunits and that the supernatant contained only the acidic subunits (Damodaran and Kinsella 1982). Using DSC, Zarins and Marshall (1990) observed only one endothermic peak when glycinin was heated in the presence of 1 % to 10 % of 2-mercaptoethanol.

The effects of cysteine was similar to those of 2-mercaptoethanol at 50 to 150 mM cysteine (Figure 6.16). At 50 mM cysteine, a large endothermic peak (91 °C) and a small shoulder were observed. Increasing cysteine concentration

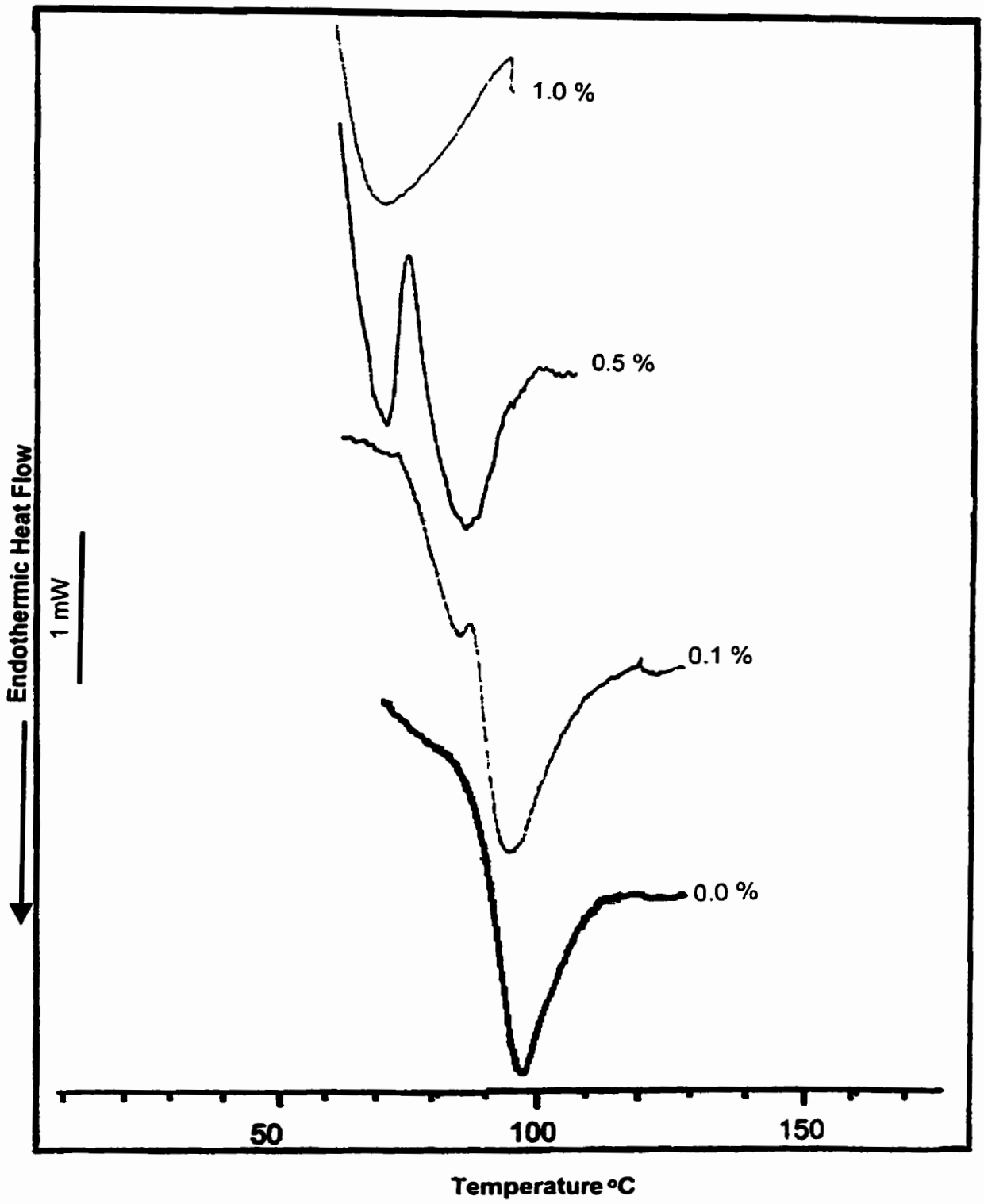


Figure 6.15: Effect of 2-mercaptoethanol concentration on T_d of NaOH-CP

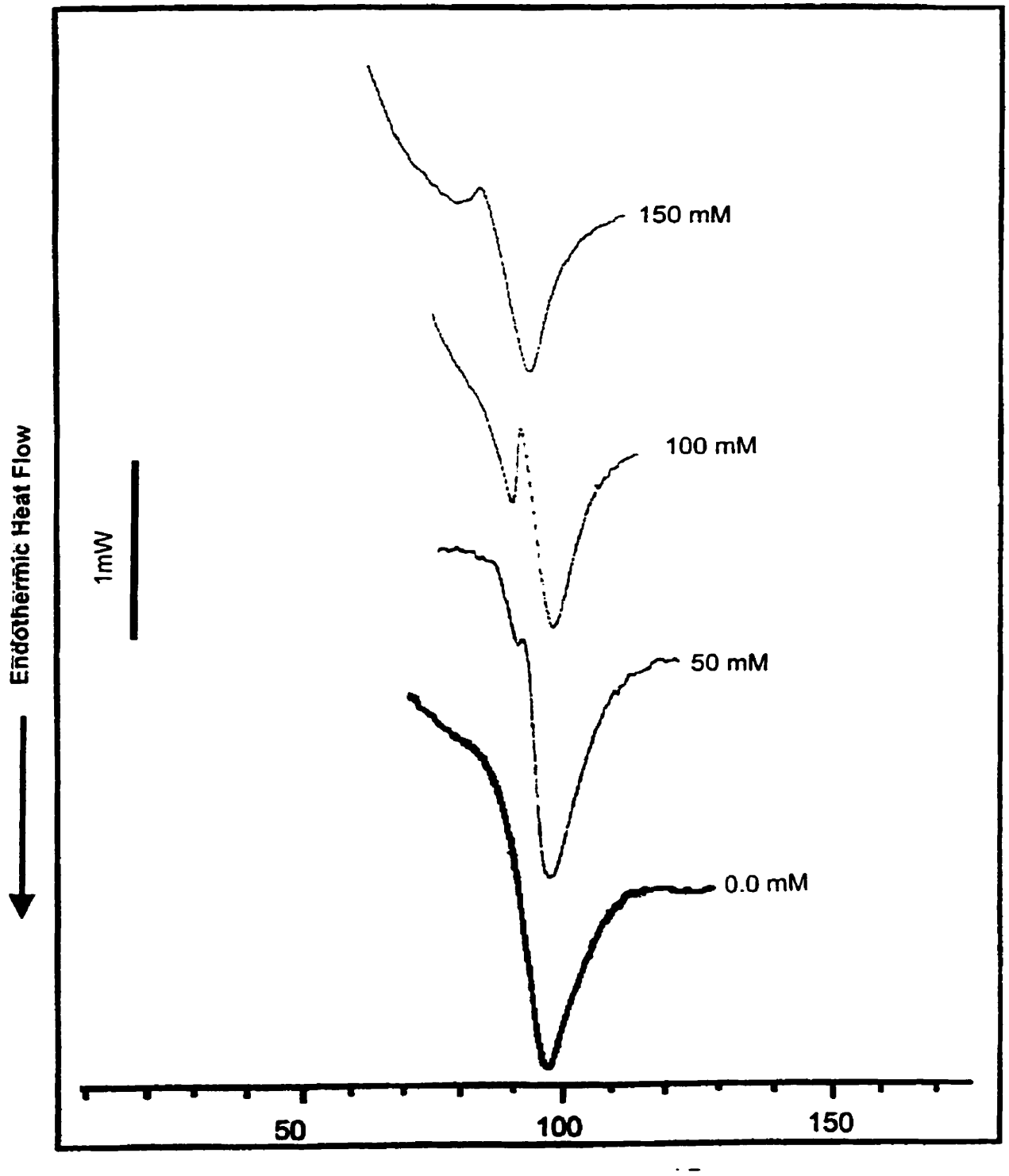


Figure 6.16: Effect of cysteine concentration on T_d of NaOH-CP

to 100 mM led to an increase in the intensity of this shoulder with denaturation temperature 83.2 °C; increasing the cysteine concentration to 150 mM led to decreased the intensity of this peak. Circle *et al.* (1964) reported that low concentrations of cysteine (0.05 %) inhibited gelation of soy isolate due to the breakdown of only the interdisulfide bonds, but cysteine at 0.5 % concentration, had no effect on gelation due to breakdown of both disulfide bonds (inter and intradisulfide bonds) which led to complete unfolding of the protein. The T_d of NaOH-CP in the presence of 2-mercaptoethanol was much lower than in the presence of cysteine. The difference in the thermal stability of NaOH-CP in the presence of 2-mercaptoethanol and cysteine could be attributed to the fact that 2-mercaptoethanol has two effects, one to break down the disulfide bond and the second to act as an alcohol; monohydric alcohol's promote destabilization of proteins by weaking hydrophobic interactions (Harwalker and Ma 1987).

ΔH decreased from 17.5 J/g to 9.1 J / g at 0.5 % 2-mercaptoethanol (Figure 6.17); ΔH was not calculated in the presence of 1 % 2-MeSH due to difficulty to obtain an accurate baseline; Zarins and Marshall (1990) reported ΔH values in the presence of 3 to 10 % 2-mercaptoethanol but not for presence of 1 and 2 % 2-mercaptoethanol due to difficulty on achieving an accurate baseline.

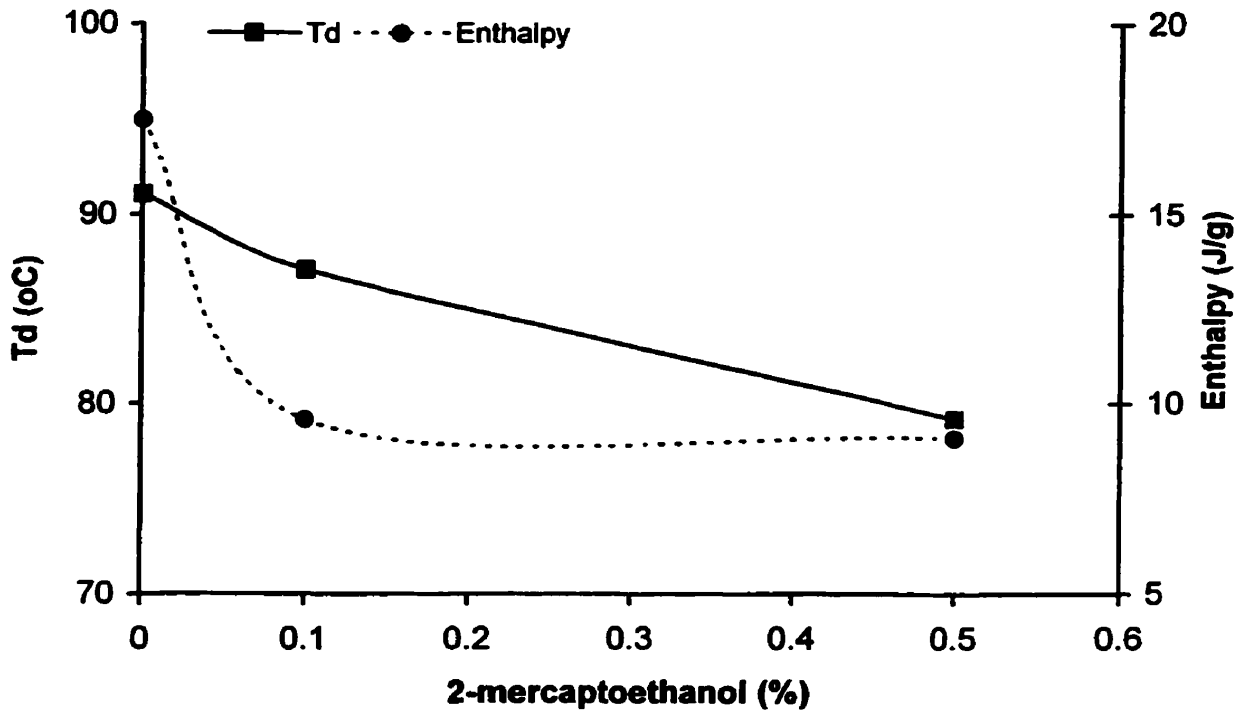


Figure 6.17: Effect of 2-mercaptoethanol concentration on T_d and ΔH of NaOH-CP

6.2.6.3 Effect of Urea

T_d in the presence of 1 M urea increased only slightly from 91.1 °C to 92.2 °C while ΔH decreased from 17.5 J/g to 13.8 J/g in the presence of the same concentration (Figure 6.18). The increase in T_d may be due to nonspecific ion effects caused by urea. Similarly, the decrease in the ΔH at the same concentration of urea may be due to the same factors responsible for a similar behavior in the presence of NaCl in (Section 6.2.5) were observed in the presence of 2 M NaCl increase of T_d and decrease of ΔH. These findings were similar to that observed for 12S canola globulin in the presence of low concentration (0.1 M) of guanidine hydrochloride (Lèger and Arntfield 1993), they attributed this effect to the capability of guanidine hydrochloride at low concentration, to exert a salt-inducing effect on proteins. Franks and Eagland (1975) reported that urea and guanidine hydrochloride have similar mechanism of action on proteins.

In the presence of 2 M urea, both T_d and ΔH decreased (Figure 6.19). Lèger and Arntifield (1993) also reported that Td and ΔH were decreased when the guanidine hydrochloride concentration was increased from 0.1 to 1.0 M. Phillips *et al.* (1994) reported that urea and guanidine hydrochloride, as denaturing agents, induce the unfolding of proteins by altering protein-protein and protein-solvent interactions. Urea also increases the dielectric permitivity

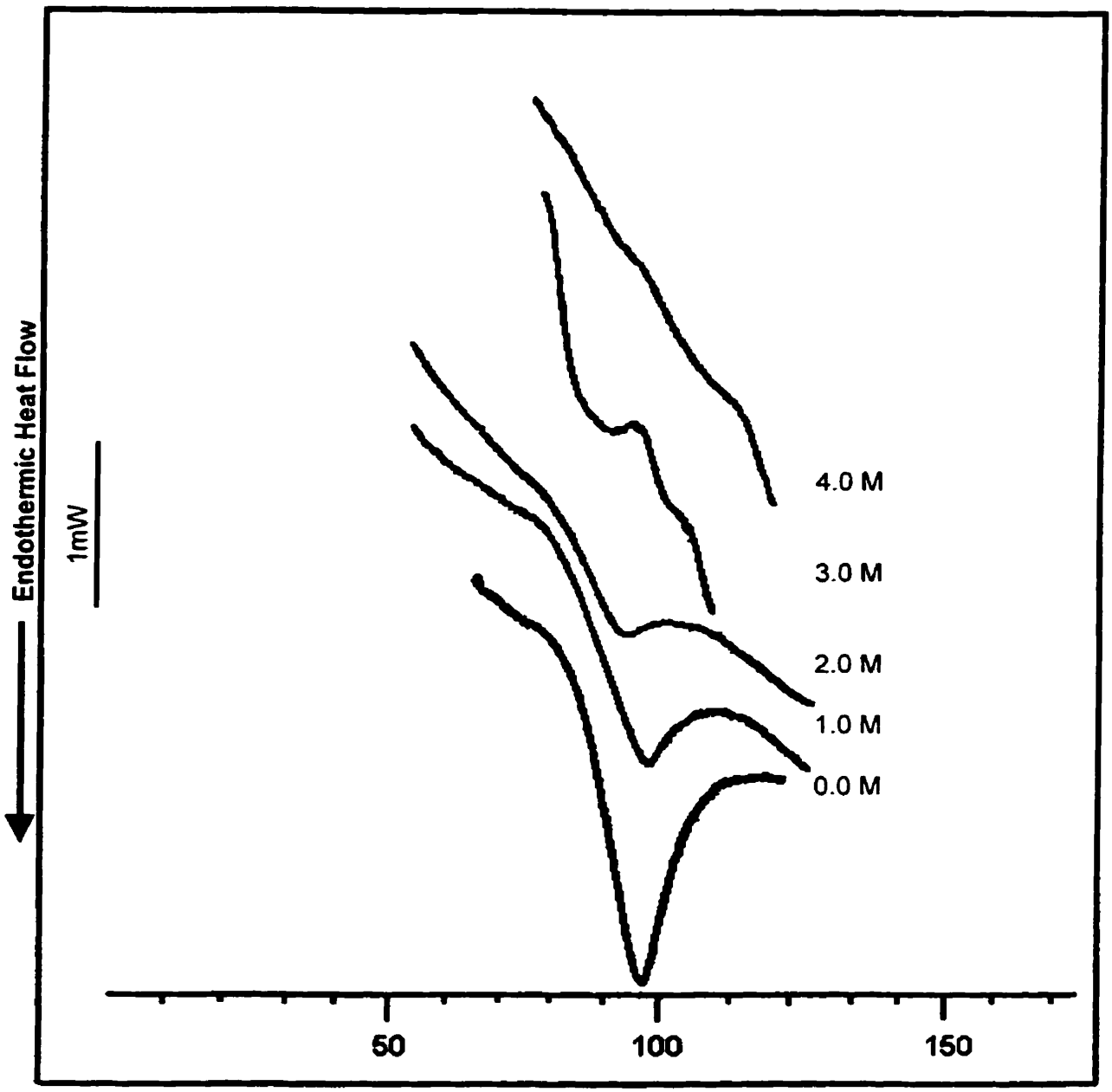


Figure 6.17: Effect of urea concentration on T_d of NaOH-CP

of water for apolar residues causing loss of protein structure and heat stability (Franks and England 1975) in hydrogen-bonded structure of water, thereby weakening hydrophobic interactions and facilitating protein unfolding. No T_d was detectable at 3 to 4 M urea; it is likely indicate that the structure of NaOH-CP was completely denaturated at these high concentrations of urea. The absence of Td in the presence of high concentrations of urea has been observed for oat globulin (Harwalkar and Ma 1987) and 12S canola globulin (Lèger and Arntfield 1993).

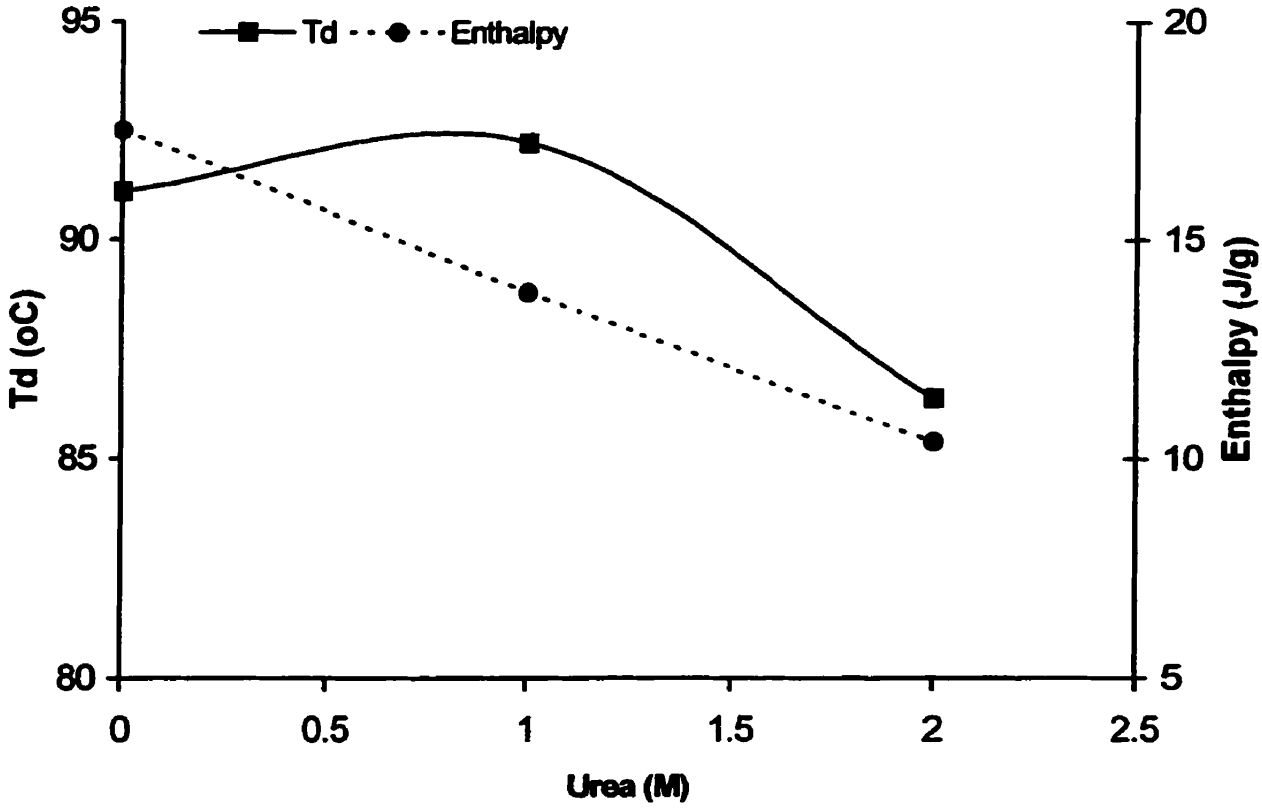


Figure 6.19: Effect of urea concentration on T_d and ΔH of NaOH-CP

GENERAL CONCLUSIONS

The results from this study suggest that cryoprecipitation from NaOH extracts and citric acid extracts can give homogeneous proteins depending on the extraction conditions. Using this technique, mainly glycinin-rich protein (NaOH-CP) can be obtained from NaOH extraction / cryoprecipitation, while mainly β-conglycinin can be obtained from citric acid extraction / cryoprecipitaion. PAGE, SDS-PAGE, SEC and DSC confirmed the homogeneity of NaOH-CP obtained by cryoprecipitation.

Temperature and pH appear to be the main factors affecting the cryoprecipitation mechanism. The yield of NaOH-CP increased with increasing temperature from 21 °C to 60 °C during the extraction; during cryoprecipitation the maximum yield was obtained after 18 h of cooling at 4 °C. The NaOH-CP could be obtained using a recycling extraction technique.

The glycinin-rich protein (NaOH-CP) was investigated for its gelling characteristics. The minimum protein concentration of NaOH-CP to form heat-induced gels was 8 %. Increasing protein concentration, heating temperature, heating time and pH (7-10) enhanced the gel formation. In cold-set gelation, increasing $CaCl_2$ concentration and protein concentration resulted with increasing the gel strength of NaOH-CP. In cold-set gelatoin, 10 % of NaOH-CP protein gave a clear white gel which had a gel strength similar to that obtained by thermal gelation of 16 % of protein concentration.

Variable-temperature FTIR spectroscopy revealed that unfolding of NaOH-CP with increasing temperature led to loss of the secondary structure and the formation of irreversible intermolecular hydrogen-bonded β–sheet structures. Increasing NaCl concentration stabilized the NaOH-CP against high temperature induced denaturation. Heating NaOH-CP in the presence of 100 mM SDS resulted in a decrease in α–helical and intramolecular β–sheet structures in favor of an increase in unordered structure and the formation of a small amount of intermolecluar β–sheet, while significant amounts of intermolecular β–sheet were formed in the presence of 2-mercaptoethanol. This suggests that disulfide bonds are not necessary in the aggregation step, but might be responsible for the increase the chain length of the polypeptide and may stabilize the gel network through the formation of the crosslinks between the aggregates.

The thermal stability of NaOH-CP was increased by increasing NaCl concentration from 0.5 to 2 M while there was no practical change in the thermal stability over a similar concentration of $CaCl_2$.

High concentrations of SDS (100 to 150mM SDS) slightly decreased thermal stability and markedly decreased enthalpy possibly due to non-specific detergent binding with NaOH-CP. 2-mercaptoethanol decreased both thermal stability and enthalpy whereas cysteine slightly affected the thermal stability of NaOH-CP. Glycinin structure was completely destroyed using urea at 3 M.

CLAIMS OF ORIGINAL RESEARCH

1) This is the first study to demonstrate that separate acid extraction and alkaline extraction followed by cryoprecipitation of the two extracts result in a β–conglycinin-rich protein and a glycinin-rich protein respectively from defatted soybean.

2) This is the first study that developed a recycling extraction procedure for preparing a glycinin-rich protein as a primary product and isoelectric precipitated protein as a secondary product from defatted soybean.

3) This is the first study to investigate the cold-set gelation characteristics of a cryoprecipitated glycinin protein and to compare these cold–set gelling characteristics with those of the thermally-induced cryoprecipitated protein.

4) This is the first study to demonstrate the differences between SEM microstructural characteristics of thermally-induced gel and cold-set gel of a cryoprecipitated glycinin protein.

5) This is the first study to use DSC and FTIR to study the structural characteristics of a cryoprecipitated glycinin protein and to show that

differences in the effects of temperature on the secondary structure of glycinin in the presence of reducing agents.

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