ISOLATION AND CHARACTERIZATION OF PROTEINS FROM CHICKPEA (*Cicer arietinum L.*) SEEDS

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ABSTRACT

Chickpea (*Cicer arietinum L.*) seed is a potential source of protein ingredients with desirable nutritional and functional properties. Knowledge of molecular characteristics of a food protein is essential before a protein can gain widespread use as a food ingredient. The objectives of this study were to prepare chickpea proteins using different extraction methods and precipitation methods and to investigate molecular characteristics using polyacrylamide gel electrophoresis (PAGE; Native and SDS), reversed phase high performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS) techniques. Proteins of ground chickpea seed were extracted with sodium hydroxide (NaOH) and with citric acid solutions and precipitated with addition of acid and by cryoprecipitation. The protein contents of the protein preparation ranged from 49% to 97%. The microstructures of chickpea protein isolates examined by scanning electron microscope (SEM) revealed the presence of starch grains in the cryoprecipitates from citric acid extraction but not in isoelectric precipitates. The globulins (legumins and vicilins), glutelins, and albumins from both citric acid and NaOH isolates were characterized by Native-PAGE. The cryoprecipitates contained mainly the globulin-rich proteins. With SDS-PAGE characterization, protein subunits were identified as follows: (i) legumin subunits: MW 40, 39, 26, 23, and 22 kDa , (ii) vicilin subunits: MW 50, 37, 33, 19, and 15 kDa, (iii) glutelin subunits: 58, 55, and 54 kDa, and (iv) albumin subunits: 10 kDa. Separation of fractions of isolated chickpea proteins by RP-HPLC showed that early eluting fractions (Rt 20-30 min) consisted of subunits of MW 6.5-31 kDa (SDS-PAGE). At elution time 30-36 min, the fractions obtained were composed mainly of mixtures of legumin and vicilin subunits (MW 14-45

kDa). The major subunits of chickpea protein fractions from both cryoprecipitates and isoelectric precipitates are legumin basic subunit (MW~ 23 kDa) and vicilin-rich proteins (MW~19, 17, 15 kDa). ESI-MS analysis of fractions separated by RP-HPLC showed MW ranging between 5.1 and 53.5 kDa. The subunits of MW 35366, 27626, 22864, 20531, 16092, and 15626 Da of fractions from ESI-MS corresponded to MW 35.3, 28.0, 24.1, 20.5, 16.1, and 15.3 kDa identified in SDS-PAGE. These fractions were identified as legumin-rich and vicilin-rich proteins.

RÉSUMÉ

La graine de pois chiche (*Cicer arietinum L.*) est une source potentielle d'ingrédients protéiques avec des propriétés alimentaires et fonctionnelles d`un grand intérêt. La connaissance des caractéristiques moléculaires d'une protéine alimentaire est un atout important avant qu'une protéine puisse gagner une utilisation répandue comme ingrédient alimentaire. Les objectifs de cette étude étaient de préparer des extraits protéiques de pois chiche, en utilisant différentes méthodes d'extraction et de précipitation, et d'étudier ses caractéristiques moléculaires par électrophorèse sur un gel de polyacrylamide (PAGE ; Native et SDS), par chromatographie liquide à haute performance à phase inverse (RP-HPLC) ainsi que par spectrophotométrie de masse à ionisation (ESI-MS). Les protéines de la graine moulue de pois chiche ont été extraites avec une solution d'hydroxyde de sodium (NaOH) et d`acide citrique, suivie d`une précipitation par un acide et une cryoprécipitation. La proportion en protéines dans l`extrait protéique varie entre 49% et 97%. L`analyse des microstructures des isolats protéiques de pois chiche par un microscope électronique à balayage (SEM) révèle la présence des grains d'amidon dans les cryoprécipités obtenus par extraction avec l`acide citrique mais pas dans les précipités isoélectriques. Les globulines (légumins et vicilines), glutélines et albumines, obtenues par extraction avec l'acide citrique et avec l'hydroxyde de sodium, ont été caractérisées par électrophorèse Native-PAGE. Les cryoprécipités contenaient principalement les protéines riches en globulines. Suite à une caractérisation par électrophorèse SDS-PAGE, les sous-unités protéiques ont été identifiées comme suite : (i) sous-unités de légumins: poids moléculaire (PM) 40, 39, 26, 23 et 22 kDa, (ii) sous-unités de vicilines: PM 50, 37, 33, 19 et 15 kDa, (iii) sous-unités de glutélines: 58, 55 et

54 kDa et (iv) sous-unités d'albumines: 10 kDa. La séparation des fractions protéiques de pois chiche par RP-HPLC a montré que les fractions éluées en premier (Temps d'élution 20-30 minutes) sont les sous-unités de PM de 6.5-31 kDa (SDS-PAGE). A un temps d'élution de 30-36 minutes, les fractions éluées sont principalement un mélange de sous-unités de légumins et de vicilines (PM 14-45 kDa). Les sous-unités majoritaires des fractions protéiques de pois chiche, obtenues par une cryoprécipitation et par une précipitation isoélectrique, sont des sous-unités de légumins (PM~ 23 kDa) et des protéines riches en vicilines (PM~19, 17 and 15 kDa). L'analyse par ESI-MS des fractions séparées par RP-HPLC montre que leur PM varient entre 5.1 et 53.5 kDa. Les sous-unités de PM 35366, 27626, 22864, 20531, 16092 et 15626 Da obtenues par ESI-MS correspondent a ceux de PM de 35.3, 28.0, 24.1, 20.5, 16.1 et 15.3 kDa identifiées par électrophorèse SDS-PAGE. Ces fractions ont été identifiées comme étant des protéines riches en légumins et en vicilines.

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Abbreviations

AAFC	Agriculture and Agri-Food Canada
AF	Fractions of C. C-E collected from RP-HPLC
C. C -CP	Chickpea / citric acid extraction / cryoprecipitaiton precipitate
C. C-E	Chickpea / citric acid extraction / extract
C. C-S	Chickpea / citric acid extraction / supernatant from cryoprecipitaiton
CF	Fractions of C. C-CP collected from RP-HPLC
C. Na-CP	Chickpea / NaOH extraction / cryoprecipitate
C. Na-CIP	Chickpea / NaOH extraction / isoprecipitate after cryoprecipitaiton
C. Na-E	Chickpea / NaOH extraction / extract
C. Na-IP	Chickpea / NaOH extraction / isoelectric precipitate
DF	Fractions of C. Na-E collected from RP-HPLC
DLA-IP	Diluted alkaline chickpea isoprecipitate
EF	Fractions of C. Na-IP collected from RP-HPLC
ESI-MS	Electrospray Ionization-Mass spectrometry
FF	Fractions of C. Na-CIP collected from RP-HPLC
GF	Fractions of C. Na-CP collected from RP-HPLC
HF	Fractions of DLA-IP isolate
kDa	Kilodalton
MW	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
RP-HPLC	Reverse phase high performance liquid chromatography
Rt	Retention time
S. C -CP	Soybean / citric acid extraction / cryoprecipitaiton precipitate
S. C-E	Soybean / citric acid extraction / extract
S. C-S	Soybean / citric acid extraction / supernatant from cryoprecipitaiton
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope

S. Na-CP	Soybean / NaOH extraction / cryoprecipitaiton precipitate
S. Na-CIP	Soybean / NaOH extraction / isoprecipitate after cryoprecipitaiton
S. Na-E	Soybean / NaOH extraction / extract
S. Na-IP	Soybean / NaOH extraction / isoelectric precipitate
T. O. F.	Time of flight

CHAPTER 1

INTRODUCTION

Chickpea (*Cicer arietinum L.*) is an ancient crop, first grown in Turkey about 7,450 B.C. and in India about 4,000 B.C. (Singh, 1997). The production of chickpea ranks third in the world, and first in the Mediterranean basin, among pulse crops (Singh and Ocampo, 1997); world production yielded 7.1 million tonnes in 2003 with India contributing a major share (58%) of this production, followed by Pakistan (9.8%) and Turkey (9%) (FAOSTAT, 2004; Maiti, 2001). By comparison, soybean is the most commonly used plant protein in the world and ranks first in volume of production (Smartt, 1990). However, soybean proteins are food allergens and a large portion of the world's soybeans have been genetically modified (Anonymous, 2004). Consequently, there is a need to find alternative sources of plant proteins. A large proportion of the world protein supply is obtained from plant sources like cereals, legumes and oilseeds. These plant protein sources are relatively inexpensive and there is a growing demand for their use in the food industry. As a result of incremental applications of plant proteins in food and non-food markets, the production of plant protein isolates is a growing industry. The European Union is eager to develop its own protein crops in order to reduce commercial dependence on soybean proteins (Chominot, 1992). Friedamn (1996) indicated that chickpea protein quality is equivalent to that of soybean meal. The more important value of chickpea seed is its relatively high protein content. Therefore there is a growing market in North American for high-quality, large-type (Kabuli) chickpeas (Maiti, 2001).

Recently, the need of chickpea has been increasing due to its potential source of high protein content (25.3~28.9%) with high protein digestibility (76~78%). In addition, it is

abundant in linoleic acid (40% in chickpea oil), fiber, vitamins and minerals (Hulse, 1991; Duke, 1981). Furthermore Sotelo et al. (1987) found that chickpea contains very low content of trypsin inhibitors.

There have been several studies (Cai, 2001; Paredes-Lopez et al., 1991; Sanchez-Vioque et al., 1999) on the comparison of chickpea protein isolate; however there is relatively little information on the molecular characteristics of chickpea proteins. This information is essential for understanding of the various uses and applications of chickpea proteins as a food ingredient.

The overall objective of this research is to isolate and characterize proteins from chickpea seed. The specific objectives are to (1) isolate proteins from chickpea seed by aqueous solvent extraction and precipitation techniques, (2) separate and characterize particular fractions of the protein by using reverse-phase high performance liquid chromatography, (3) characterize individual fractions by electrophoresis and (4) identify the molecular weights of proteins and subunits of chickpea isolates by electrophoresis and electrospray ionization mass spectrometry (ESI-MS).

CHAPTER 2

LITERATURE REVIEW

2.1 Botanical Characteristic

Chickpea (*Cicer arietinum L.*), is a member of the family Fabaceae (or Leguminosae), and is widely grown in tropical, subtropical, and temperate regions. The common names for chickpea are bengal gram (Indian), garbanzo (Latin America), hommes and hamaz (Arab world), nohud and lablabi (Turkey), and shimbra (Ethiopia) (Muehlbauer and Abebe Tullu, 1997). Two categories of chickpea are recognized, desi (colored, small seeded, angular and fibrous) and kabuli (beige, large seeded, rams-head shaped with lower fiber content) types. The Kabuli type (Mediterranean and Middle Eastern origin) is grown in temperate regions while the desi type (Indian origin) chickpea is grown in the semi-arid tropics (Malhotra et al., 1987; Muehlbauer and Singh, 1987).

The chickpea seed is developed from an ovule after fertilization and contains the embryo, endosperm, and the protective layer, the testa (Smith, 1984). Approximately 1000 seeds are equivalent to 259.6g by weight (Cai et al., 2001). The seed coat contributes 14.5 to 16.4% of the seed weight. The cotyledons contribute 82.9 to 84% and germ contributes 1.2 to 1.5 % of the seed weight. (Chavan et al., 1989)

2.2 Uses and Applications

The chickpea seed is considered as a nutritious food and it is a common source of vegetarian diets. Generally, it is not only a feature of Asian (especially in India), Mediterranean and Mexican cuisines but also is popular in salads and may be canned in

brine or used to produce fermented food (Sotelo and Adsule, 1996).

Chickpea seeds are processed into a variety of products before consumption. Normally, dehusking into "dhal" followed by milling is the most prevalent processing of chickpea flour. Despite the use of chickpea flour to prepare numerous traditional products, it also has been widely used to manufacture enfant weaning foods and bakery products (Chavan et al., 1989). In Chile, a cooked chickpea-milk (4:1) mixture is used for feeding infants, effectively controlling diarrhea. In many developing countries, the chickpeas are usually used to feed animals (Duke, 1981).

In medical uses, chickpea has the potential to be a hycholesteremic agent since as germinated chickpea was shown to be effective in controlling cholesterol levels in rats (Geervani, 1991). As a result of its high carbohydrate content (60%), chickpea is a source of starch (21%); and is suitable for textile sizing, giving a light finish to silk, wool, and cotton cloth (Duke, 1981).

2.3 Gross Composition

As with most other legumes, the cotyledon is a major part of chickpea seed and contains about 96% of the proteins. Although the embryo is abundant in protein, fat, and minerals, its contribution is much less than the cotyledons due to the minor proportion of the total seed weight. The seed coat comprises mainly of nondigestible carbohydrate and relatively high proportion of calcium (Esh, 1959).

Chickpea seed contains 38-59% carbohydrate, 14.9-24.6% protein, 2-11.7% fiber, 0.8-6.4% lipids, 2-4.8% ash and 0.14-0.44% calcium (Duke, 1981; Huisman and van der Poel, 1994). The composition of raw whole seeds is given in Table 2.1.

Table 2.1: Chickpea seed composition.

Constituent	Content (/100g seeds)
Proximate	
Food Energy, cal	357
Protein (g)	14.9-24.6
Total lipids (g)	0.8-6.4
Carbohydrate (g)	38-59[A]; 50.6-70.9[B]
Fiber (g)	2.1-11.7
Ash (g)	2.0-4.8
Moisture (g)	4.5-15.6
Minerals	
Calcium (mg)	140-440
Phosphorous (mg)	190-382
Iron (mg)	5.0-23.9
Vitamins	
β-carotene (μg)	0-225
Thiamine (mg)	0.21-1.1
Riboflavin (mg)	0.12-0.33
Niacin (mg)	1.3-2.9

Compiled form A: Duke, 1981; B: Chavan et al., 1989; Huisman and van der Poel, 1994.

2.3.1 Chickpea Oil

Chickpea is high in monounsaturated and polyunsaturated acids, and low in saturated fatty acids (Cai et al., 2001).Table 2.2 shows the fatty acid composition of the oil.

Table 2.2: Fatty acid composition of chickpea (Kabuli type) seed oil.

Fatty acid		**Abundance in oil (%)**	
Myristic acid	(C14:0)	2.3[A]	-
Palmitic acid	(C16:0)	5.1[A]	9.4[B]
Stearic acid	(C18:0)	2.1[A]	1.5[B]
Arachidic acid	(C20:0)	0.1[A]	-
Oleic acid	(C18:1)	50.3[A]	42.0[B]
Linoleic acid	(C18:2)	40.0[A]	43.6[B]
Linolenic	(C18:3)	-	1.8[B]

[A] Duke, 1981; [B] Cai et al., 2001.

The composition and quality of chickpea lipids can be influenced by differences in cultivars and environmental factors such as soil conditions, temperature, water, fertilizers and diseases (Maiti et al., 2001).

2.3.2 Chickpea Fibre

Although the carbohydrate composition is the major component of chickpea seed (50.6-70.9%) (Chavan et al., 1989), the seed is also a good source of crude fiber content. Table 2.3 illustrates the carbohydrate and fiber composition of chickpea seeds.

Table 2.3: Carbohydrate composition of chickpea seed.

Constituent	%
Total carbohydrates	**50.6-70.9**
Starch	37.2-50.8
Amylose (% of total starch)	31.8-45.8
Total sugars	4.8-9.3
Reducing sugars	0.1
Sucrose	0.7-2.9
Raffinose	Trace-3.0
Verbascose	Trace-4.5
Stachyose	0.5-6.48
Manninotriose	1.6-3.1
Crude Fiber	**7.1-13.5**
Cellulose	7.1-9.7
Hemicellulose	3.5-8.7
Pectin substances	1.5-3.8
Lignin	2.2-5.9
Dietary fiber	**19.9-22.7**

(Chavan et al., 1989)

Starch is the main component of total carbohydrates of chickpea seeds ranging from 37.2 to 50.8%; non-reducing sugars are the major soluble sugars in chickpeas (Chavan et al., 1989); Sosulski et al. (1982) reported that chickpeas contain higher amount of raffinose, stachyose, and verbascose (especially high in stachyose and manninotriose) compared to cowpeas, field beans, horse gram, lentils, lima beans, and mung beans. It is reported that these oligosaccharides are involved in flatulence production (Chavan et al., 1989).

Singh (1984[a]) reported that crude fiber (CF), acid detergent fiber (ADF), neutral detergent fiber (NDF), and dietary fiber were significantly higher in seeds of "desi" cultivars than in seeds of "kabuli" cultivars. Singh et al. (1983) showed that the chickpea seed coat had the highest hypocholesterolemic effect, followed by black gram, green gram, and lentil fibers.

2.3.3 Vitamins and Minerals in Chickpea Seed

The main vitamin in chickpea is niacin followed by thiamin, riboflavin, and β -carotene (Table 2.1). Sathe et al. (1984) reported that chickpea contained relatively high level of B-group vitamins therefore the chickpea seed can be a good source of those vitamins.

Chickpea is a satisfactory source of dietary minerals, such as calcium, phosphorous, magnesium, iron, and potassium; most of the calcium is found in the seed coat (Chavan et al., 1989). These minerals are known to associate with other components such as proteins, phytic acid, oxalic acid, and polyphenols, and complex polysaccharides such as starch, fiber, and lignin (O'Dell, 1969; Erdman, 1981).

2.3.4 Anti-nutrition Factors

Protease inhibitors, α-amylase inhibitors, oligosaccharides, phytic acid, saponins, phenolic compounds, and tannins are considered as anti-nutritional factors that are present in chickpea (Wesche-Ebeling et al., 2001). The effects of these anti-nutritional factors are reduced by many processing techniques such as cooking, germination, and fermentation (Chavan et al., 1989).

Chymotrypsin and trypsin inhibitors are the main protease inhibitors in chickpea. Sumathi and Pattabiraman (1976) reported that the chymotrypsin inhibitor was more heat resistant than trypsin inhibitor and both required heating in acidic conditions for inactivation.

Marquez and Alonso (1999) optimized the conditions for inactivation of trypsin inhibitor in chickpea, by soaking the chickpea in water, citric acid solution, and sodium carbonate solution separately, and using different conditions of heat treatment. Their results (Table 2.4) showed that soaking lowered the trypsin inhibitor activity irrespective of the pH of the soaking solution; the trypsin inhibitor activity was significantly reduced by boiling the seeds in water, and was totally removed after 5 min of treatment.

Singh (1984[b]) observed that tannins and polyphenols lowered the bioavailability of vitamins and minerals. Rao and Deosthale (1982) found that the whole seeds contained 80% more tannins compared with the cotyledons only. Substantial variations in the polyphenol content of chickpea cultivars having varying seed coat color have been reported (Singh, 1984[b]); the darker the color of seed coat, the greater the quantity of polyphenols. Dehusking is one of the processing treatments to remove tannins in chickpea seeds for bean consumption (Reddy et al., 1985; Price, 1980).

Table 2.4:

Trypsin inhibitor activity and protein content of the untreated and treated chickpea.

Processing conditions for chickpea	Trypsin inhibitor[a] (mg/g dry sample)	Protein content (g/100g dry sample)	
		Total	Soluble
Raw with husk	8.4	23.4	19.3
Raw without husk	6.6	24.3	20.2
Soaked in water	5.4	24.0	19.8
Soaked in 1% w/v $C_6H_8O_7$	5.4	24.2	16.0
Soaked in 1% w/v Na_2CO_3	4.5	24.0	18.9
Soaked in 2% w/v Na_2CO_3	4.7	24.0	15.6
Boiled in water, 30s	3.7	25.1	15.0
Boiled in water, 60s	2.2	25.1	13.7
Boiled in water, 120s	1.1	25.1	13.1
Boiled in water, 300s	0.0	25.0	11.3
Average S D[b]	0.1	0.1	0.1
Average V C[c]	35.5×10^{-3}	2.5×10^{-3}	5.9×10^{-3}

[a] (mg/100g dry sample).

[b] Standard Deviation, [c] Variation Coefficient.

(Marquez and Alonso, 1999)

2.4 Chickpea Proteins

The protein content of chickpea seeds ranges from 14.9 to 24.6 % with an average of 21.5% (Duke, 1981). The storage proteins of chickpea seed have been fractionated into

globulin (salt soluble), albumin (water soluble), prolamin (alcohol soluble), glutelin (acid/alkali soluble), and residual proteins (Chavan et al., 1989). The comparison of these Osborne-fractions showed that albumins and glutelins have better functional properties than the globulin fractions (Tömösközi et al., 1999).

Singh and Jambunathan (1982) reported that globulin comprise the major storage protein (56.0%), followed by glutelin (18.1%), albumin (12.0%), and prolamin (2.8%). Table 2.5 (Singh and Jambunathan, 1982) shows the amino acid composition of the protein fractions; globulin is deficient in methionine and cystine while albulmin and glutein contain slightly higher amount of those amino acids.

Although the globulin fraction of chickpea is low in the sulphur containing amino acids, chickpea is still considered to be a source of a high quality of protein. Table 2.6 shows the essential amino acids of chickpea compared to wheat, rice, and corn; the chemical scores indicate that the quality of chickpea protein is higher than that of corn and wheat, and equivalent to that of rice (Sotelo and Adsule, 1996).

2.4.1 Globulins and Glutelins

Globulins represent about 70% of legume seed proteins and are composed of two major groups, characterized by their sedimentation coefficients, the 11S (320-400 kDa) or legumin, and the 7S (145-190 kDa) or vicilin (Casey et al., 1993). Legumin is a major storage protein representing a source of energy, carbon, and reduced nitrogen for germination and seedling growth in chickpea. Legumin represents around 64% of the total protein content and 97% of the globulins in chickpea seed (Plietz et al., 1980).The approximate molecular weight of legumin is 360 kDa; it is made up of six α-β subunits

Table 2.5: Amino acid composition (g/16g N) of seed protein fractions of chickpeas.

Amino acid	Albumin	Globulin	Glutelin	Prolamin
Lysine	10.8	6.4	6.8	2.3
Histidine	2.3	2.6	2.9	2.6
Arginine	5.6	10.7	6.8	4.8
Aspartic acid	13.8	12.7	10.1	10.3
Threonine	5.4	3.5	5.7	2.2
Serine	5.2	5.2	5.6	1.9
Glutamic acid	18.4	15.2	16.6	17.7
Glycine	5.4	3.7	4.7	3.1
Alanine	5.3	4.3	4.9	2.3
Cystine	3.5	1.0	1.4	0.6
Valine	4.5	4.2	5.7	2.1
Methionine	1.8	0.8	1.2	0.9
Isoleucine	5.1	4.4	5.4	2.3
Leucine	9.8	7.5	9.1	1.6
Tyrosine	4.2	2.9	3.7	2.3
Phenylalanine	5.1	6.1	4.4	3.4

(Singh and Jambunathan, 1982)

Table 2.6:

Essential amino acid and chemical scores content of wheat, rice, corn and chickpea.

Amino acid (g/ 100g protein)	***Content (g/ 100g protein)***			
	Chickpea	***Wheat***	***Rice***	***Corn***
Methionine + Cystine	2.61	3.63	3.32	3.04
Tryptophan	1.00	1.29	1.23	0.69
Lysine	7.47	1.93	3.25	2.75
Isoleucine	4.76	3.39	3.07	3.14
Phenylalanine + Tyrosine	9.43	3.69	8.19	6.34
Valine	4.84	4.36	4.88	4.88
Threonine	4.23	2.52	3.06	3.17
Leucine	7.18	6.79	8.46	14.8
Chemical score[a]	60 (Met + Cys)	35 (Lys)	60 (Lys)	51(Lys)

(Sotelo and Adsule, 1996)

[a] FAO (1970); amino acids in parentheses are limiting.

held together as a triagonal antiprism by non-covalent bonds. Each α chain is linked to β chains by disulfide bonds. The β chains are the hydrophobic heart of the protein, and the hydrophilic α chains are at the exterior of the molecular (Plietz et al., 1980; Sanchez-Vioque et al., 1999).

Sanchez-Vioque et al. (1999) characterized the chickpea proteins by SDS-PAGE and identified the major fractions with the MW of 46.5, 39.8, 25.3, and 24.3 kDa. These

fractions can be related to the polypeptide α and β chains of 11S protein (Vairinhos and Murray, 1982).

Although vicilin is also a part of the storage globulin, the structure and conformation of vicilin have been studied much less than legumin. Boulter (1983) defined the vicilin group as the protein fraction which consists mainly of 50 kDa subunits and a range of lower molecular weight polypeptide chains (33kDa, 29kDa, 13kDa, and 12.5 kDa). The MW of chickpea vicilin polypeptides is 50, 35, 33, 19, 15, 13, and 12.5 kDa based on SDS-PAGE (Gueguen, 1991).

Glutelins, which are found mostly in cereals, belong to the 11–12S globulin family of proteins; the proportion of glutelins domains around 18.1% of the total proteins in chickpea (Singh and Jambunathan, 1982). Glutelins comprise subunits of around 55 kDa, and they are post-translationally cleaved to give acidic (33 kDa in oats, 28–31 kDa in rice) and basic (23 kDa and 20–22 kDa, respectively) polypeptide chains linked by a single disulphide bond (Shotwell, 1999; Takaiwa et al., 1999).

2.4.2 Albumins

The albumin fraction is more heterogeneous and is more abundant in sulfur amino acids comparing to the globulin fraction, thus the albumins in legume proteins may naturally complement the amino acid pattern of globulin. In general, most albumin proteins have some physiological functions, such as the enzymatic activities of lipoxygenases, glycosidases, or proteases involved in the degradation of storage proteins. Other albumins, such as protease inhibitors or lectins, are implicated in defensive mechanisms (Gueguen, 1991; Rubio et al., 1994).

There are diverse two types of albumins, the PA2 albumin and the PA1 or 2S albumins (Casey et al., 1993). PA2 albumin from pea has been purified and characterized (Croy et al., 1984; Schroeder, 1984). Vioque et al. (1998) reported that 2S albumin has been described as a lectin which is capable of agglutinating papainized human erythrocytes in pea and chickpea. Vioque et al. (1999) studied the 2S albumins of chickpea protein. A chickpea 2S albumin was prepared and purified by solubilization in 60% methanol followed by ion-exchange chromatography. Under denaturing conditions, it is composed of two peptides of 10 and 12 kDa.

2.4.3 Prolamins

The name "Prolamin" was originally based on the observation that these proteins are generally rich in proline and amide nitrogen derived from glutamine. Prolamins are generally defined as soluble in alcohol/water mixtures (e.g. 60–70% (v/v) ethanol), but some prolamin occur as alcohol-insoluble polymers (Shewry and Tatham, 1990). Nevertheless, all individual prolamin polypeptides are alcohol-soluble in the reduced state; the molecular weights of prolamins vary extensively from 10 to almost 100 kDa. Consequently, prolamin storage proteins are much more variable in structure than those of the 7S and 11/12S globulins, and it is possible that the major groups of prolamins in the Triticeae family (wheat, barley, and rye) and the Panicoideae family (maize, sorghum, and millets) have separate evolutionary origins (Shewry et al., 1995).

Most prolamins share two structural characteristics: firstly, they have distinct regions or domains with different structures and may have different origins; secondly, the amino acid sequences consist of repeated blocks based on one or shorter peptide motifs,

enriched in specific amino acid residues, such as methionine. These features are responsible for the high proportions of glutamine, proline and other specific amino acids (e.g. histidine, glycine, methionine, phenylalanine) in some prolamin groups (Shewry and Halford, 2002).

2.5 Functional Properties of Chickpea Proteins

Tradtionally, it has been considered that functional properties of plant proteins are inferior to those of animal proteins. Most plant protein sources have been used as animal feed to produce animal proteins, such as egg, milk and meat proteins which have desirable foaming, emulsification, gelling, heat coagulation, and binding/ adhesion properties (Damodaran, 1999). More recently, many plant proteins have been shown to also have desirable functional properties.

Chickpea has been considered as a source of relatively high quality protein with desirable functional properties (Hulse, 1991). Paredes-Lopez et al. (1991) and Sanchez-Vioque et al. (1999) studied the functional properties of the chickpea protein isolates (Table 2.7 and Table 2.8); the results show that chickpea proteins have desirable solubility, water absorption, fat absorption, emulsion capacity and foam stability properties.

2.5.1 Solubility

Protein solubility is one of the most important functional properties of commercial food protein preparations. Solubility can affect the thermodynamic reactions of the equilibrium between protein-protein and protein-solvent interactions under a given set of environmental conditions, and is related to the net free energy change arising from the

Table 2.7: Functional properties of protein isolates at pH 7.0.

Functional properties	MPI*	IPI*	SPI*
Nitrogen solubility (%)	72.5 ± 0.8	60.4 ± 0.9	21.2 ± 0.5
Water absorption (ml/g protein)	4.9 ± 0.3	2.4 ± 0.3	5.7 ± 0.3
Oil absorption (ml/g protein)	2.0 ± 0.2	1.7 ± 0.1	1.9 ± 0.0
Emulsifying activity (%)	63.7 ± 1.0	72.9 ± 1.4	50.8 ± 1.2
Emulsion stability (%)	94.3 ± 0.9	85.0 ± 2.8	99.7 ± 0.5
Foam expansion (%)	43.3 ± 2.0	47.5 ± 2.5	41.8 ± 2.5
Foam stability (%)	59.2 ± 3.4	66.6 ± 1.6	53.2 ± 2.4

* MPI = Micelle protein isolate; IPI = isoelectric protein isolate; SPI = Soy protein isolate (commercial)

(Paredes-Lopez et al., 1991), data are the mean ± SD of three analyses

Table 2.8: Functional properties of chickpea flour (CF), Isolate-A (IA), and Isolate-B (IB). Data are the mean ± SD of three analyses.

	CF	IA	IB
Solubility[a]	31.8 ± 1.1	26.6 ± 0.9	**46.3** ± 3.2
Water absorption[b]	178.8 ± 2.4	**343.7** ± 30.1	199.5 ± 4.9
Fat absorption[c]	134.8 ± 6.1	**409.4** ± 24.9	125.7 ± 11.2
Emulsion capacity [d]	94.7 ± 0.7	48.1 ± 5.7	**76.9** ± 2.2

[a] Percentage of soluble nitrogen in 0.1 M NaCl solution at pH 7

[b] Grams of water absorbed per 100g sample

[c] Grams of fat absorbed per 100g sample

[d] Percentage of fat emulsified (% weight)

*IA and IB were from alkaline extraction, with (IB), and without (IA) sodium sulphite,and precipitation of proteins at isoelectric point (pI = 4.3)

(Sanchez-Vioque et al., 1999)

interactions of hydrophobic and hydrophilic residues of the protein with the surrounding aqueous solvent; it can be affected by pH, ionic strength, ion types, temperature, solvent polarity, and processing conditions (Damodaran, 1996). Solubility also affects other functional properties such as emulsification or foaming properties, thus a highly soluble protein is required to acquire optimal functionality (Kinsella, 1976).

Sanchez-Vioque et al. (1999) determined the solubility of chickpea protein by measuring the soluble nitrogen content of chickpea proteins at various pH values. The solubility curve of chickpea proteins is shown in Figure 2.1.

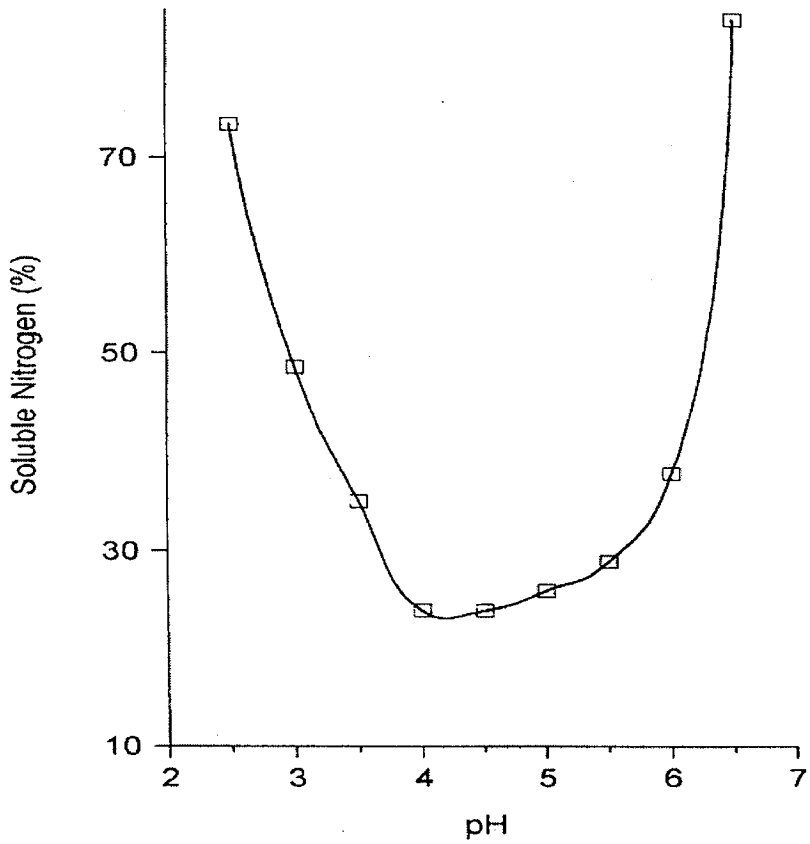

Figure 2.1: Chickpea proteins solubility curve (Sanchez-Vioque et al., 1999).

2.5.2 Emulsifying and Foaming Properties

Table 2.7 shows that emulsifying and foaming properties of chickpea isolates are comparable to those of commercial soy isolates (Paredes-Lopez et al., 1991). Damodaran (1996) reported that some functional properties of proteins are related to their amino acid composition. The quantities of acidic/basic and hydrophobic /hydrophilic amino acids determine the net charge at a given pH and solubility characteristics, water-binding potential and surfactant properties of proteins. Moreover the free sulfhydryl amino acids, which affect emulsifying and foaming properties, also play an important role in inter- and intra-molecular disulfide bond formation during thermal processing.

2.5.3 Modification of Chickpea Functionality

By chemical or enzymatic manipulation methods, protein functional properties such as solubility, emulsifying and rheological properties can be modified intentionally (Campbell et al., 1992; Hamada, 1992; Lahl and Braun, 1994).

Liu and Hung (1998) reported that acetylation improved the solubility of chickpea protein at high alkaline pH (pH>8), but decreased solubility at low pH (pH 2-7); acetylation also increased water and oil absorption capacities but decreased emulsion stability of chickpea protein compared to native chickpea protein.

2.6 Isolation and Characterization of Chickpea Proteins

Alkaline (pH 8-10) extraction to solubilize protein from seeds and subsequent precipitation of the proteins at or near the isoelectric point is the most widely used technique to prepare proteins for use in the food industry; this process was patented by Anson and Pader (1957). After an alkaline solubilization of the proteins, the insoluble

material is removed by centrifugation; this is followed by the addition of acid to the supernatant until the proteins precipitated at isoelectric point (Gueguen, 1991).

Paredes-Lopez et al. (1991) used both the alkaline extraction/acid precipitation procedure and a micellization process (Murray et al., 1978) to isolate chickpea proteins; the micellization process involved precipitation of the proteins from a neutral salt extract by dilution in cold water. The micellization has been suggested as a milder approach for the recovery of protein isolate because of less denaturation of the protein structure compared with isoelectric precipitation (Murray et al., 1981).

Liu et al. (1994) proposed a pilot scale (Figure 2.2) for isolating chickpea proteins. Two protein concentrates (CPC-7, CPC9) and two isolates (CPI-7, CPI-9) were derived from the extracts at pH 7 and pH 9, separately. The protein concentrate CPC-9 contained 89% protein.

Due to the irreversible denaturation of protein isolates, it is not generally recommended to isolate the proteins by carrying out the precipitation procedures such as selective cryoprecipitation, heat coagulation, or addition of metals. There is an interest in separating proteins by chromatographic techniques (Li-Chan, 1996).

Reverse phase high performance liquid chromatography (RP-HPLC) is a commonly used analytical technique for isolation and purification of food proteins and peptides because it is more selective to separate proteins by different hydrophobic characteristics. Yust et al., (2003) reported that several fractions contained bioactive peptides from chickpea legumin hydrolysates were separated using RP-HPLC and the results showed the fractions constituted more hydrophobic amino acids possessed more angiotensin I-converting emzyme (ACE) inhibitory activity.

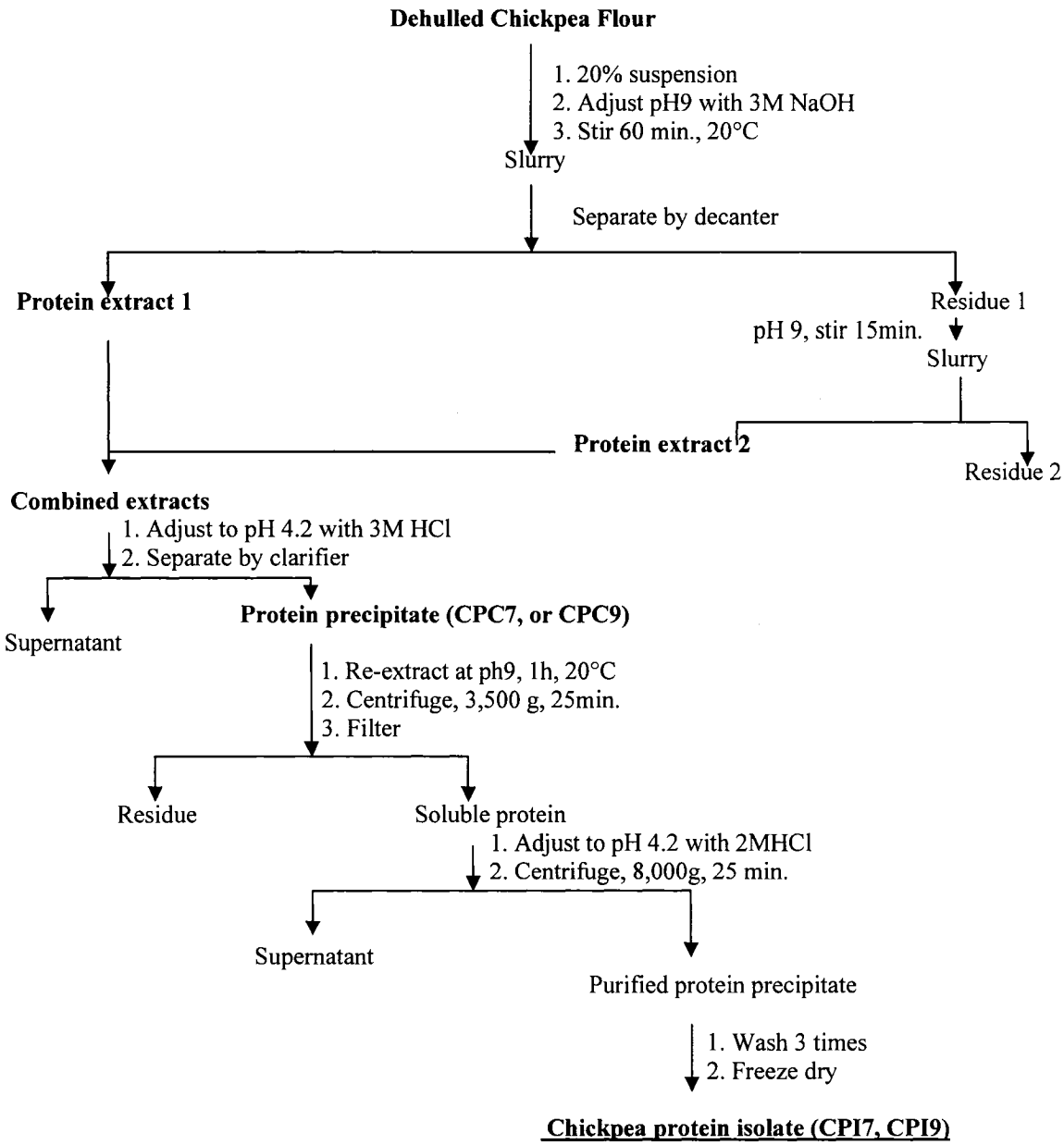

Figure 2.2: Diagram of chickpea protein isolate procedure (Liu and Hung, 1998).

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

Commercial chickpea seeds and a chickpea protein isolate (DLA-IP) were provided by Dr. J. Boye (Food Research and Development Centre, Agriculture and Agri-Food Canada). Sample of dried chickpea seeds and soybean seeds were ground by a micro sample mill (Braun, KSM2 Type 4041, CA) and preserved in air-tight plastic containers at room temperature until they were used. All chemical reagents were analytical grade.

3.2 Protein Extraction and Precipitation

3.2.1 Sodium Hydroxide Extraction /Acid Precipitation /Cryoprecipitation

Proteins were extracted using the procedure of Fan and Sosulski (1974) as adapted by Alli and Baker (1980). A sample (20 g) was mixed with NaOH solution (200 mL, 0.02%, pH 11.5). The mixture was allowed to stand with intermittent stirring for 1 h and centrifuged (8000×g, 10 min). The residue was discarded and the extract was filtered through fine glass wool. The filtrate was divided into three parts, as follows: (i) the extract (C.Na-E) freeze-dried directly; (ii) the pH of extract was adjusted to pH 4.5 (2N HCl) and the precipitate (C.Na-IP) was recovered by centrifugation (8000×g, 10 min) and lyophilization; (iii) the extract was refrigerated (4°C, 18 h) and the precipitated proteins (C.Na-CP) were recovered by centrifugation (8000×g, 10 min) followed by lyophilization; the supernatant was used for another acid precipitation as described in (ii) above where

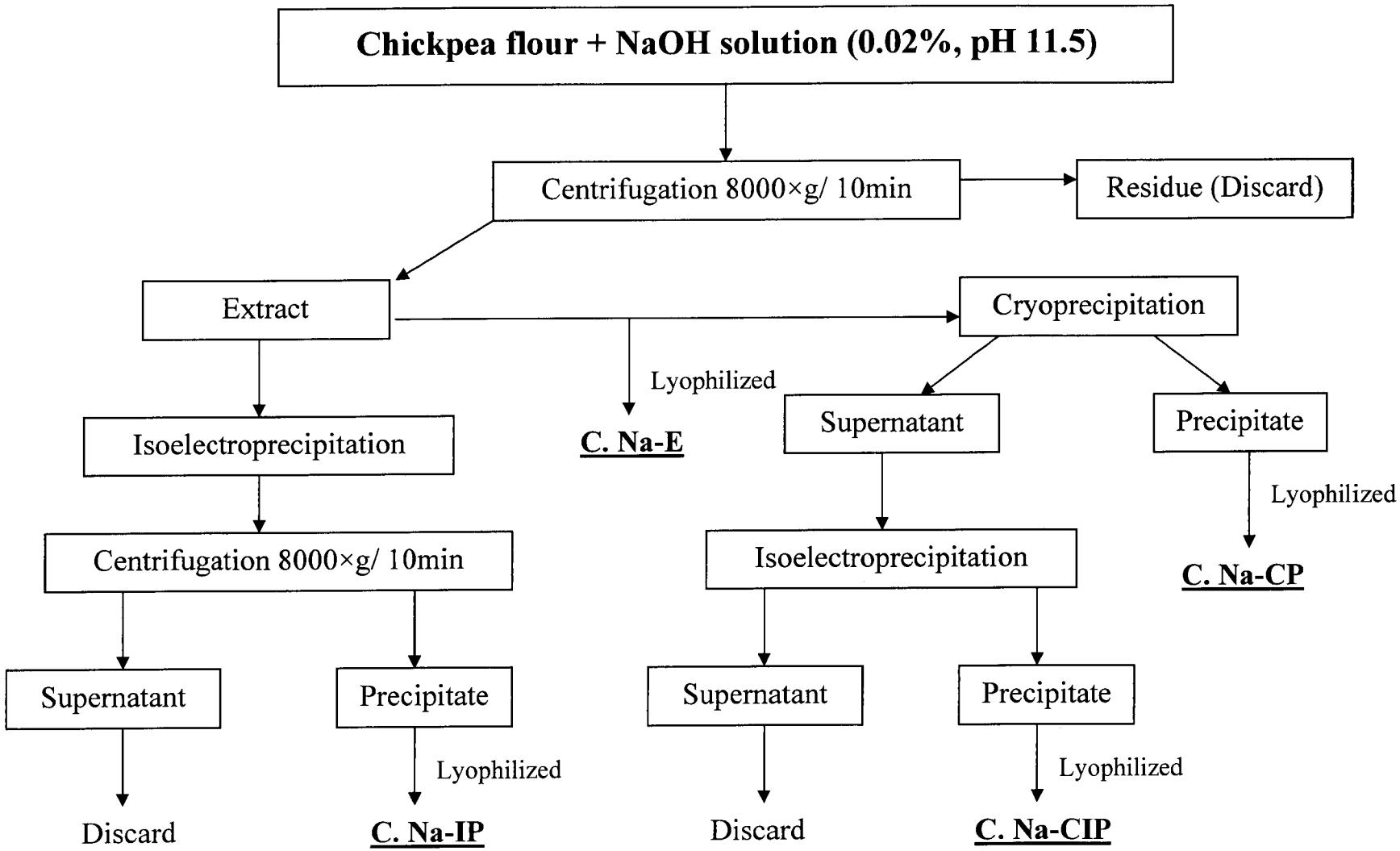

Figure 3.1: Diagram of NaOH extraction and precipitations of chickpea isolates.

the precipitate (C.Na-CIP) was recovered by centrifugation and then lyophilized. The flow chart of the NaOH extraction is shown in Figure 3.1.

The chickpea isolate DLA-IP was prepared by extraction with a dilute alkaline solution. The extracts were maintained at pH 8.5, 55℃ with intermittent stirring for 45-60 min then dilute acid was used to adjust the pH to 4.5 and centrifuged (8000×g, 10 min); the isoelectric precipitate was recovered by freeze drying.

3.2.2 Citric Acid Extraction

Citric acid soluble proteins were extracted according to procedures described by Melnychyn (1969) and modified by Alli and Baker (1980). Figure 3.2 illustrates the process of citric acid extraction of chickpea proteins. Chickpea flour (20 g) was mixed with citric acid solution (200 mL, 0.4 N, pH 3.5) and allowed to stand for 1 h with intermittent stirring. The mixture was centrifuged (8000×g, 10 min) and then the extract was filtrated through glass wool; the residue was discarded. The extract was separated into two parts, as follows (i) the isolate (C.C-E) was obtained by lyophilization of the filtrate directly; (ii) the filtrate was refrigerated (4 °C, 18 h) and the precipitate (C.C-CP) and supernatant (C.C-S) were recovered by centrifugation (8000×g, 10 min) and then freeze-dried.

3.3 Examination of Chickpea Protein Isolates by Scanning Electron Microscope (SEM)

For investigation of microstructures of the protein isolates, the freeze-dried samples of isolates C. Na-CP, C. Na-IP, C. Na-CIP and C. C-CP were directly deposited on an aluminium stub covered with a carbon tape, sputter coated with gold (10-15 nm)

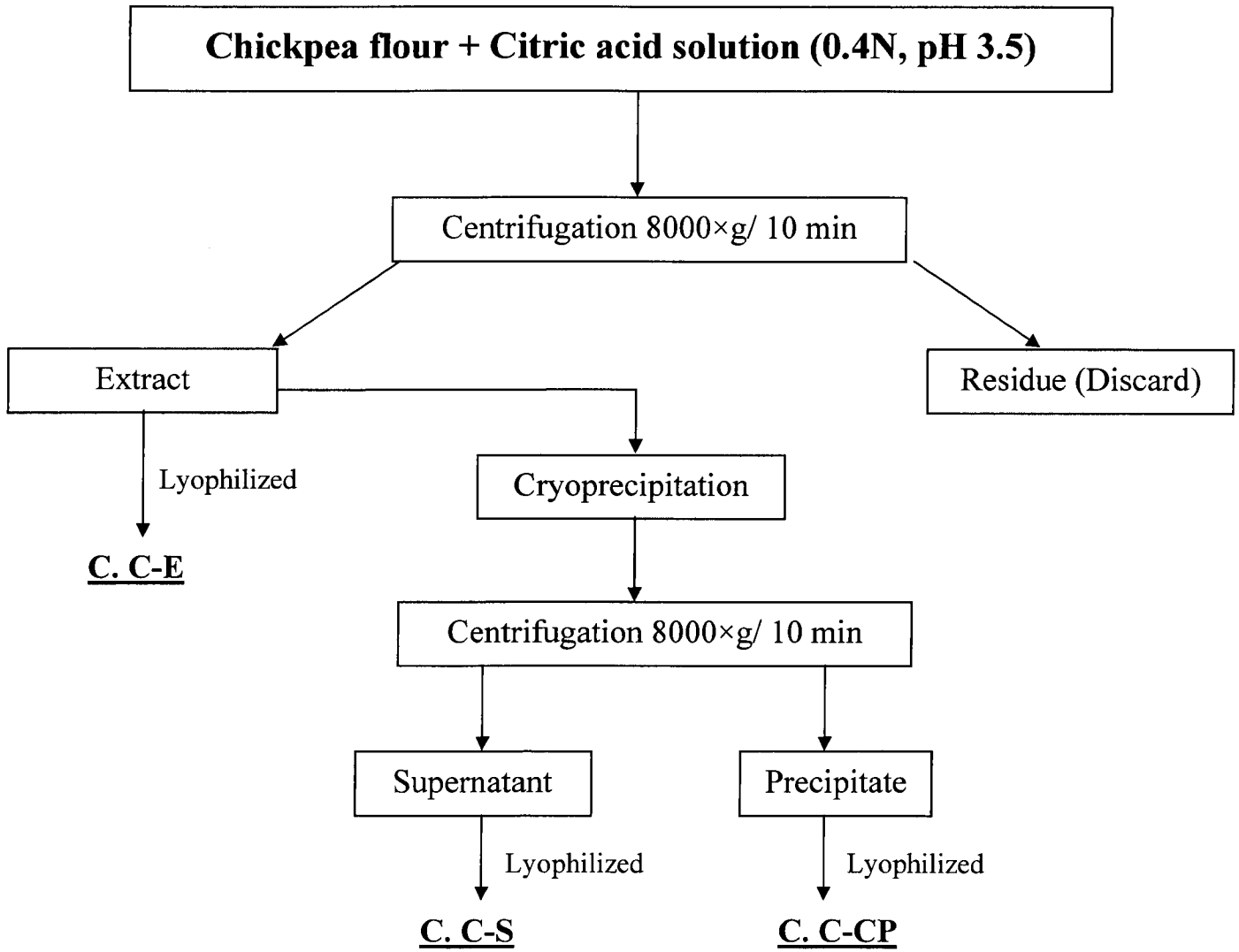

Figure 3.2: Diagram of citric acid extraction and precipitations of chickpea isolates.

and observed with a scanning electron microscope (model Hitachi S-3000N) at Food Research and Development Centre, Agriculture and Agri-Food Canada (Saint-Hyacinthe, Qc).

3.4 Determination of Protein Content

The protein contents of the isolates were measured by micro-Kjeldahl method (A.O.A.C., 1980); a conversion factor of 6.25 was used to convert the nitrogen content to protein content. All the analyses were conducted in triplicate.

3.4.1 Calculation of Yield

Yields were calculated on the basis of the protein weight of the chickpea isolates against the protein weight of chickpea flour using the equation below.

$$\text{Yield \%} = \frac{\text{Weight of chickpea protein isolates} \times \text{Protein content (chickpea isolates)} \times 100}{\text{Weight of chickpea protein samples} \times \text{Protein content (chickpea flour)}} \%$$

3.5 Preparation of Soybean Isolates

Soybean protein isolates were prepared using the same extraction and precipitation techniques as for chickpea isolates, in order to identify the chickpea proteins as globulins and albumins; this was necessary since there are no reference standards for these proteins and the globulins and albumins from soybean are well characterized.

3.6 Protein Characterization

3.6.1 Native-Polyacrylamide Gel Electrophoresis (Native-PAGE)

Native-PAGE was performed according to the method of Davis (1964). A Mini-Protean II Electrophoresis Cell unit (Bio-Rad, Hercules, CA) was used with a 4% acrylamide stacking gel and a 10% separation gel. Sample solutions (20 µl) were prepared from 8-20 mg of freeze-dried protein extracts, supernatants or precipitates dissolved in 1 ml sample buffer (distilled water, 1.5 M Tri-HCl pH 8.8, glycerol and 1% bromophenol blue), and injected into each sample well. High molecular weight calibration kit (Amersham Bioscience, UK), the standard protein markers were thyroglobulin (MW= 669,000 Da), ferritin (MW= 440,000 Da), catalase (MW= 232,000 Da), lactate dehydroxygenase (MW= 140,000 Da) and albumin (MW= 66,000 Da). The migration of proteins was carried out for approximately 3.5 hours at constant current (7.5 mA/ gel). Gels were fixed with fixing solution (water: methanol: acetic acid/ 700ml: 200ml: 100ml) for 30 min and then stained with Coomassie Brilliant Blue R-250 for 1h. The stained gels were destained by changing the fixing solution until the excess stain disappeared.

3.6.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the technique reported by Laemmli (1970); 4% stacking gel and 12% resolution gel were used to separate the proteins. Sample solutions (15-20µl) were prepared from 8-20 mg of freeze-dried protein extracts, supernatants or precipitates dissolved in 1 ml sample buffer (distilled water, 0.5 M Tri-HCl pH 6.8, glycerol 10% SDS, 1% bromophel blue and β- mercaptoethanol) heated at 98 ℃ for 10

min, then applied to the sample wells. The standard protein marker (broad range molecular weight, Bio-Rad Hercules, CA) which contained myosin (MW= 200,000 Da), β-galactosidase (MW= 116,250 Da), phosphorylase b (MW=97,400 Da), serum albumin (MW=66,200 Da), ovalbumin (MW= 45,000 Da), carbonic anhydrase (MW= 31,000 Da), trypsin inhibitor (MW= 21,500 Da), lysozyme (MW= 14,400 Da) and aprotinin (MW= 6,500 Da), was used to prepared a standard curve for molecular weight estimation. Electrophoretic migration was monitored at constant current (14mA/ gel) for 1.5 to 2 h. The gels were fixed with fixing solution (water: methanol: acetic acid/ 700ml: 200ml: 100ml) for 30 min and then stained with Coomassie Brilliant Blue R-250 for 1h. The stained gels were destained by changing the fixing solution until the excess stain disappeared.

3.7. Fractionation of Protein Isolates by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

3.7.1 Sample Preparation

A quantity (20-40 mg) of the lyophilized chickpea protein extracts or isolates was dissolved in 1ml of the trifluoroacetic acid (TFA) solution (0.1%, water: acetonitrile = 9:1) with a heat treatment (40℃) in water bath (Precision Scientific, USA) for 30 min and then centrifuged (micro-centrifuge, Fisher, USA), then filtered through a membrane filter (0.45 μm, Osmonics Inc., USA).

3.7.2 Sample Injection

The filtrates were subjected to RP-HPLC using the procedure reported by Alli et al. (1993) with modifications; the equipment was a Beckman model liquid

chromatography system (Beckman, CA, USA). Samples injected manually using a 100 µl loop (Life Science, CA). Separation was performed using a guard column (RP-HPLC Guard Column 4.6 × 50 mm, J. T. Baker, USA) followed by a reversed phase analytical column (C_{18} 5 micron pore size, 4.6 × 250 mm; J. T. Baker, USA). A gradient solvent system was monitored by a Programmable Solvent Module (model 126) within a gradient dual pump system for the high pressure delivery with a flow rate 1 ml/min. The two solvents (A and B) to gradient elution system A comprised of 0.1% trifluoroacetic acid (TFA) in distilled, deionized water and B consisted of 0.1% trifluoroacetic acid (TFA) in acetonitrile /distilled deionized water (70:30). A linear gradient elution (30% to 70% solvent B 30 min) was used for separation then re-equilibrated (10 min) to initial conditions. Elution profiles were detected by a programmable detector module (model 166) at 210nm and fractions which showed relatively high response were collected using Water Fraction Collector (NE, USA), dried in a speed-vac concentrator (Savant, NY). Chromatographic data were analyzed by Beckman Gold System (version V810, USA) then translated from print (PRN) format to Microsoft Excel© worksheet. Fractions obtained from RP-HPLC were subjected to Native-PAGE, SDS-PAGE and positive ion electrospray mass spectrometry (ESI-MS) for further analysis and characterization.

3.8 Eletrospray Ionization Mass Spectrometry (ESI-MS)

3.8.1 Sample Preparation

Fractions collected from RP-HPLC were dissolved in 500 µl 0.2% formic acid (Anachemia, N.Y.) and passed though a membrane filter (0.45 µm, Osmonics Inc., USA).

All elutes were placed in 1.5 ml micro-centrifuge tubes and stored at 4℃ until they were analysed.

3.8.2 Sample Injection

All ESI-MS analyses were performed at Biotechnology Research Institute (BRI), National Research Council Canada (NRC). Sample solution (1 μl) was injected into the ESI-MS (Waters Micromass QTOF Ultima Global, Micromass, Manchester, UK); hybrid mass spectrometer equipped with a nanoflow electrospray source, operated in positive ionisation mode (+ESI), at 3.80 kV; source temperature was 80 ℃, desolvation temperature was 150 ℃. The TOF was monitored at an acceleration voltage of 9.1 kV, a cone voltage of 100 V, and a collision energy of 10 eV (for MS survey). For the MS survey mass range, m/z, was 400-1990 and scanned continuously over the chromatographic run. The mass spectrometer was tuned and calibrated with [Glu]-Fibrinopeptide B (Sigma Chemicals; St. Louis MO). Instrumental control and data analysis were manipulated by using software-MassLynx V4.0.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Protein Contents and Yields of Chickpea Isolates

Table 4.1 shows the protein contents and yields of chickpea protein isolates. Protein contents of isolates from sodium hydroxide (NaOH) extraction are higher than those from citric acid extraction. Based on solubility curve (Figure 2.2) of chickpea proteins reported by Sanchez-Vioque et al. (1999), chickpea proteins have relatively high solubility (approximately 70% soluble nitrogen) above pH 7. The protein content and yield of the citric acid cryoprecipitate (C. C-CP) were 44.5% and o.1%, respectively; for NaOH cryoprecipitate (C. Na-CP), the protein content and yield were 71.6% and 2.3%, respectively; for NaOH isoelectric precipitate (C. Na-IP), the protein content and yield were 97.6% and 67.2%, respectively. Paredes-Lopez et al. (1991) and Sanchez-Vioque et al. (1999) reported that the protein contents of isoelectric precipitates from chickpea proteins were 84.8% and 78%, respectively. The yields of isolates from sodium hydroxide are higher than those of isolates from citric acid extraction and the yields of cryoprecipitated proteins were lower than isoprecipitated proteins.

4.2 SEM Microstructures of Chickpea Isolates

Figure 4.2.1 shows the electron micrographs of cryoprecipitates (C. C-CP and C. Na-CP) from citric acid extraction and NaOH extraction. In photographs 1 and 3 (magnification, 1mm), there was no apparent difference of the microstructures between C. C-CP and C. Na-CP but at higher magnification (50 μm, photographs 2 and 4) differences

Table 4.1: Proteins contents and yields of chickpea isolates.

Chickpea Isolates	Protein Content %	Yield %
Citric acid extraction		
C. C-E	13.3 ± 1.56	21.7 ± 0.66
C. C-S	12.8 ± 1.54	17.5 ± 0.48
C. C-CP	44.5 ± 2.29	0.1 ± 0.24
Sodium hydroxide extraction		
C. Na-E	49.8 ± 3.05	53.7 ± 1.07
C. Na-IP	97.9 ± 1.21	67.2 ± 2.53
C. Na-CIP	96.2 ± 4.81	65.8 ± 2.05
C. Na-CP	71.6 ± 5.25	2.3 ± 0.11
DLA-IP	87.2 ± 2.79	ND

% Protein = % Kjeldahl nitrogen x 6.25.
Results are means of triplicate determinations with standard deviation.

$$\text{Yield \%} = \frac{\text{Weight of chickpea protein isolates} \times \text{Protein content (chickpea isolates)} \times 100}{\text{Weight of chickpea protein samples} \times \text{Protein content (chickpea flour)}} \%$$

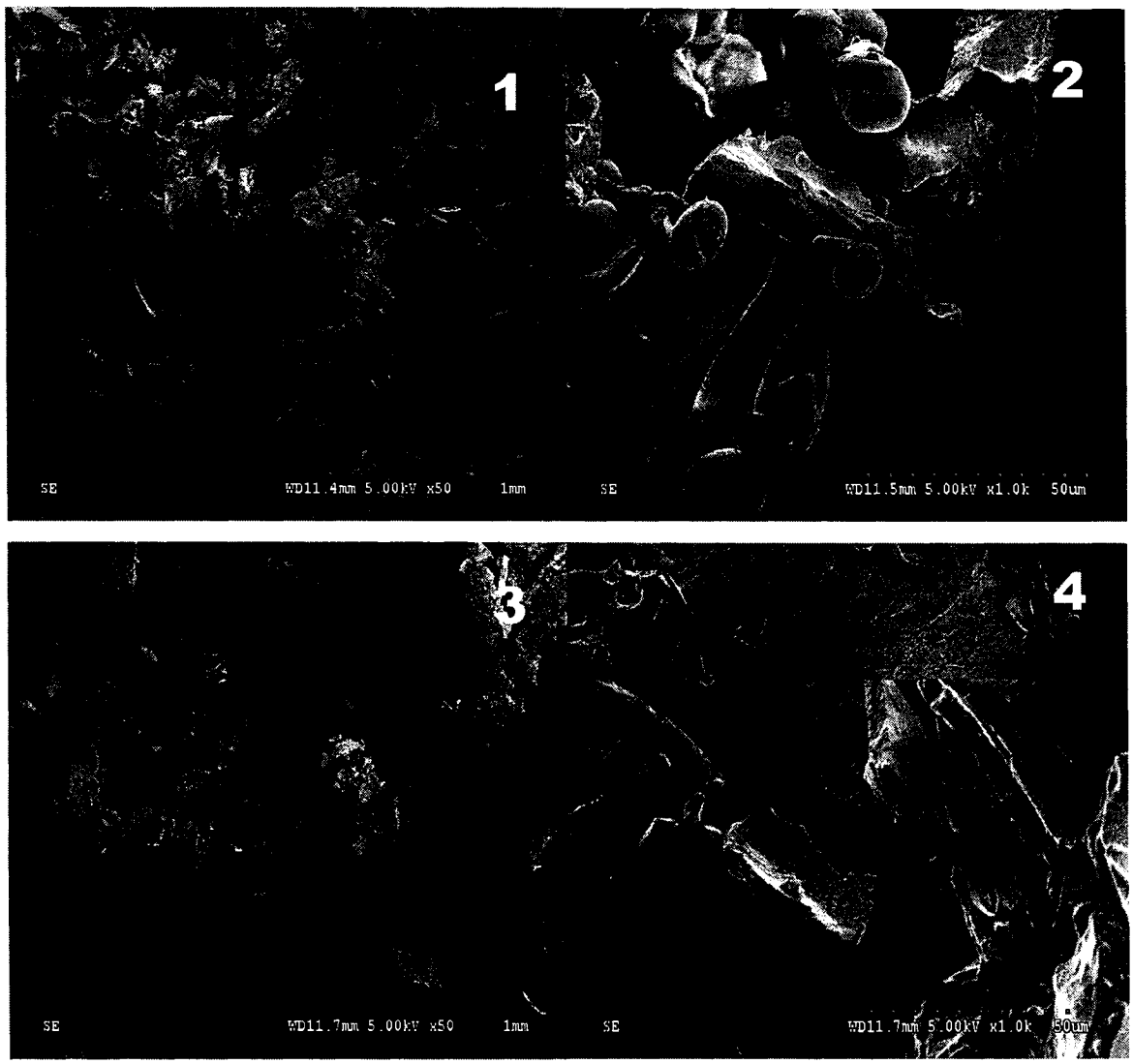

Figure 4.2.1: Electron photomicrographs of freeze-dried protein of chickpea cryo-precipitate (C. C-CP; 1 and 2) from citric acid extraction and chickpea cryoprecipitate (C. Na-CP; 3 and 4) from NaOH extraction. Magnification: (1) 1 mm (2) 50 μm (3) 1 mm (4) 50 μm.

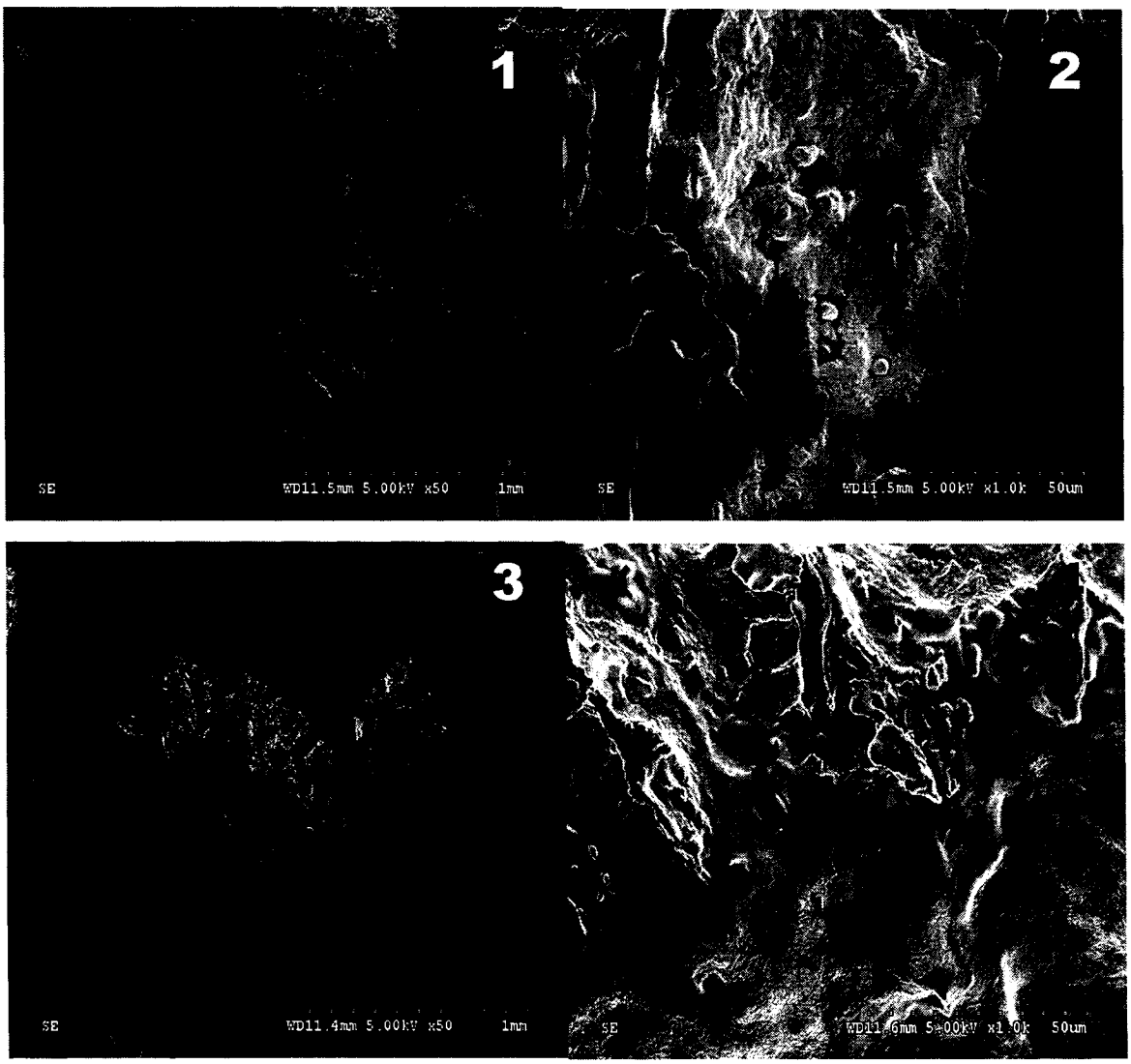

Figure 4.2.2: Electron photomicrographs of freeze-dried protein of chickpea iso-precipitate C. Na-IP (1 and 2) and C. Na-CIP (3 and 4) from sodium hydroxide extraction. Magnification: (1) 1 mm (2) 50 μm (3) 1 mm (4) 50 μm.

in microstructures were observed. Spherical bodies and the irregular shaped structure, with sharp edges in photo 2 are seen in the citric acid cryoprecipitate (photographs 2); the spherical bodies are similar to starch granules reported by Marconi et al. (2000) in raw chickpea flour and by Alli (1979) in kidney bean protein isolate from citric acid extraction and cryoprecipitation. In photograph 4, the microstructure of the cryoprecipitate C. Na-CP from NaOH extraction is characterized as a complete and flat platform with sharp edges. The protein contents of isolate C. C-CP and C. Na-CP were 44.5 and 71.6%, respectively (Table 4.1); the presence of starch grains in the citric acid cryoprecipitate (C. C-CP) reflects its relatively low protein content.

The electron photomicrographs of the two chickpea isoelectric precipitates (C. Na-IP and C. Na-CIP) from sodium hydroxide extraction are shown in Figure 4.2.2. At both low and high magnification (1 mm, photographs 1 and 3; 50 μm, photographs 2 and 4), the microstructures of these two isolates are similar. No starch grains were observed in the isoelectric precipitates; this reflects the higher protein contents of isolates (C. Na-IP, 97.5 % and C. Na-CIP, 96.2 %; Table 4.1).

4.3 PAGE-Characterization of Chickpea Isolates

4.3.1 Native-PAGE

Estimation of molecular weight of the protein fractions separated by Native-PAGE was determined using the high molecular weight standard; this was carried out only to tentatively identify the protein fractions as globulins, albumins, and glutelins. SDS-PAGE was used to determine MW of protein subunits. Since there is no reference of

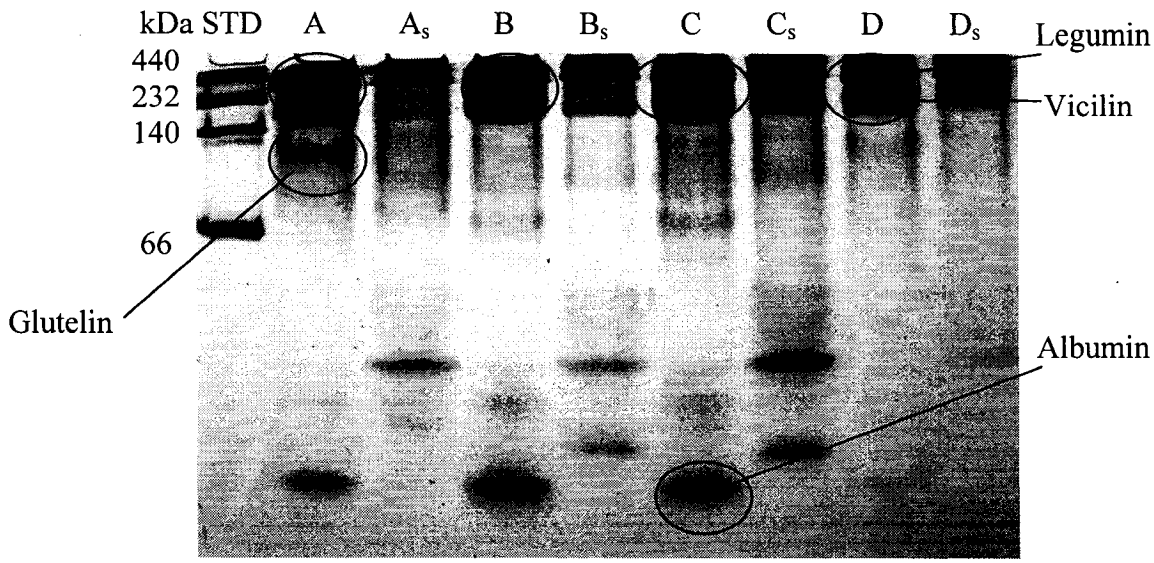

Figure 4.3.1: 12% Native-PAGE of chickpea and soybean isolates from NaOH extraction. **A**: extract from chickpea (C. Na-E), $\mathbf{A_s}$: extract from soybean (S. Na-E), **B**: isoprecipitate from chickpea (C. Na-IP), $\mathbf{B_s}$: isoprecipitate from soybean (S. Na-IP), **C**: isoprecipitate after cryoprecipitation from chickpea (C. Na-CIP), $\mathbf{C_s}$: isoprecipitate after cryoprecipitation from soybean (S. Na-CIP), **D**: cryoprecipitate from chickpea (C. Na-CP) and $\mathbf{D_s}$: cryoprecipitate from soybean (S. Na-CP).

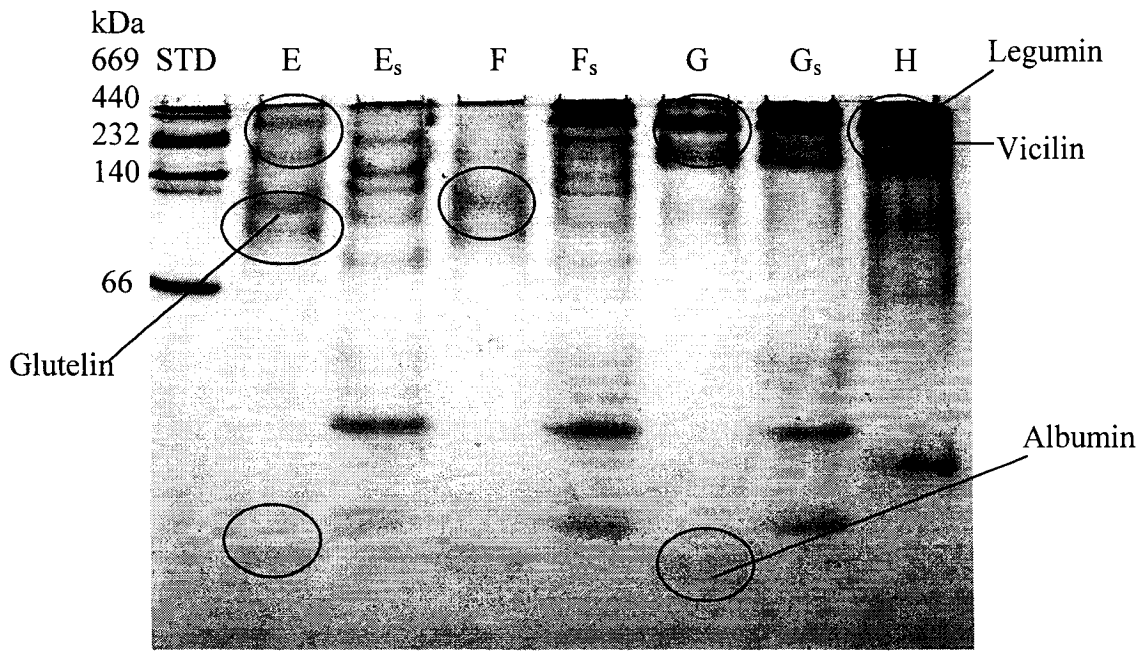

Figure 4.3.2: 12% Native-PAGE of chickpea and soybean isolates from citric acid extraction. **E**: extract from chickpea (C. C-E), $\mathbf{E_s}$: extract from soybean (S. C-E), **F**: supernatant from chickpea (C. C-S), $\mathbf{F_s}$: supernatant from soybean (S. C-S), **G**: cryoprecipitate from chickpea (C. C-CP), $\mathbf{G_s}$: cryoprecipitate from soybean (S. C-CP) and **H**: chickpea isoelectric precipitate from AAFC (DLA-IP).

chickpea proteins for globulins, albumins, and glutelins, soybean proteins were used as reference in the PAGE characterization.

Under non-denaturing conditions (Figure 4.3.1 and Figure 4.3.2), two major bands with MW estimated in range 320~400 kDa and 140~195 kDa were found in all chickpea isolates; these bands could represent the globulin legumins (11S) and vicilins (7S), respectively (Casey et al., 1986). The cryoprecipitated protein C. Na-CP (Figure 4.3.1: D) and C. C-CP (Figure 4.3.2: G) showed mainly the globulins compared with the other isolates. The presence of glutelins in chickpea seed is of interest since this group of proteins is considered to be absent among most leguminous seeds. In soybean proteins, the main proteins are mostly composed of 15S, 11S, 7S and 2S proteins (Wolf et al., 1970) but no glutelins. The band estimated as MW 110 kDa is considered to be glutelin; this band was found in chickpea but not in soybean. Glutelins are generally soluble in acidic and basic solutions (Chavan et al., 1989) and this could explain the presence of glutelin bands in chickpea isolates from citric acid and sodium hydroxide extractions but not in the isolates from corresponding soybean extraction.

Singh and Jambunathan (1982) reported that chickpea globulins represent the major storage protein (56.0%), followed by glutelin (18.1%), albumin (12.0%) and small amounts of prolamin. The relatively intense band with the greatest migration distance in the chickpea isolates is considered to be albumins (Figure 4.3.1). Vioque et al. (1999) and Clemente et al. (2000) reported that intense bands representing 2S albumin fraction with MW ranging from 20-26 kDa.

4.3.2 SDS-PAGE

Determination of molecular weight of the subunits of the proteins was performed using SDS-PAGE. Subunits separated from chickpea proteins and soybean proteins are shown in Figure 4.3.3 and Figure 4.3.4. The estimated MW of subunits of chickpea isolates from sodium hydroxide extraction is summarized in Table 4.3.1. In present work, the estimated molecular weights of subunits from NaOH extraction as the main subunits of legumins, were 40, 39, 23, and 22 kDa and these subunits are similar to those of 11S protein subunits (47, 40, 25 and 24 kDa) reported by Sanchez-Vioque et al. (1999); the estimated molecular weights of subunits from NaOH extraction as subunits of vicilins, were 50, 37, 35, 33, 19, and 15 kDa and these subunits are comparable to 7S protein subunits (50, 35, 33, 19, 15 and 13 kDa) reported by Gueguen (1991). Two minor bands, 54 and 10 kDa, which could be subunits of glutelins and 2S albumin were also found in isolates from NaOH extraction. A minor band with estimated MW 7 kDa appeared in all NaOH isolates could be a peptide of 2S albumin with MW 4-10 kDa (Shewry et al., 1995). Isolate C. Na-E (Figure 4.3.3: A) contained subunits of MW 96 kDa which could be the chickpea lipoxygenase (92 kDa) reported by Clemente et al. (2000); similar MW of lipoxygenase (93.3 kDa) reported by Sathe et al. (1987) was observed in soybean isolates (Figure 4.3.3) S. Na-E (A_S), S. Na-IP (B_S), S. Na-CIP (C_S), and S. Na-CP (D_S).

The estimated molecular weights of subunits of isolate C. C-CP (Table 4.3.1) were 42, 39, 35, 33, 24 and 23 kDa. These subunits are similar to the subunits of 11S and 7S proteins reported by Sanchez-Vioque et al. (1999) and Gueguen (1991), and suggest that isolate C. C-CP is comprised mainly the subunits of globulins. The estimated MW of subunits of isolate C. C-S were 55, 52, 39, 35 and 33 kDa; a minor band with estimated

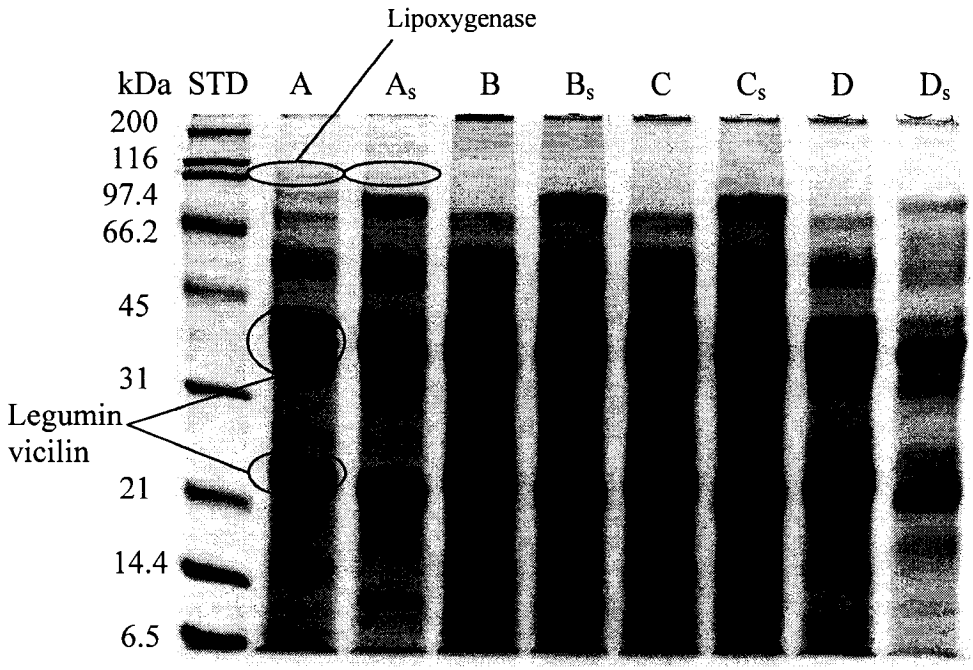

Figure 4.3.3: 12% SDS-PAGE of chickpea and soybean isolates from NaOH extraction. **A**: extract from chickpea (C. Na-E), **A_s**: extract from soybean (S. Na-E), **B**: isoprecipitate from chickpea (C. Na-IP), **B_s**: isoprecipitate from soybean (S. Na-IP), **C**: isoprecipitate after cryoprecipitation from chickpea (C. Na-CIP), **C_s**: isoprecipitate after cryoprecipitation from soybean (S. Na-CIP), **D**: cryoprecipitate from chickpea (C. Na-CP) and **D_s**: cryoprecipitate from soybean (S. Na-CP).

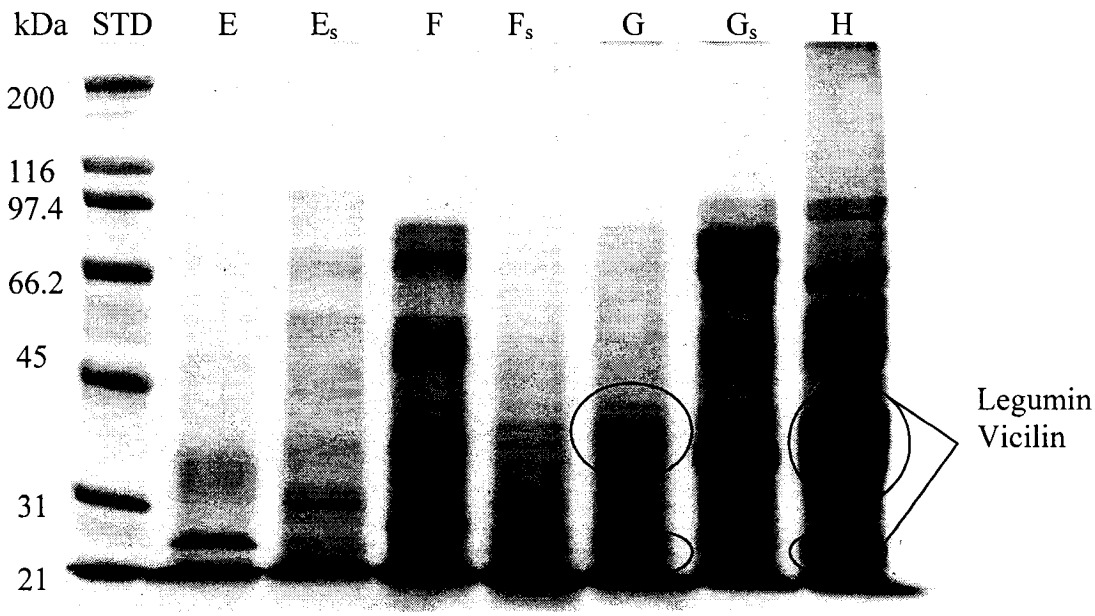

Figure 4.3.4: 12% SDS-PAGE of chickpea and soybean isolates from citric acid extraction. **E**: extract from chickpea (C. C-E), **E_s**: extract from soybean (S. C-E), **F**: supernatant from chickpea (C. C-S), **F_s**: supernatant from soybean (S. C-S), **G**: cryoprecipitate from chickpea (C. C-CP), **G_s**: cryoprecipitate from soybean (S. C-CP) and **H**: diluted alkaline isoprecipitate from chickpea (DLA-IP).

Table 4.3.1: The molecular weight (kDa) of subunits of chickpea proteins.

Protein / Subunits	MW reported by previous researchers (KDa)	MW obtained in present work from NaOH extraction					MW obtained in present work from citric acid extraction		
		C. Na-E	**C. Na-IP**	**C. Na-CIP**	**C. Na-CP**	**DLA-IP**	**C. C-E**	**C. C-S**	**C. C-CP**
Lip-oxygenase	92[1]	96^{B}	-	-	-	90^{B}	-	-	-
Legumin	47[2]	-	-	-	-	-	-	-	-
(11S)	40[2]	39^{A}, 40^{A}	39^{A}, 40^{A}	39^{A}, 40^{A}	39^{A}, 40^{A}	40^{A}	-	39^{A}	42^{B}, 39^{A}
	25[2]	26^{B}	26^{B}	26^{B}	26^{B}	-	26^{A}	25^{A}	-
	24[2]	23^{A}, 22^{A}	23^{A}, 22^{A}	23^{A}, 22^{A}	23^{A}, 22^{A}	23^{A}, 24^{A}	-	-	24^{A}, 23^{A}
Vicilin	50[3]	50^{B}	50^{B}	50^{B}	50^{B}	52^{A}	-	52^{A}	
(7S)	35[3]	35^{A}, 37^{A}	35^{A}, 37^{A}	35^{A}, 37^{A}	35^{A}, 37^{A}	37^{A}	-	35^{A}	35^{A}
	33[3]	33^{A}	33^{A}	33^{A}	-	33^{A}	-	33^{A}	33^{A}
	19[3]	19^{B}	19^{B}	19^{B}	19^{B}	-	-	-	-
	15[3]	15^{B}	15^{A}	15^{A}	15^{B}	-	-	-	-
	13[3]	-	-	-	-	-	-	-	-
Glutelin	55[4]	54^{B}	54^{B}	54^{B}	54^{B}	58^{B}	-	55^{B}	-
Albumin	12[5]	-	-	-	-	-	-	-	-
(2S)	10[5]	10^{B}	10^{B}	10^{B}	10^{B}	-	-	-	-
Unknown		84^{B}, 73^{B}, 7^{B}	73^{B}, 7^{B}	73^{B}, 7^{B}	73^{B}, 7^{B}	70^{B}	-	84^{B}, 75^{B}	-

1: Clemente et al., 2000.
2: Sanchez-Vioque et al., 1999.
3: Gueguen, 1991.
4: Takaiwa et al., 1999.
5: Vioque et al., 1999.
A: Major band, B: Minor band.

C. Na-E: extract from NaOH extraction.
C. Na-IP: isoelectric precipitate from NaOH extraction.
C. Na-CIP: isoelectric precipitate from NaOH extraction after cryoprecipitation.
C. Na-CP: cryoprecipitate from NaOH extraction.
DLA-IP: isoelectric precipitate from diluted NaOH extraction.
C. C-E: extract from citric acid extraction.
C. C-S: supernatant from citric acid extraction.
C. C-CP: cryoprecipitate from citric acid extraction.

MW 55 kDa could represent the glutelin subunits reported by Takaiwa et al. (1999); similar bands were also found in chickpea isolates from NaOH extraction.

4.4 Characterization of Chickpea Isolates by RP-HPLC, PAGE and ESI-MS

(i) Citric acid cryoprecipitate (C. C-CP) and NaOH cryoprecipitate (C. Na-CP)

RP-HPLC of the cryoprecipitate (C. C-CP) from citric acid extraction (Figure 4.4.1) gave 6 fractions as follows: CF1 (Rt: 22.2 min), CF2 (Rt: 23.8 min), CF3 (Rt: 25.5), CF4 (Rt: 28.0 min), CF5 (Rt: 30.2 min) and CF6 (Rt: 33.9 min); the cryoprecipitate (C. Na-CP) from NaOH extraction (Figure 4.4.2) gave 6 fractions: GF1 (Rt: 6.5 min), GF2 (Rt: 14.0 min), GF3 (Rt: 23.0 min), GF4 (Rt: 25.4 min), GF5 (Rt: 27.5 min) and GF6 (Rt: 33.2 min). The major peaks of cryoprecipitate C. C-CP and C. Na-CP appeared after Rt 30 min.

Fractions obtained from RP-HPLC were subjected to Native-PAGE, SDS-PAGE and ESI-MS techniques. Figure 4.4.3 and Figure 4.4.4 show the Native-PAGE patterns of fractions from cryoprecipitate C. C-CP and C. Na-CP, respectively. Bands were observed in fractions CF2 and CF6; a band on the top of gel in CF3 to CF6 could be protein aggregates which could not pass through the gel. Figure 4.4.4 shows a relatively intense band (MW under 66 kDa) in GF3, GF4 and GF5; a prominent band of MW 66 kDa was observed in fraction GF5. Figure 4.4.5 and Figure 4.4.6 show the SDS-PAGE electrophoretic patterns of fractions from cryoprecipitate C. C-CP and C. Na-CP, respectively. ESI-MS analysis of fractions (CF) from cryoprecipitate C. C-CP showed an intensive background noise in the m/z spectrum and the data not shown could not be interpreted. Four interpreted m/z spectra of fractions (GF3, GF4, GF5 and GF6) were

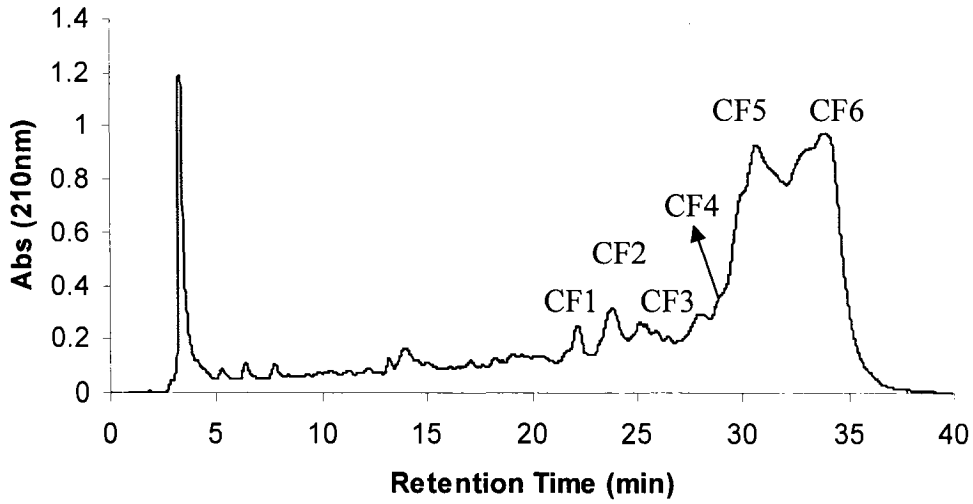

Figure 4.4.1: RP-HPLC chromatogram of chickpea cryoprecipitate (C. C-CP) from citric acid extraction.

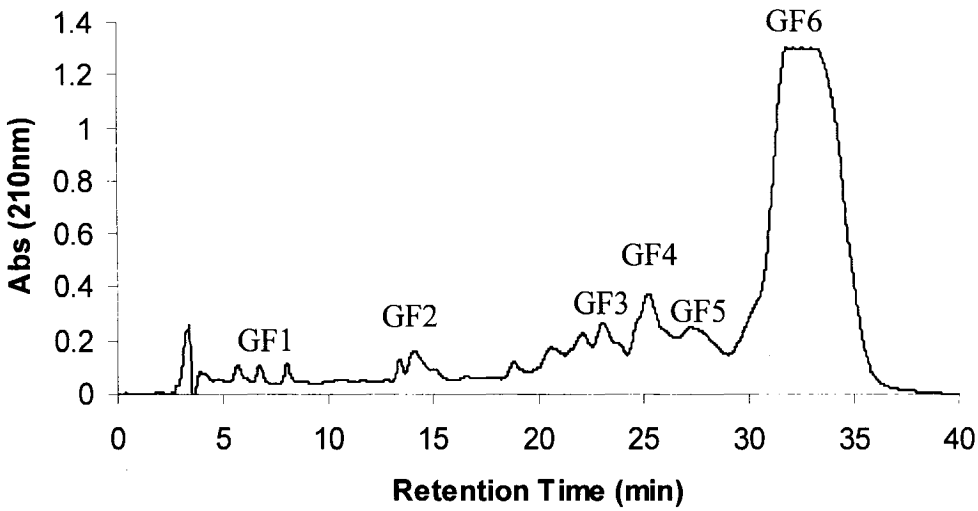

Figure 4.4.2: RP-HPLC chromatogram of chickpea cryoprecipitate (C. Na-CP) from NaOH extraction.

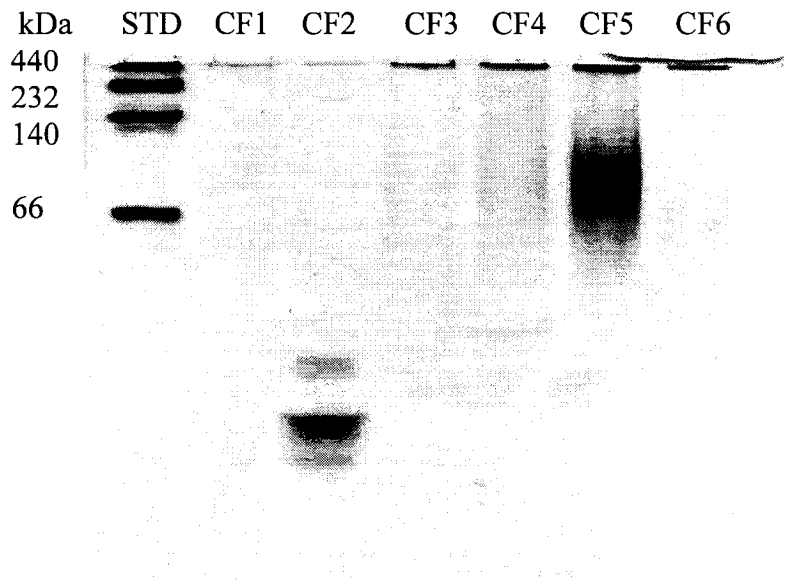

Figure 4.4.3: Native PAGE of RP-HPLC fractions (CF) of cryoprecipitate (C. C-CP) from citric acid extraction. STD = molecular weight standard markers.

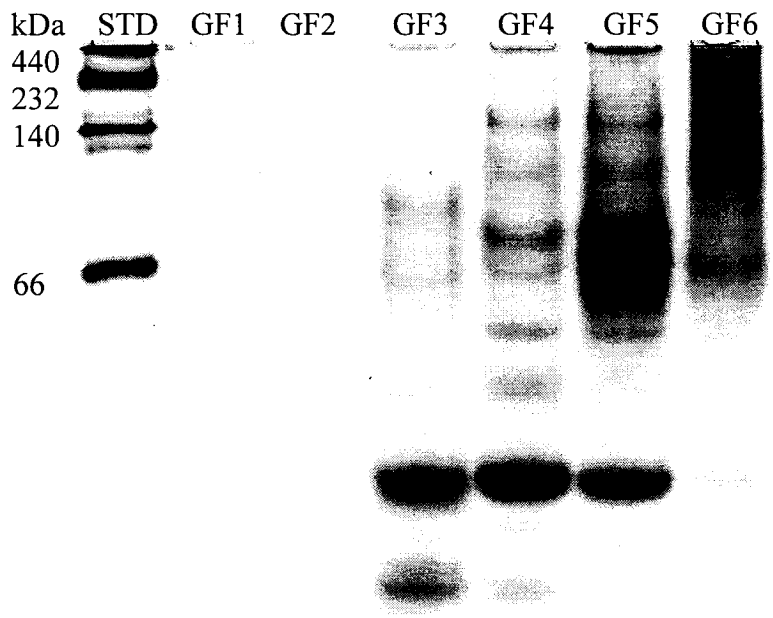

Figure 4.4.4: Native PAGE of RP-HPLC fractions (GF) of cryoprecipitate (C. Na-CP) from NaOH extraction. STD = molecular weight standard markers.

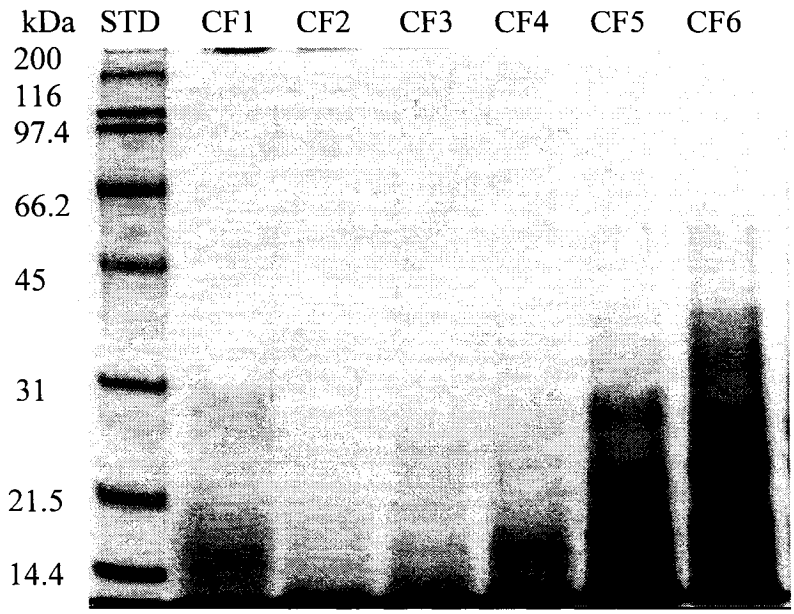

Figure 4.4.5: SDS-PAGE of RP-HPLC fractions (CF) of cryoprecipitate (C. C-CP) from citric acid extraction. STD = molecular weight standard markers.

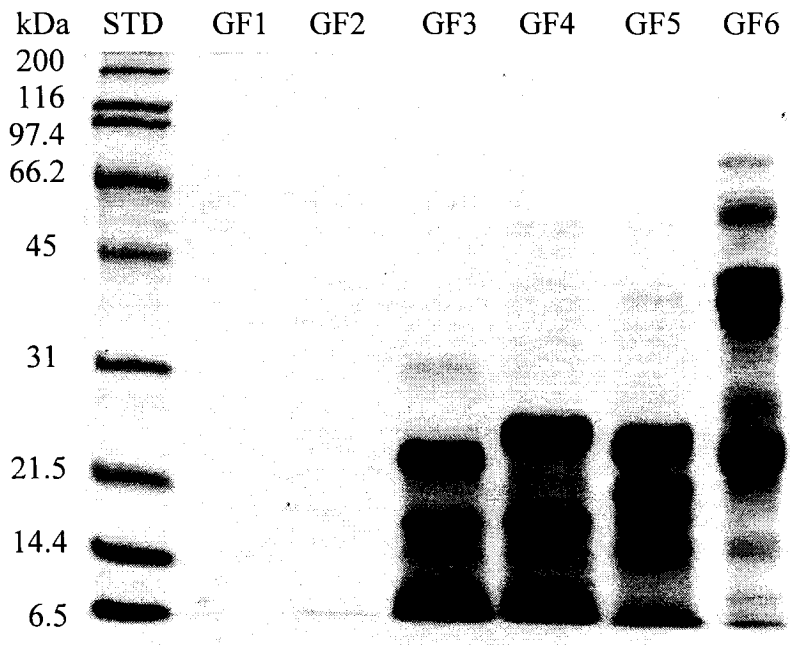

Figure 4.4.6: SDS-PAGE of RP-HPLC fractions (GF) of cryoprecipitate (C. Na-CP) from NaOH extraction. STD = molecular weight standard markers.

shown in Figure 4.4.7-Figure 4.4.10.

The combined information from characterization of chickpea cryoprecipitate C. C-CP and C. Na-CP including the RP-HPLC retention times of fractions, the estimated MW of subunits of fractions from SDS-PAGE and the interpreted MW of subunits of fractions from ESI-MS were summarized in Table 4.4.1. The estimated molecular weights of major subunits from SDS-PAGE (Figure 4.4.5) of fraction CF were 15.8 kDa (CF4), 18.9 and 22.1 kDa (CF5), and 15.8, 19.3 and 22.8 kDa (CF6); these subunits represent the subunits of legumin and vicilin proteins. The CF fractions contained relatively high amounts of 7S subunits with MW ranging from 15.8~19.3 kDa. This is similar to the cryoprecipitate from citric acid extraction which comprised mainly 7S subunits (Abdolgader, 2000) from soybean cryoprecipitated proteins obtained from a similar extraction method.

In Figure 4.4.6, the major subunits of fraction GF3, GF4 and GF5 are located at MW 22.0 (GF3), 23.1 (GF4) and 22.4 (GF5) representing the basic subunits of legumins (25~23 kDa; Vairinhos and Murray, 1982); several minor subunits of vicilins can be found in the MW ranging from 15.2 to 19.0 kDa. These results suggest that the fractions obtained from C. Na-CP are vicilin-rich and legumin-rich proteins. The major subunits of fraction GF6 have MW 41.1, 37.9, 35.3, 25.7 and 21.5 kDa which are similar to the subunits of 11S and 7S proteins reported by Sanchez-Vioque et al. (1999) and Gueguen (1991). In ESI-MS analysis for GF fractions, the interpreted MW (14013 Da) from fraction CF3 (Figure 4.4.7) could be a subunit of vicilin which appeared in SDS-PAGE (MW 15.2 kDa); in fraction GF4 and GF5, the interpreted MW of 16092 and 16094 Da could correspond to vicilin subunits whereas the same subunits appeared at MW 17.0 kDa in SDS-PAGE; in fraction GF6, the interpreted MW of 53549, 35466, and 14648 Da were

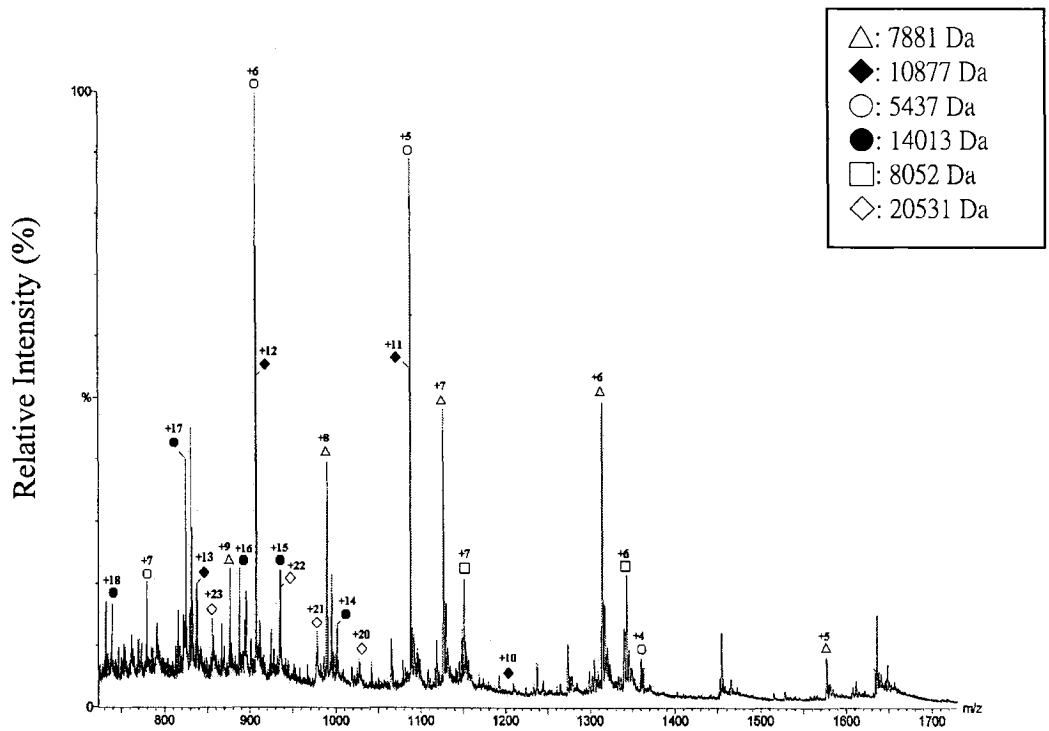

Figure 4.4.7: Interpreted ESI-MS spectrum (m/z) of fraction GF3 of cryoprecipitate (C. Na-CP) from NaOH extraction.

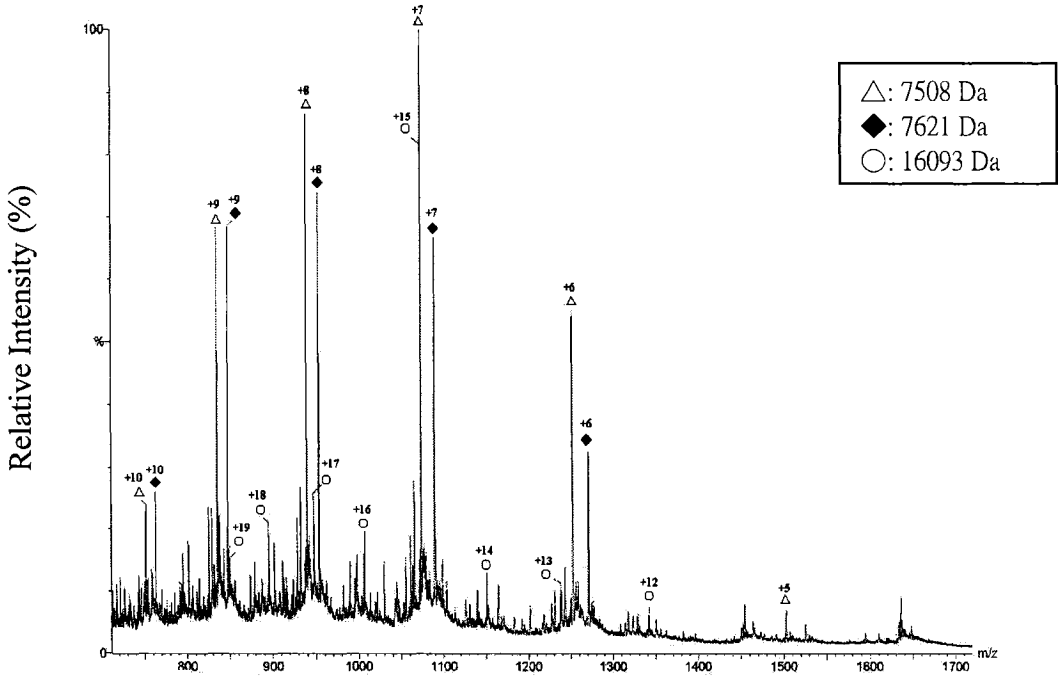

Figure 4.4.8: Interpreted ESI-MS spectrum (m/z) of fraction GF4 of cryoprecipitate (C. Na-CP) from NaOH extraction.

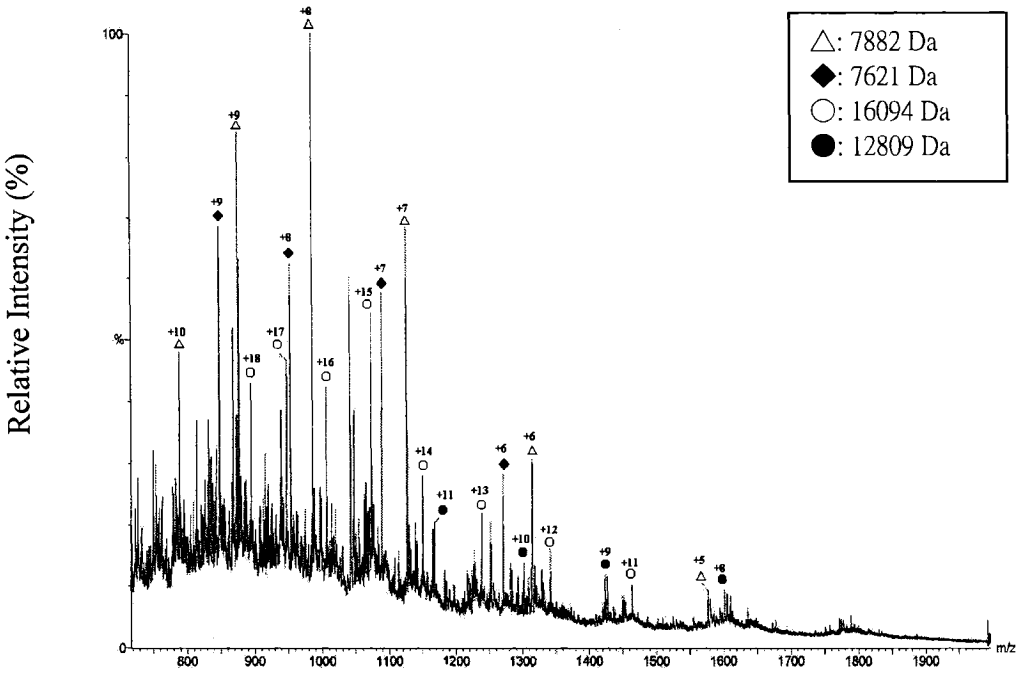

Figure 4.4.9: Interpreted ESI-MS spectrum (m/z) of fraction GF5 of cryoprecipitate (C. Na-CP) from NaOH extraction.

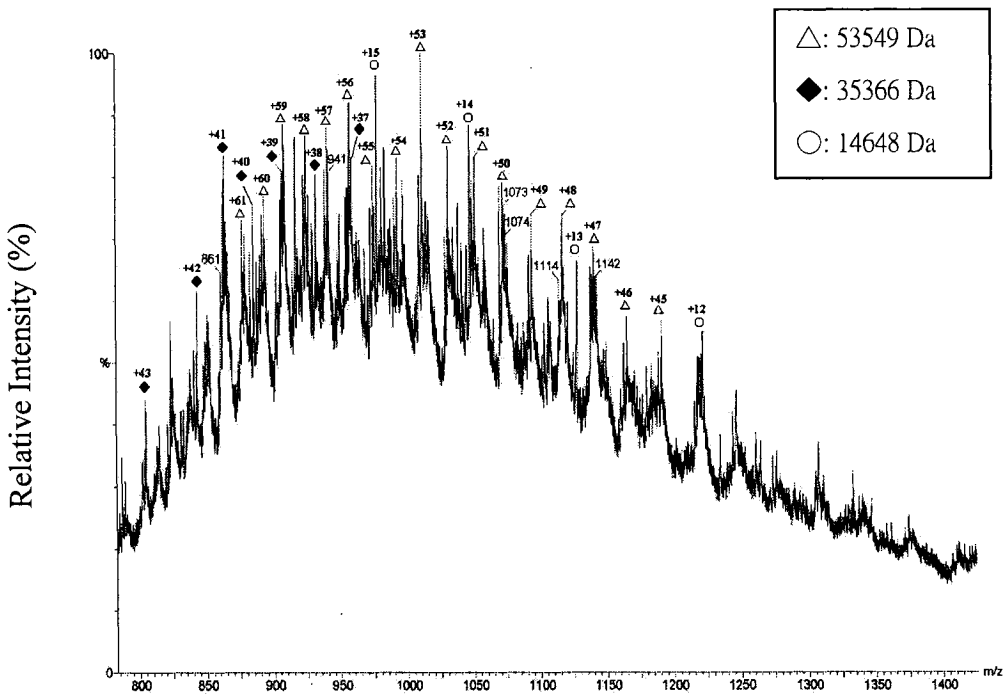

Figure 4.4.10: Interpreted ESI-MS spectrum (m/z) of fraction GF6 of cryoprecipitate (C. Na-CP) from NaOH extraction.

Table 4.4.1: Characterization of chickpea protein isolates C. C-CP and C. Na-CP.

Isolate	RP-HPLC Fractions	Rt (min)	Estimated MW of subunits from SDS-PAGE (kDa)	Interpreted MW of subunits from ESI-MS (Da)	Reported subunits (kDa)
C. C-CP	CF4	28.0	15.8[A], 13.6	ND	Legumin 47[1]
	CF5	30.2	28.1, 22.1[A], 18.9[A]	ND	40[1] 25[1]
	CF6	33.9	32.9, 27.1, 22.8[A], 19.3[A], 15.8[A]	ND	24[1]
C. Na-CP	GF3	23.0	29.1, 22.0[A], 17.1, 15.2, 8.6[A]	20531, 14013, 10877, 8052, 7881, 5437	Vicilin 50[2]
	GF4	25.4	49.2, 23.1[A], 17.0, 15.2, 8.6, 6.8	16093, 7621, 7508	35[2] 33[2]
	GF5	27.5	37.9, 22.4[A], 19.0, 17.0, 15.2	16094, 12809, 7882, 7621	19[2] 15[2]
	GF6	33.2	63.1, 51.3, 41.1[A], 37.9[A], 35.3[A], 25.7[A], 21.5[A], 15.2,	53549, 35366, 14648	13[2]

1: Sanchez-Vioque et al., 1999.
2: Gueguen, 1991.
A: Major band.
ND: Not determined.

C. Na-CP: cryoprecipitate from NaOH extraction.
C. C-CP: cryoprecipitate from citric acid extraction.

noted in SDS-PAGE as 51.3, 35.3 and 15.2 kDa, respectively. These subunits could represent the subunits from vicilins reported by Gueguen (1991).

(ii) NaOH isoelectric precipitate (C. Na-IP) and NaOH isoelectric precipitate after cryoprecipitation (C. Na-CIP)

The RP-HPLC chromatogram of isoelectric precipitate (C. Na-IP) from NaOH extraction (Figure 4.4.11) was similar to that of the isoelectric precipitate (C. Na-CIP) from NaOH extraction after cryoprecipitation (Figure 4.4.12); both gave 4 fractions: EF1 (Rt: 23.4 min), FF1 (Rt: 23.6 min), EF2 (Rt: 25.6 min), FF2 (Rt: 25.5 min), EF3 (Rt: 27.8 min), FF3 (Rt: 28.3 min), EF4 (Rt: 33.0 min) and FF4 (Rt: 33.7 min). Native-PAGE also demonstrated similarities in the fractions of isolate C. Na-IP and C. Na-CIP (Figure 4.4.13 and Figure 4.4.14); for fractions EF4 and FF4, they showed protein aggregates that could not migrate in the gel.

Figure 4.4.15 and Figure 4.4.16 show the SDS-PAGE of fractions from isoelectric precipitate (C. Na-IP) and isoeletric precipitate (C. Na-CIP) after cryoprecipitation. The interpreted ESI-MS spectra of fractions EF and FF from isoelectric precipitate C. NaIP and C. NaCIP are shown in Figure 4.4.17-Figure 4.4.24; Table 4.4.2 summarizes the results of RP-HPLC of fractions, the MW of subunits of fractions from SDS-PAGE and the interpreted MW from ESI-MS analysis. SDS-PAGE demonstrated very faint bands in fraction EF1 and FF1. One major subunit of MW 24.1 kDa and 24.6 kDa from fractions EF2 and FF2 respectively can be considered as a subunit of 11S legumins; several major bands from fraction EF3 and FF3 can be considered as subunits of vicilins (MW 17.3 and 15.3 kDa from fraction EF2 and MW 18.3 kDa from fraction FF2). The estimated MW of major bands from fraction EF4 and FF4 were 41.3 (EF4), 42.5 (FF4), 37.4 (FF4), 33.2

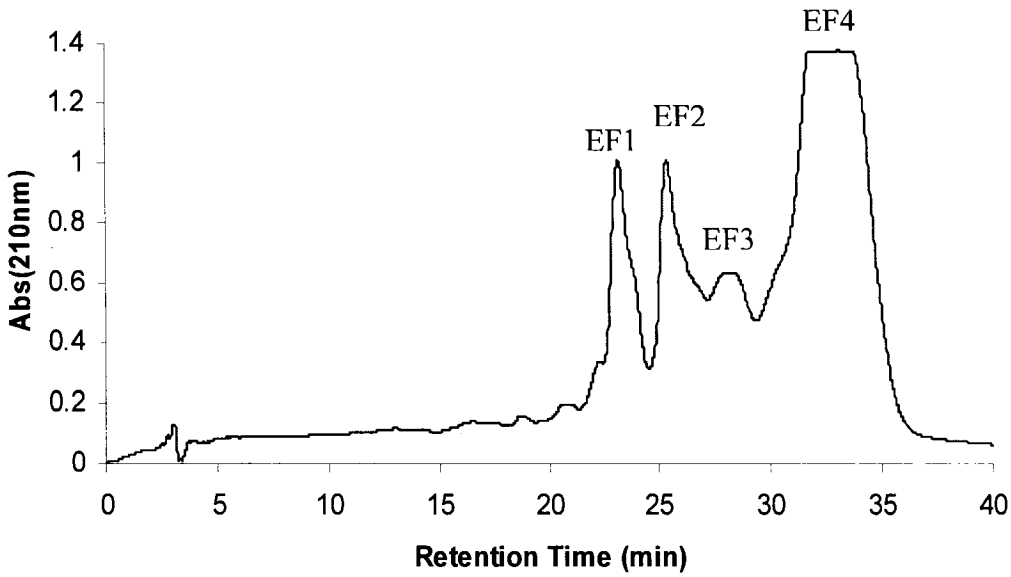

Figure 4.4.11: RP-HPLC chromatogram of chickpea isoelectric precipitate (C. Na-IP) from NaOH extraction.

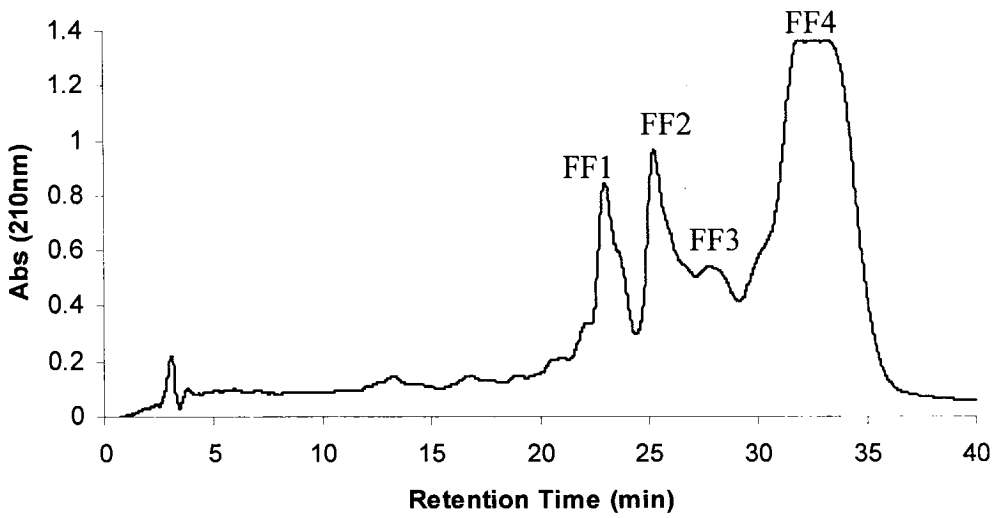

Figure 4.4.12: RP-HPLC chromatogram of chickpea isoelectric precipitate (C. Na-CIP) from NaOH extraction after cryoprecipitation.

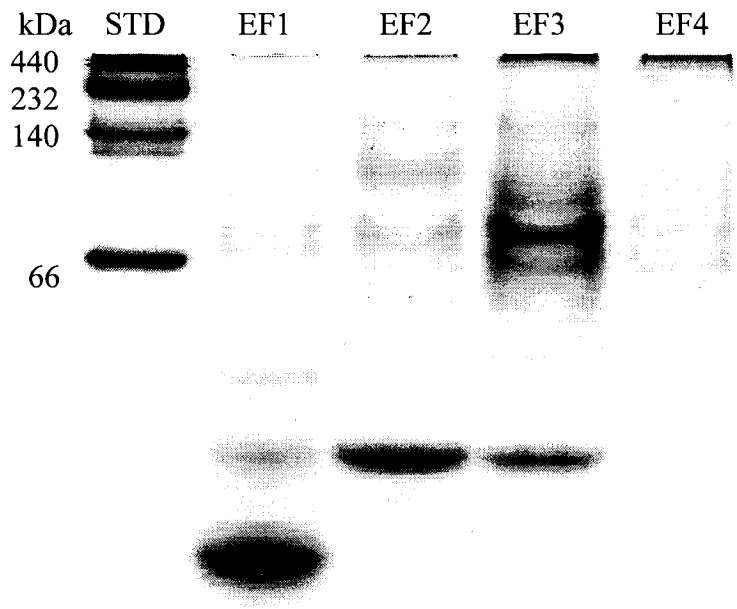

Figure 4.4.13: Native PAGE of RP-HPLC fractions (EF) of isoelectric precipitate (C. Na-IP) from NaOH extraction. STD = molecular weight standard markers.

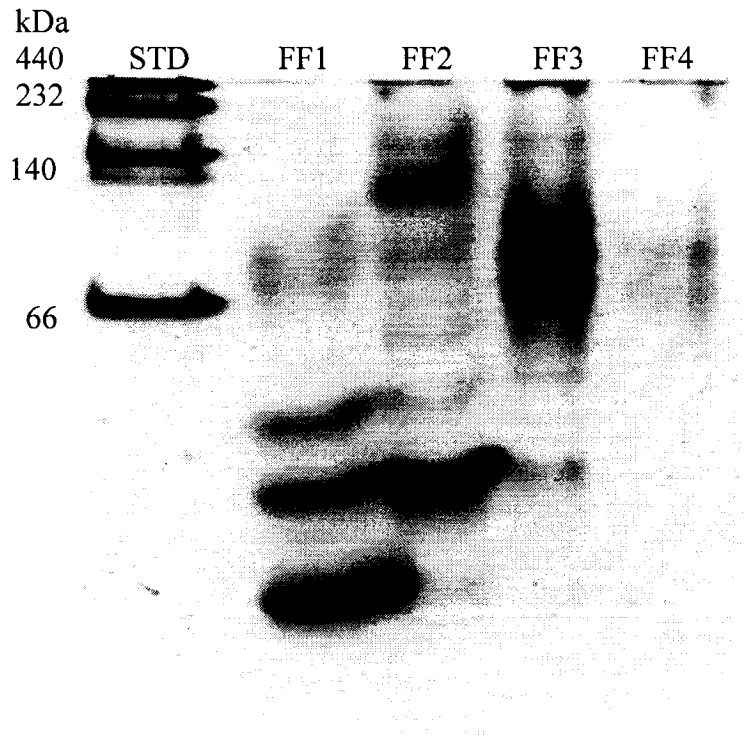

Figure 4.4.14: Native PAGE of RP-HPLC fractions (FF) of isoelectric precipitate (C. Na-CIP) from NaOH extraction after cryoprecipitation. STD = molecular weight standard markers.

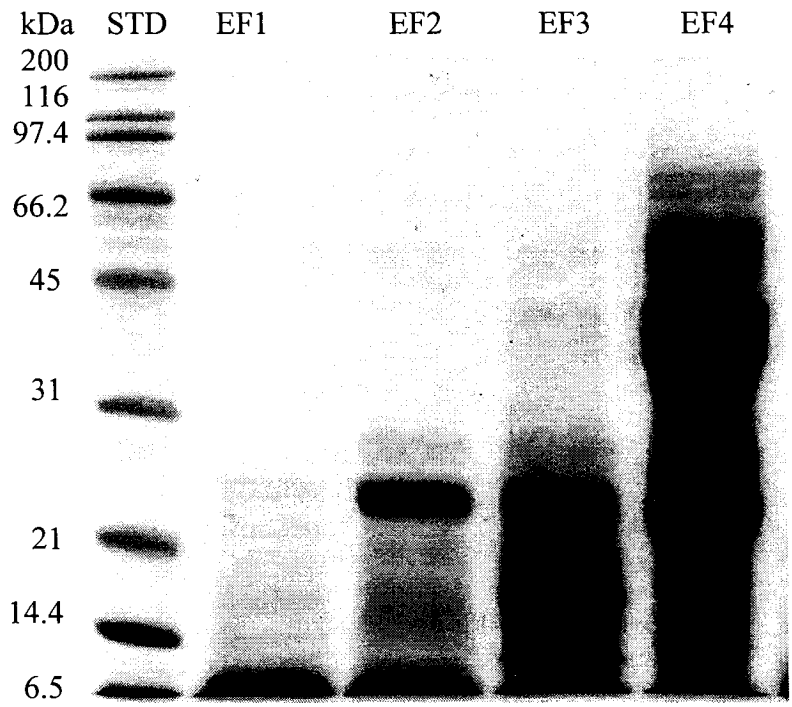

Figure 4.4.15: SDS-PAGE of RP-HPLC fractions (EF) of isoelectric precipitate (C. Na-IP) from NaOH extraction. STD = molecular weight standard markers.

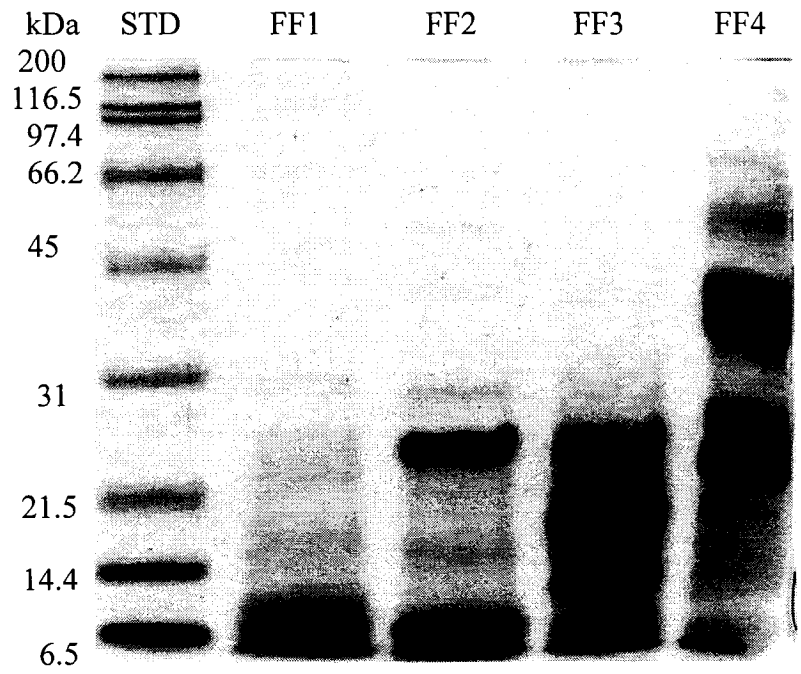

Figure 4.4.16: SDS-PAGE of RP-HPLC fractions (FF) of isoelectric precipitate (C. Na-CIP) from NaOH extraction after cryoprecipitation. STD = molecular weight standard markers.

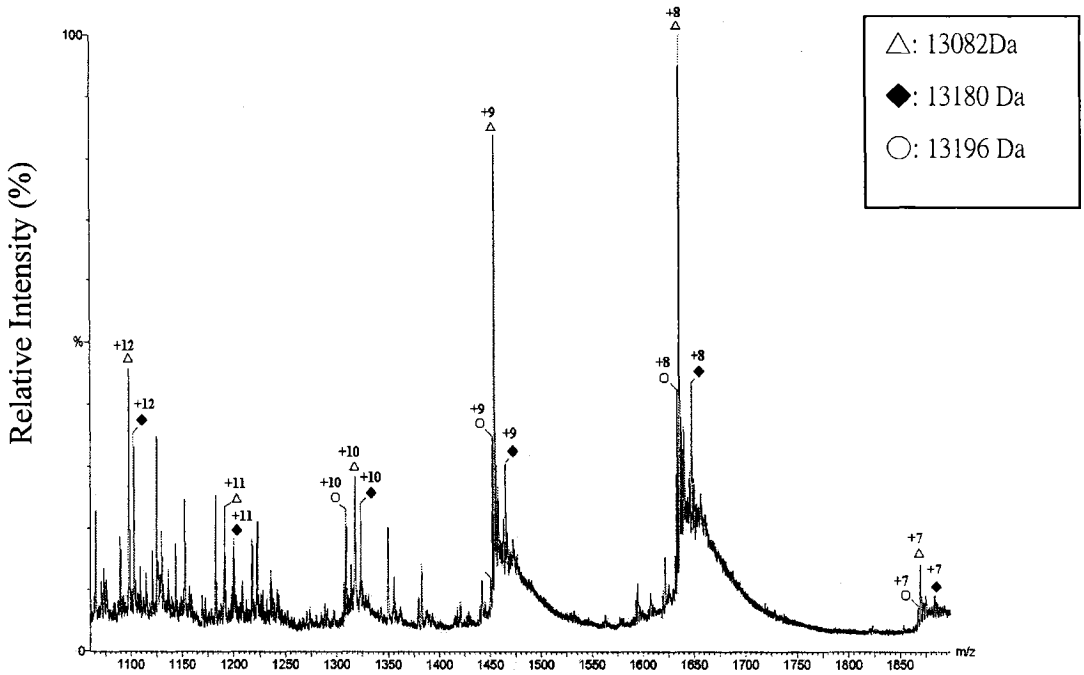

Figure 4.4.17: Interpreted ESI-MS spectrum (m/z) of fraction EF1 of isoelectric precipitate (C. Na-IP) from NaOH extraction.

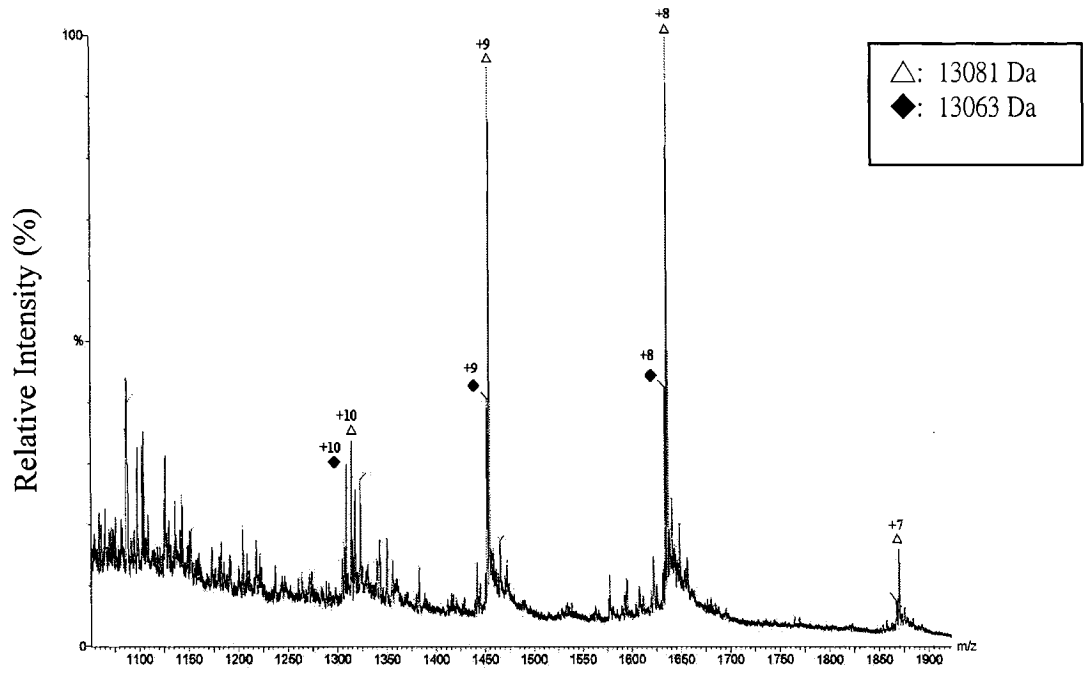

Figure 4.4.18: Interpreted ESI-MS spectrum (m/z) of fraction FF1 of isoelectric precipitate (C. Na-CIP) after cryoprecipitation from NaOH extraction.

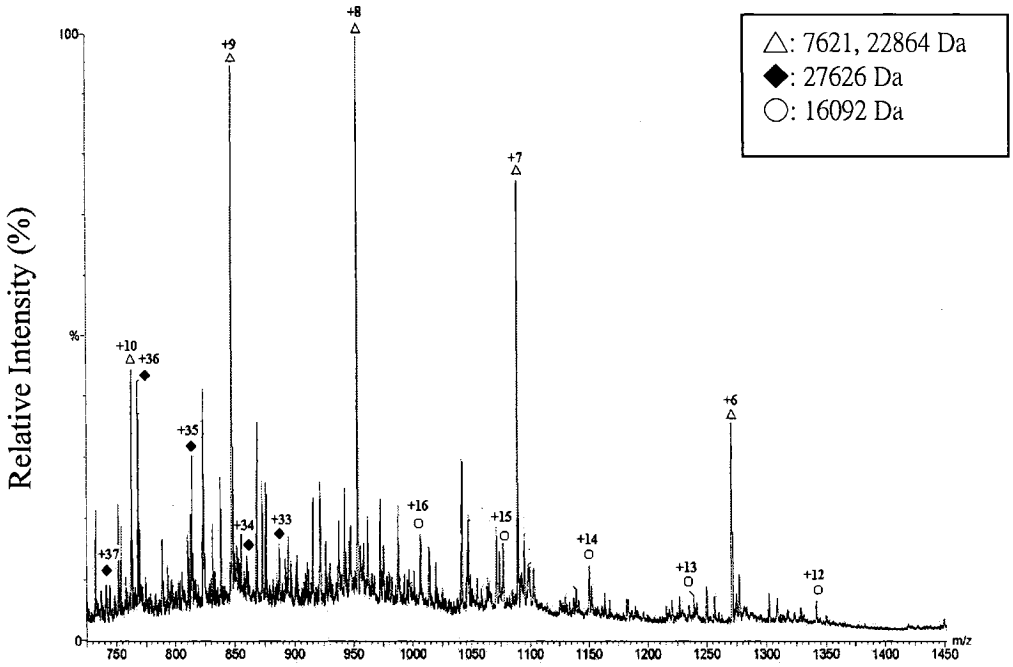

Figure 4.4.19: Interpreted ESI-MS spectrum (m/z) of fractioin EF2 of isoelectric precipitate (C. Na-IP) from NaOH extraction.

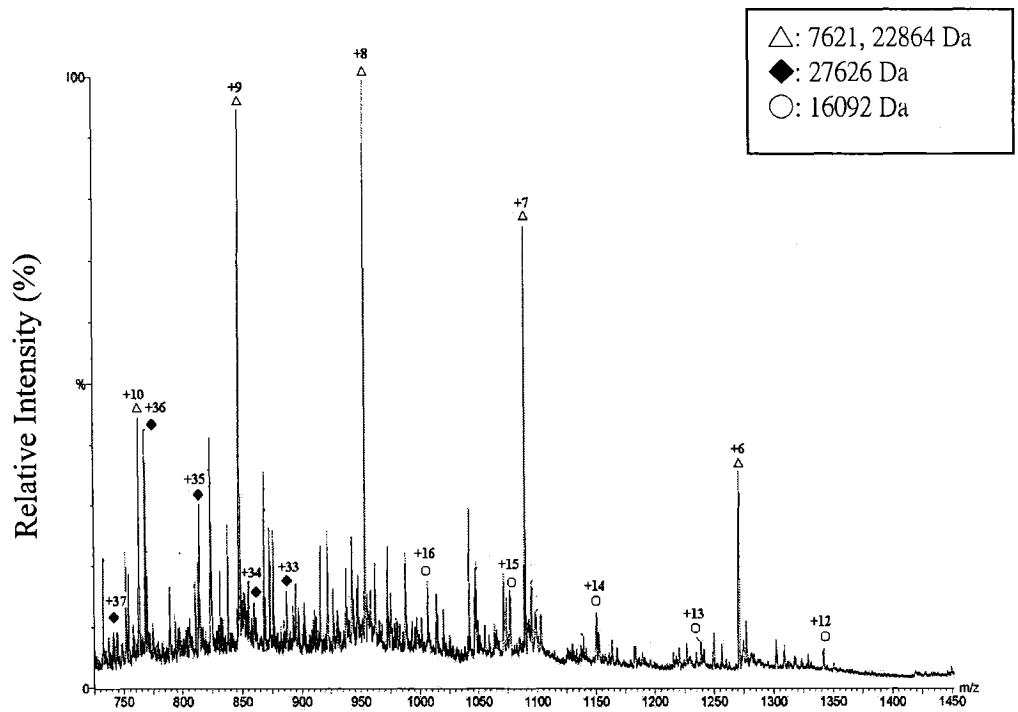

Figure 4.4.20: Interpreted ESI-MS spectrum (m/z) of fraction FF2 of isoelectric precipitate (C. Na-CIP) after cryoprecipitation from NaOH extraction.

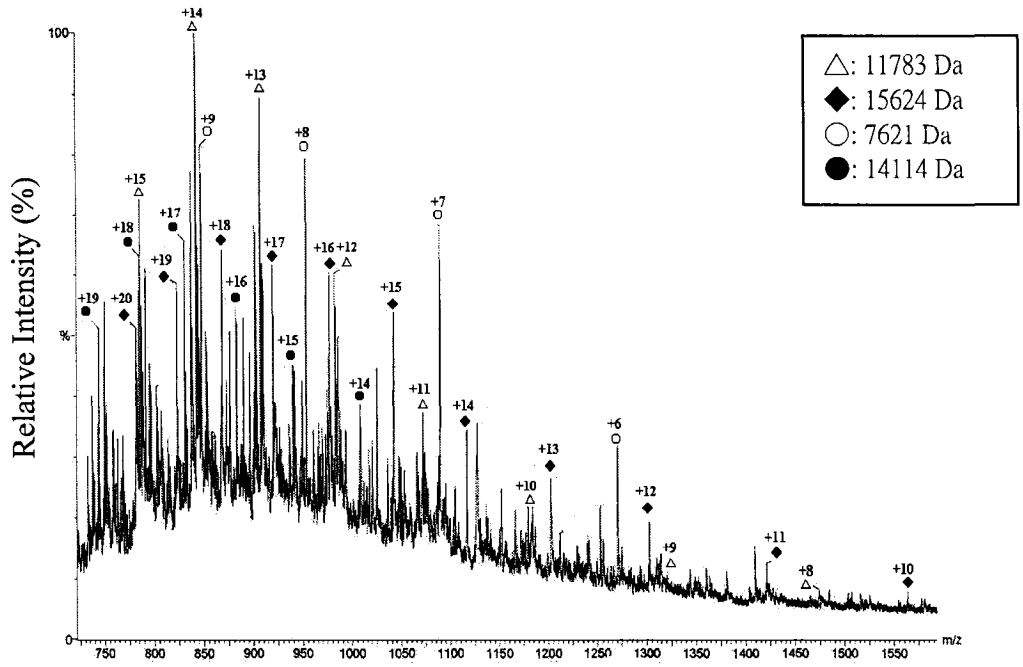

Figure 4.4.21: Interpreted ESI-MS spectrum (m/z) of fraction EF3 of isoelectric precipitate (C. Na-IP) from NaOH extraction.

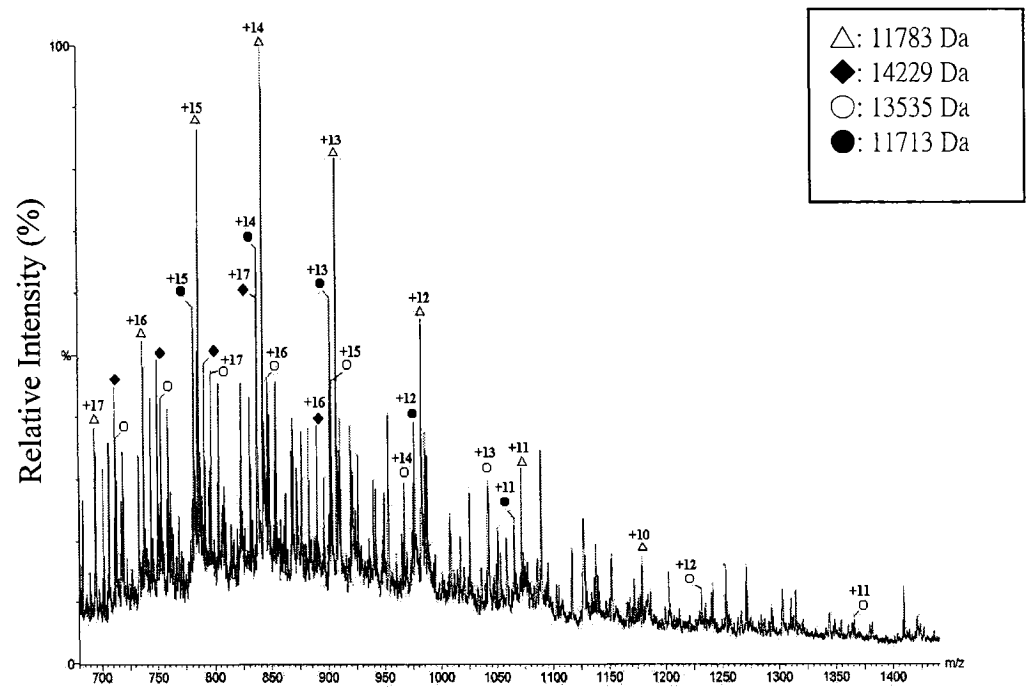

Figure 4.4.22: Interpreted ESI-MS spectrum (m/z) of fraction FF3 of isoelectric precipitate (C. Na-CIP) after cryoprecipitation from NaOH extraction.

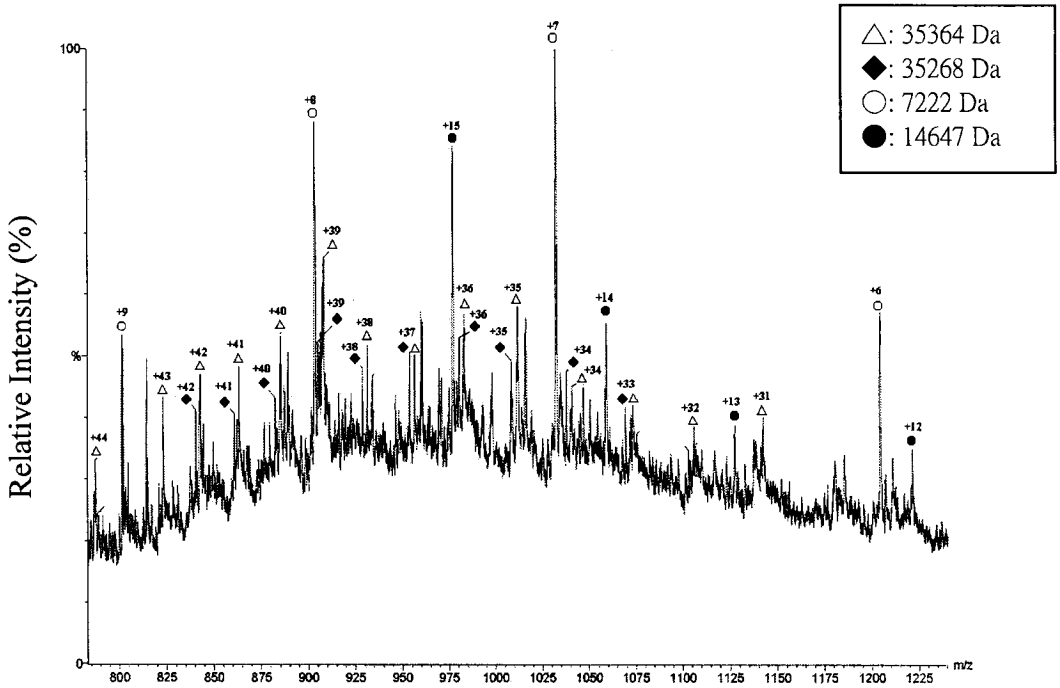

Figure 4.4.23: Interpreted ESI-MS spectrum (m/z) of fraction EF4 of isoelectric precipitate (C. Na-IP) from NaOH extraction.

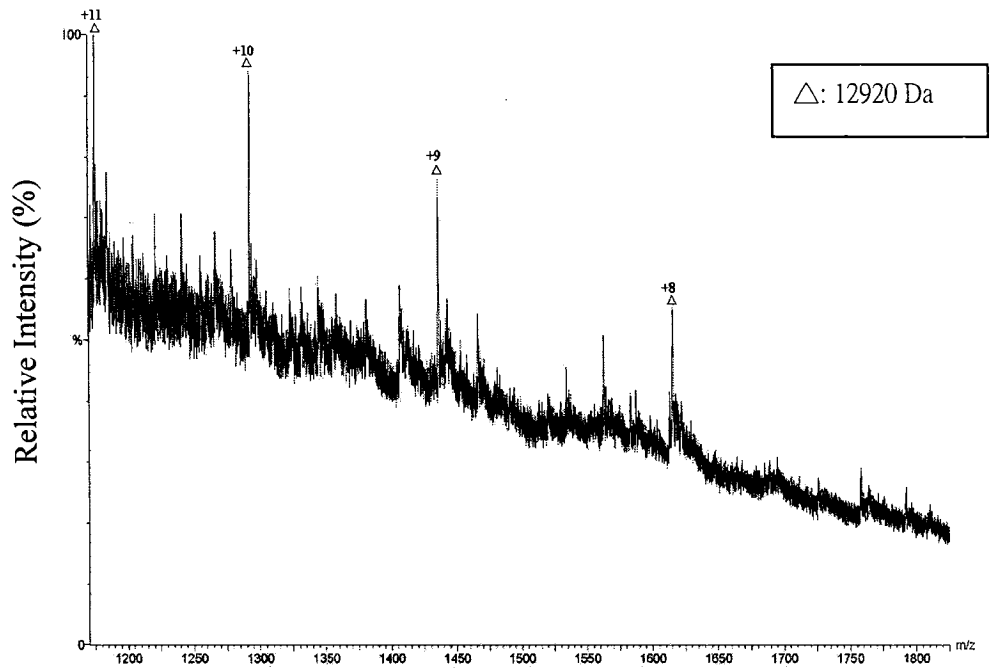

Figure 4.4.24: Interpreted ESI-MS spectrum (m/z) of fraction FF4 of isoelectric precipitate (C. Na-CIP) after cryoprecipitation from NaOH extraction.

Table 4.4.2: Characterization of chickpea protein isolates C. Na-IP and C. Na-CIP.

Isolate	RP-HPLC Fractions	Rt (min)	Estimated MW of subunits from SDS-PAGE (kDa)	Interpreted MW of subunits from ESI-MS (Da)	Reported subunits (kDa)
C. Na-IP	EF1	23.4	24.6, 22.2, 17.0, 13.5	13196, 13180, 13082	Legumin 47[1]
	EF2	25.6	28.0, 24.1[A], 20.8, 15.3, 7.4	27626, 22864, 16092, 7621	40[1] 25[1]
	EF3	27.8	28.0,24.1, 20.8,17.3[A], 15.3[A], 10.8[A]	15624, 14114, 11783, 7621	24[1]
	EF4	33.0	72.9, 51.5, 41.3[A], 33.2[A], 27.7, 24.6[A], 21.5[A], 18.4, 14.8, 11.4	35364, 35268, 14647, 7222	
C. Na-CIP	FF1	23.6	23.1, 16.9, 7.1	13081, 13063	Vicilin 50[2]
	FF2	25.5	29.0, 24.6[A], 15.9, 7.1	27626, 22864, 16092, 7621	35[2] 33[2]
	FF3	28.3	24.5, 18.3[A], 10.9[A]	14229, 13535, 11783, 11713	19[2] 15[2]
	FF4	33.7	73.4, 54.7, 42.5[A], 37.4[A], 26.8, 23.1[A], 19.8,	12920	13[2]

1: Sanchez-Vioque et al., 1999.
2: Gueguen, 1991.
A: Major band.

C. Na-IP: isoelectric precipitate from NaOH extraction.
C. Na-CIP: isoelectric precipitate from NaOH extraction after cryoprecipitation.

(EF4), 23.1 (FF4), 24.6 (EF4) and 21.5(EF4) kDa; these bands could represent the subunits of 11S and 7S proteins reported by Sanchez-Vioque et al. (1999) and Gueguen (1991).

In Figure 4.4.17 and Figure 4.4.18, the ESI-MS interpretation for both fraction EF1 and FF1 suggested that a MW 13081 ±1 Da which might be a subunit of vicilins or albumins; this subunits was not detected by SDS-PAGE. The ESI-MS MW 27626, 22864, 16092 and 7621 Da from fraction EF2 and FF2 can be related to the MW 28.0, 24.1, 15.3 and 7.4 kDa (EF2) or MW 29.0, 24.6, 15.9 and 7.kDa (FF2) subunits obtained by SDS-PAGE (Table 4.4.2). The ESI-MS interpreted MW of fraction EF3 were different from those of fraction FF3; the only common subunit in the two fractions is MW 11783 Da which can be related to the MW 10.9 kDa obtained by SDS-PAGE. Fraction EF3 also contained a subunit of MW 15624 Da which was also found in SDS-PAGE with a MW 15.3 kDa. The ESI-MS interpreted MW 35364 and 35268 from fraction EF4 can be related to the major MW 33.2 kDa obtained by SDS-PAGE.

(iii) NaOH isoelectric precipitate (C. Na-IP) and NaOH cryoprecipitate (C. Na-CP)

RP-HPLC, SDS-PAGE and ESI-MS results from NaOH isoelectric precipitate (C. Na-IP) and NaOH cryoprecipitate (C. Na-CP) are summarized in Table 4.4.3. Results from Native-PAGE (Figure 4.4.13 and Figure 4.4.4) showed that fractions EF1, EF2, EF3 and EF4 showed basically similar bands as fractions GF3, GF4, GF5 and GF6. These fractions presented similar retention time in RP-HPLC separation (Figure 4.4.11 and Figure 4.4.2). In SDS-PAGE (Figure 4.4.14 and Figure 4.4.6), fractions GF3 and GF4 contained more major subunits of MW 23.1, 22.0 and 8.6 kDa (Table 4.4.3) than those of fractions EF1 and EF2 (major subunits of MW 24.1) in similar Rt; the common ESI-MS

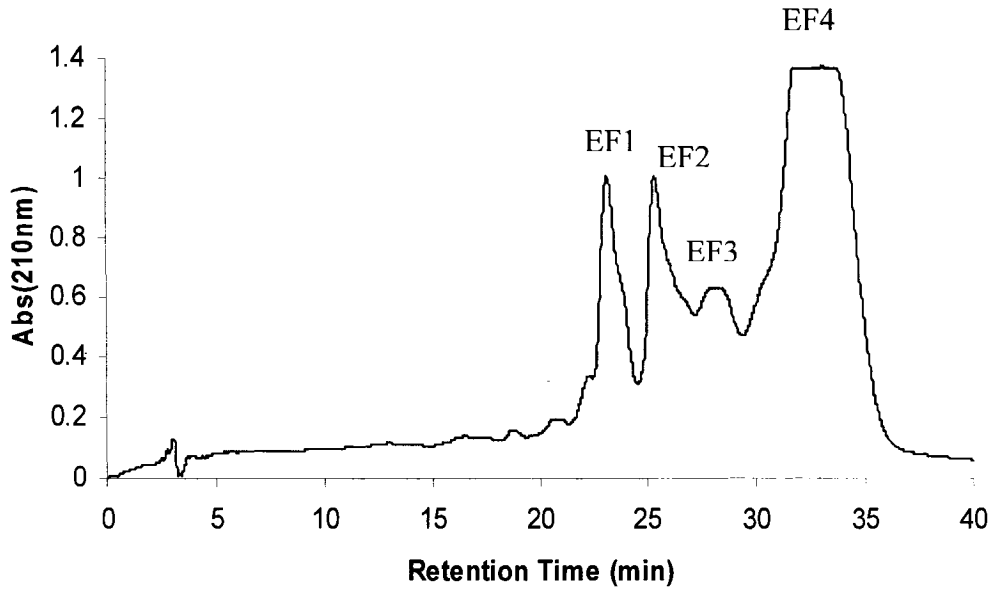

Figure 4.4.11 (page 50): RP-HPLC chromatogram of chickpea isoelectric precipitate (C. Na-IP) from NaOH extraction.

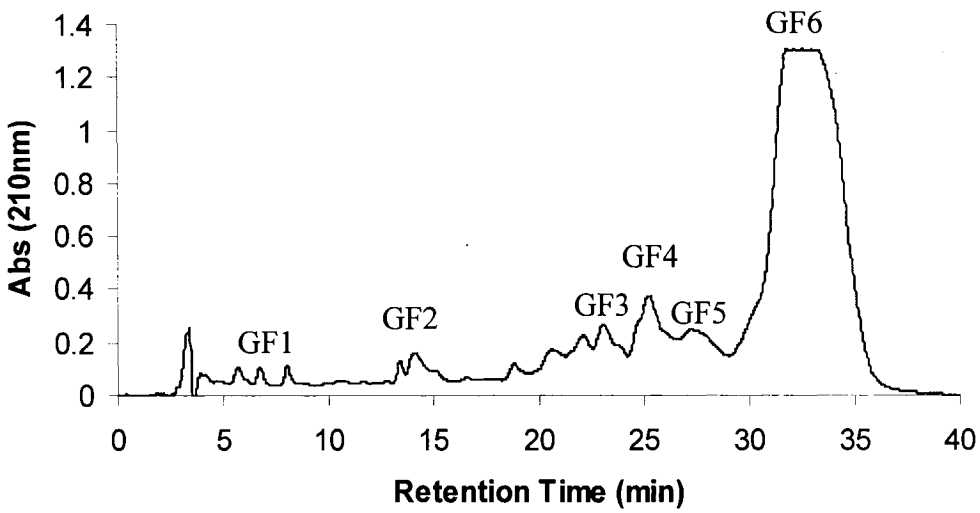

Figure 4.4.2 (page 42): RP-HPLC chromatogram of chickpea cryoprecipitate (C. Na-CP) from NaOH extraction.

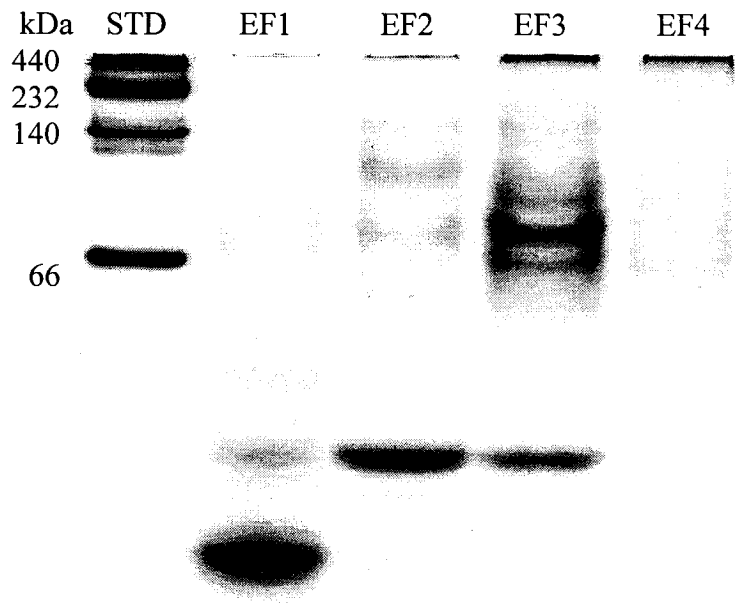

Figure 4.4.13 (page 51): Native PAGE of RP-HPLC fractions (EF) of isoelectric precipitate (C. Na-IP) from NaOH extraction. STD = molecular weight standard markers.

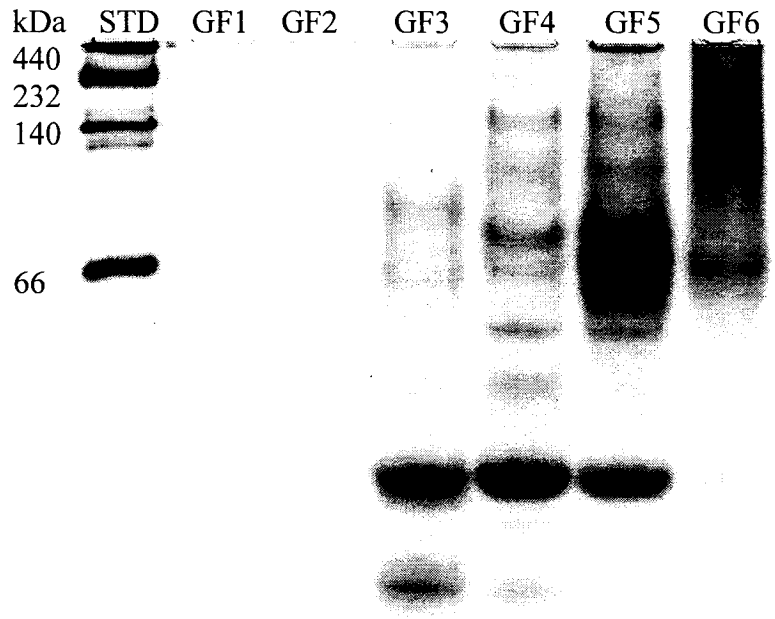

Figure 4.4.4 (page 43): Native PAGE of RP-HPLC fractions (GF) of cryoprecipitate (C. Na-CP) from NaOH extraction. STD = molecular weight standard markers.

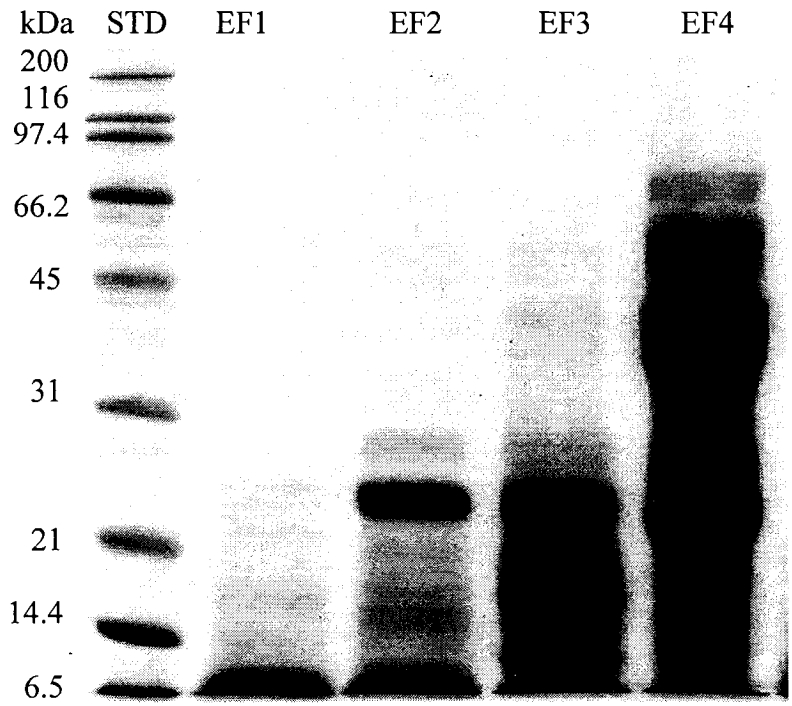

Figure 4.4.15 (page 52): SDS-PAGE of RP-HPLC fractions (EF) of isoelectric precipitate (C. Na-IP) from NaOH extraction. STD = molecular weight standard markers.

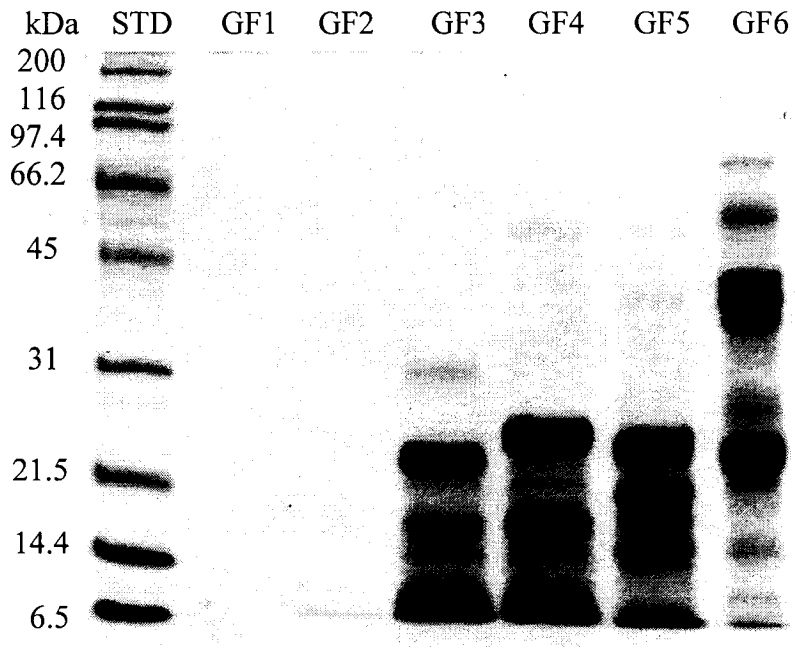

Figure 4.4.6 (page 44): SDS-PAGE of RP-HPLC fractions (GF) of cryoprecipitate (C. Na-CP) from NaOH extraction. STD = molecular weight standard markers.

Table 4.4.3: Characterization of chickpea protein isolates C. Na-IP and C. Na-CP.

Isolate	RP-HPLC Fractions	Rt (min)	Estimated MW of subunits from SDS-PAGE (kDa)	Interpreted MW of subunits from ESI-MS (Da)	Reported subunits (kDa)
C. Na-IP	EF1	23.4	24.6, 22.2, 17.0, 13.5	13196, 13180, 13082	Legumin 47[1]
	EF2	25.6	28.0, 24.1[A], 20.8, 15.3, 7.4	27626, 22864, 16092, 7621	40[1] 25[1]
	EF3	27.8	28.0,24.1, 20.8,17.3[A], 15.3[A], 10.8[A]	15624, 14114, 11783, 7621	24[1]
	EF4	33.0	72.9, 51.5, 41.3[A], 33.2[A], 27.7, 24.6[A], 21.5[A], 18.4, 14.8, 11.4	35364, 35268, 14647, 7222	
C. Na-CP	GF3	23.0	29.1, 22.0[A], 17.1, 15.2, 8.6[A]	20531, 14013, 10877, 8053, 7881, 5437	Vicilin 50[2]
	GF4	25.4	49.2, 23.1[A], 17.0, 15.2, 8.6, 6.8	16092, 7621, 7508	35[2] 33[2]
	GF5	27.5	37.9, 22.4[A], 19.0, 17.0, 15.2	16094, 12809, 7882, 7621	19[2] 15[2]
	GF6	33.2	63.1, 51.3, 41.1[A], 37.9[A], 35.3[A], 25.7[A], 21.5[A], 15.2,	53549, 35366, 14648	13[2]

1: Sanchez-Vioque et al., 1999.
2: Gueguen, 1991.
A: Major band.

C. Na-IP: isoelectric precipitate from NaOH extraction.
C. Na-CP: cryoprecipitate from NaOH extraction.

interpreted MW of subunit of fractions EF2 and GF4 is MW 16092 Da which can be related to MW 15.2 obtained by SDS-PAGE. At similar Rt~28 min, fractions EF3 and GF5 (Table 4.4.3) showed different major subunits of MW from SDS-PAGE (EF3: MW 17.3, 15.3 and 10.8 kDa; GF5: 22.4 kDa); the ESI-MS interpreted MW were also different. The bands in fraction EF4 have more intensity than those noted in fraction GF6 which could be due to the different treatment that each isolates received during their preparation. Fractions EF4 and GF5 (Rt~33 min) demonstrated similar major subunits of MW (EF4: 41.3, 33.2, 24.6 and 21.5 kDa; GF6: 41.1, 37.9, 35.3, 25.7 and 21.5 kDa); a MW of 35364 (EF4) or 35366 (GF6) Da from ESI-MS interpretation which can be a subunit of vicilin was identified as MW 35.3 kDa in SDS-PAGE.

(iv) NaOH isoelectric precipitate (C. Na-IP) and NaOH isoelectric precipitate (DLA-IP)

Figure 4.4.25 and Figure 4.4.11 (as shown previously) show the RP-HPLC chromatograms of diluted NaOH isoelectric precipitate (DLA-IP) and NaOH isoelectric precipitate (C. Na-IP). Isolate DLA-IP gave 5 fractions: HF1 (Rt: 18.5 min), HF2 (Rt: 21.6 min), HF3 (Rt: 25.6 min), HF4 (Rt: 26.7 min) and HF5 (Rt: 28.1 min); isolate C. Na-IP gave 4 fractions: EF1 (Rt: 23.4 min), EF2 (Rt: 25.6 min), EF3 (Rt: 27.8 min) and EF4 (Rt: 33.0 min). Both isolates were obtained from the same extraction (NaOH solution); however for isolate DLA-IP the extraction temperature was 55℃. This could explain the difference observed from RP-HPLC. Native-PAGE (Figure 4.4.26 and Figure 4.4.13) of fractions from isolate DLA-IP and fractions from C. Na-IP also demonstrated the differences between these two isolates. The SDS-PAGE of fractions from these two isolates (Figure 4.4.27 and Figure 4.4.15) showed that fractions HF contained subunits

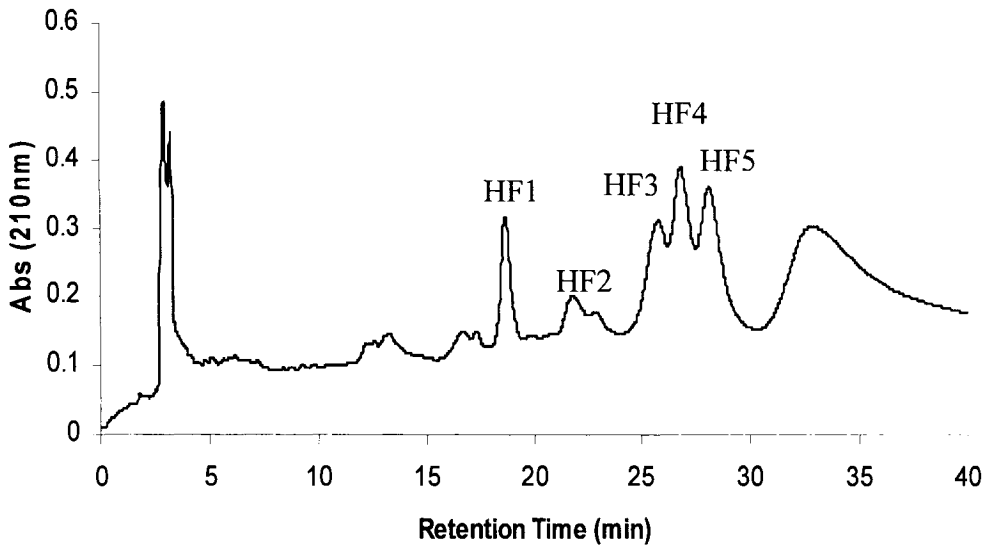

Figure 4.4.25: RP-HPLC chromatogram of chickpea isoelectric precipitate (DLA-IP) from diluted alkaline extraction at 55°C.

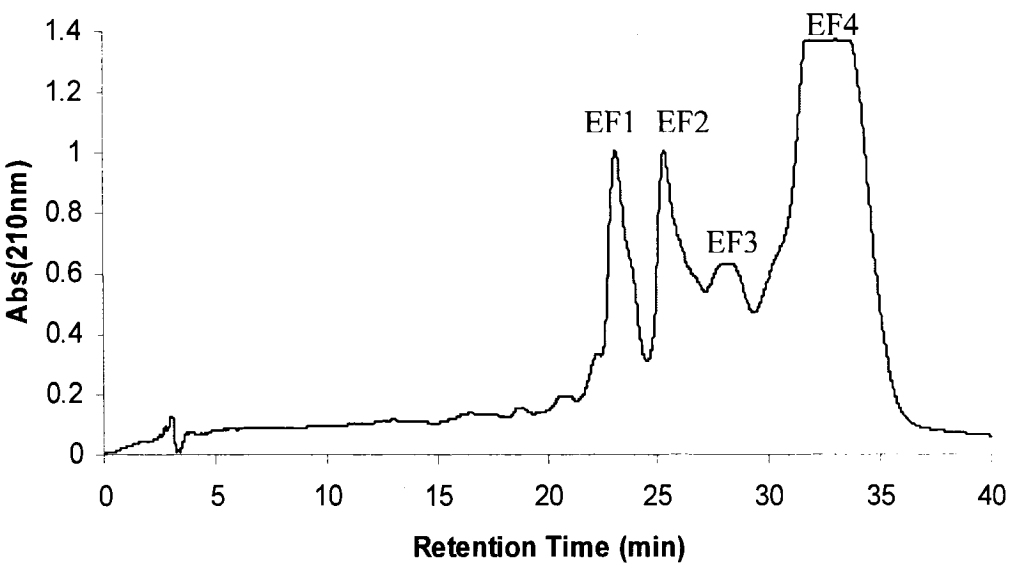

Figure 4.4.11 (page 50): RP-HPLC chromatogram of chickpea isoelectric precipitate (C. Na-IP) from NaOH extraction.

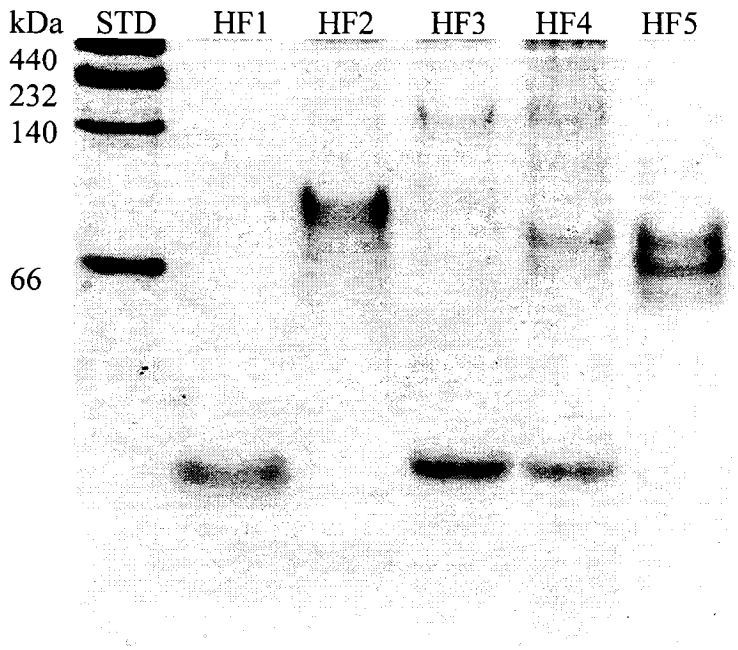

Figure 4.4.26: Native PAGE (10%) of RP-HPLC fractions (HF) of isoelectric precipitate (DLA-IP) from diluted NaOH extraction. STD = molecular weight standard markers.

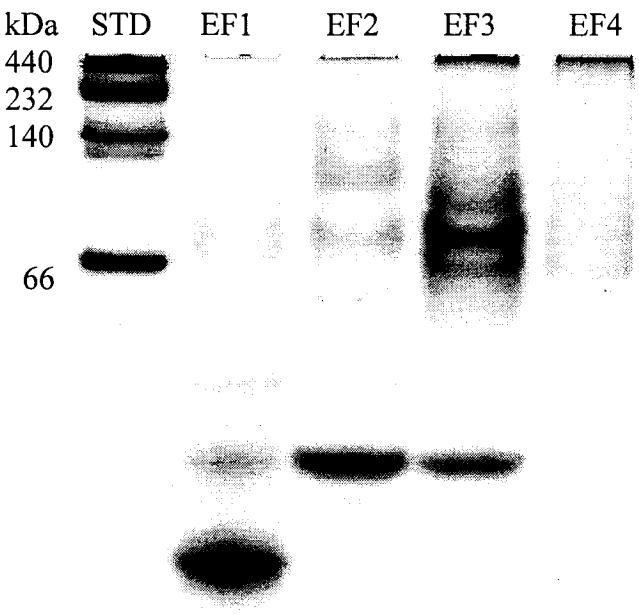

Figure 4.4.13 (page 51): Native PAGE of RP-HPLC fractions (EF) of isoelectric precipitate (C. Na-IP) from NaOH extraction. STD = molecular weight standard markers.

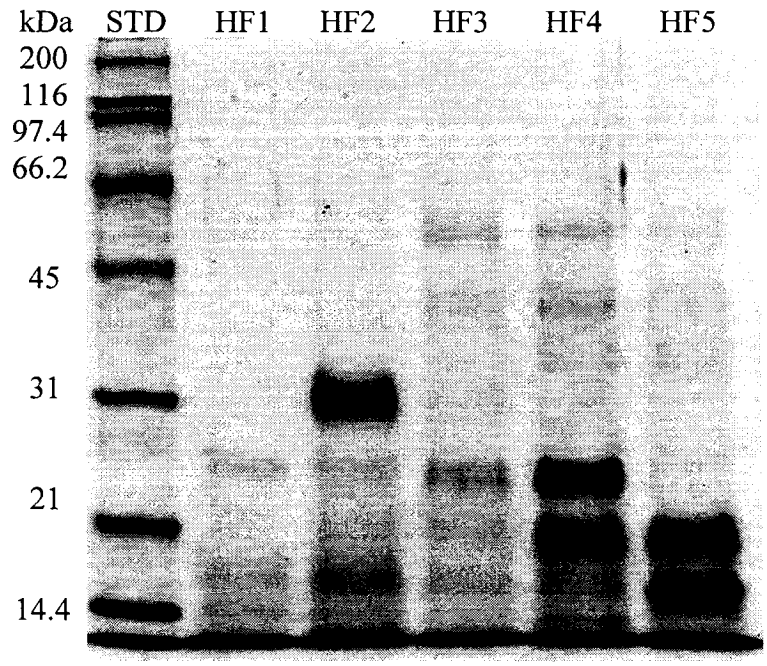

Figure 4.4.27: SDS-PAGE of fractions (HF) of isoelectric precipitate (DLA-IP) from diluted NaOH extraction. STD = molecular weight standard markers.

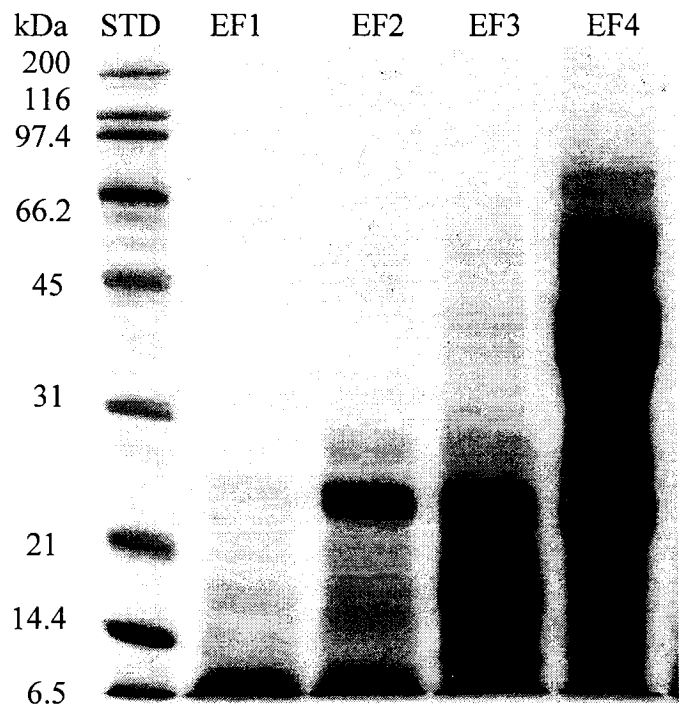

Figure 4.4.15 (page 52): SDS-PAGE of RP-HPLC fractions (EF) of isoelectric precipitate (C. Na-IP) from NaOH extraction. STD = molecular weight standard markers.

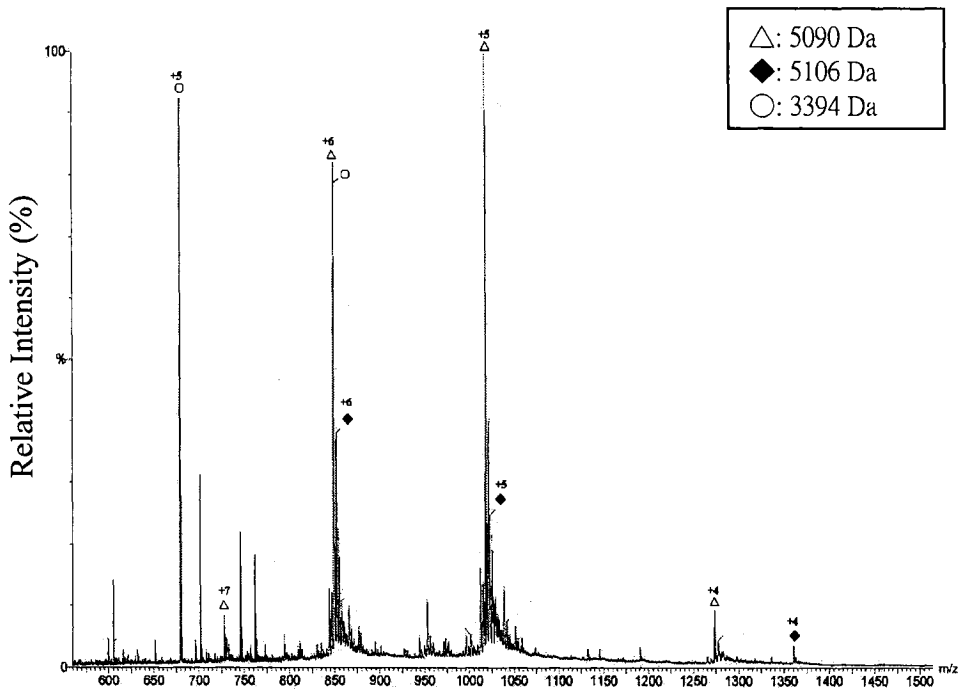

Figure 4.4.28: Interpreted ESI-MS spectrum (m/z) of fraction HF1 of isoelectric precipitate (DLA-IP) from diluted NaOH extraction at 55 °C.

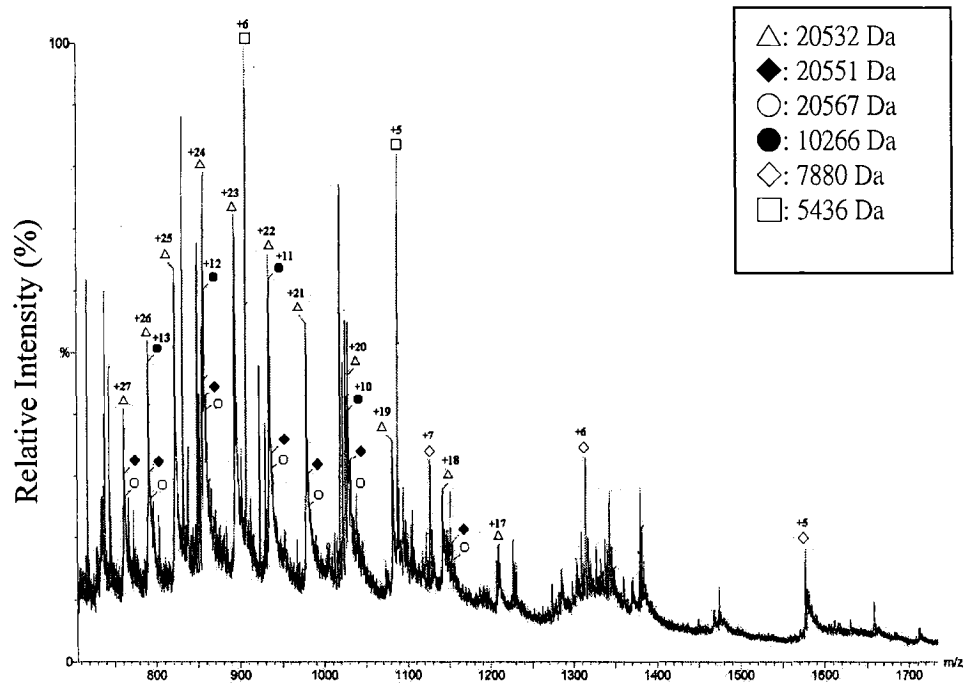

Figure 4.4.29: Interpreted ESI-MS spectrum (m/z) of fraction HF2 of isoelectric precipitate (DLA-IP) from diluted NaOH extraction at 55 °C.

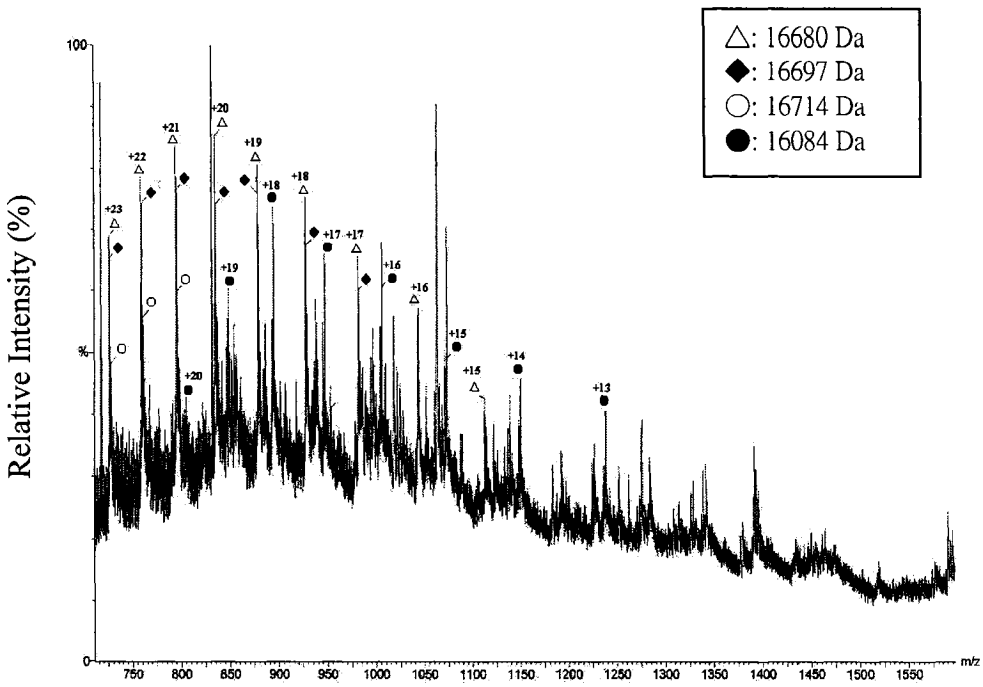

Figure 4.4.30: Interpreted ESI-MS spectrum (m/z) of fraction HF3 of isoelectric precipitate (DLA-IP) from diluted NaOH extraction at 55 °C.

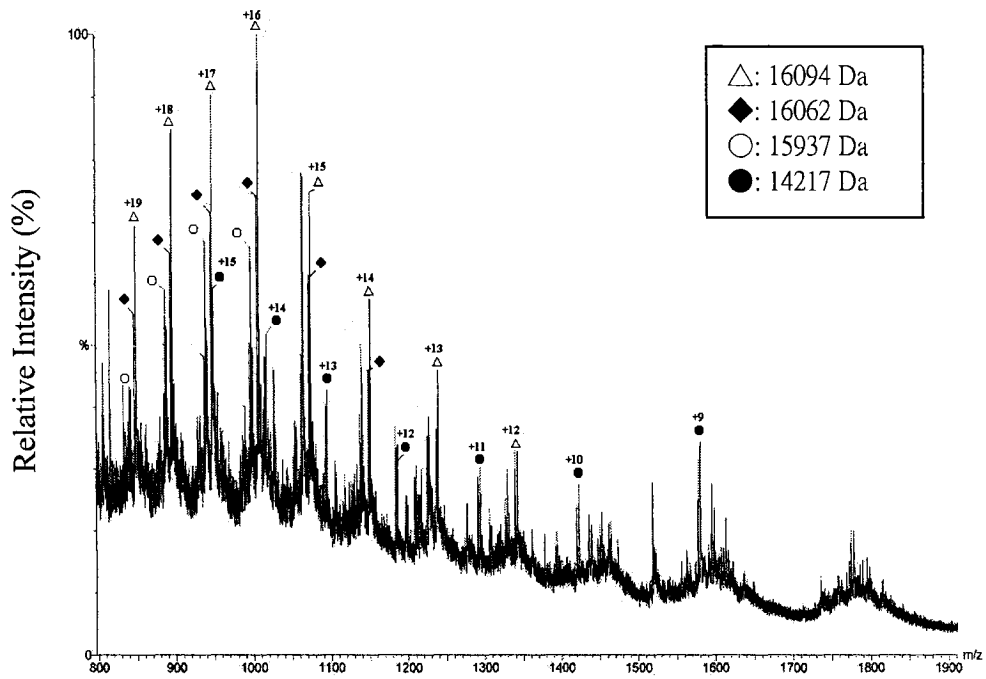

Figure 4.4.31: Interpreted ESI-MS spectrum (m/z) of fraction HF4 of isoelectric precipitate (DLA-IP) from diluted NaOH extraction at 55 °C.

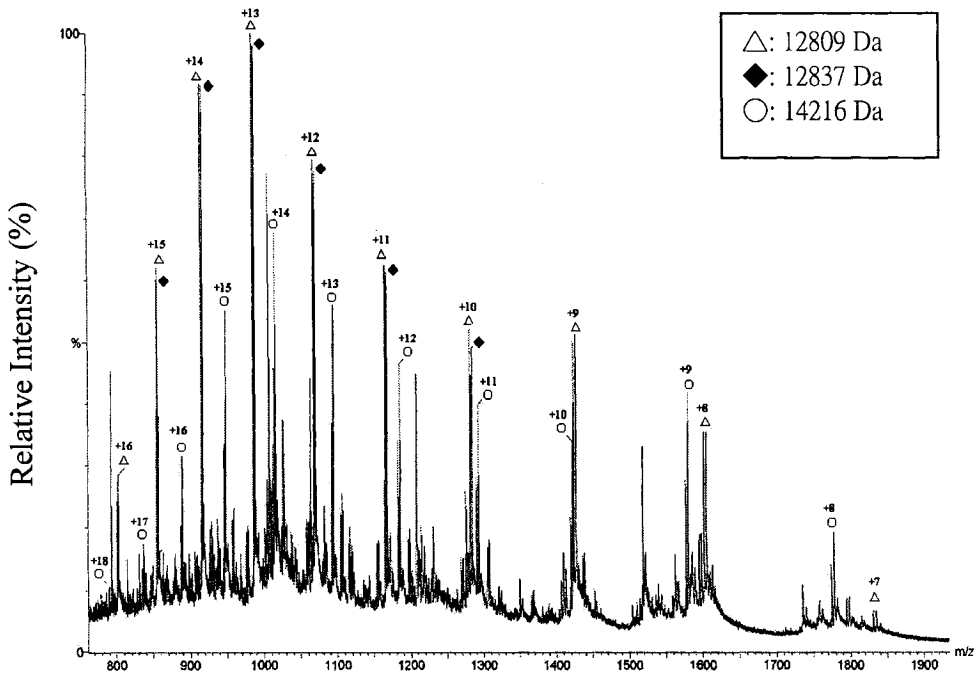

Figure 4.4.32: Interpreted ESI-MS spectrum (m/z) of fraction HF5 of isoelectric precipitate (DLA-IP) from diluted NaOH extraction at 55 °C.

Table 4.4.4: Characterization of chickpea protein isolates C. Na-IP and DLA-IP.

Isolate	RP-HPLC Fractions	Rt (min)	Estimated MW of subunits from SDS-PAGE (kDa)	Interpreted MW of subunits from ESI-MS (Da)	Reported subunits (kDa)
C. Na-IP	EF1	23.4	24.6, 22.2, 17.0, 13.5	13196, 13180, 13082	Legumin 47[1]
	EF2	25.6	28.0, 24.1[A], 20.8, 15.3, 7.4	27626, 22864, 16092, 7621	40[1] 25[1]
	EF3	27.8	28.0,24.1, 20.8,17.3[A], 15.3[A], 10.8[A]	15624, 14114, 11783, 7621	24[1]
	EF4	33.0	72.9, 51.5, 41.3[A], 33.2[A], 27.7, 24.6[A], 21.5[A], 18.4, 14.8, 11.4	35364, 35268, 14647, 7222	
DLA-IP	HF1	18.5	24.6, 16.5, 14.9	5106, 5090, 3394	Vicilin 50[2]
	HF2	21.6	49.2, 30.6[A], 16.5, 14.9	20532, 20551, 20567, 10266, 7880, 5436	35[2] 33[2]
	HF3	25.6	54.1, 42.9, 23.3[A], 19.5, 16.5	16714, 16680, 16697, 16084	19[2] 15[2]
	HF4	26.7	54.8, 42.3, 23.4[A], 19.0[A], 16.1, 14.3	16094, 16062, 15937, 14217	13[2]
	HF5	28.1	55.5, 42.9, 19.2[A], 16.1[A]	14216, 12837, 12809	

1: Sanchez-Vioque et al., 1999.
2: Gueguen, 1991.
A: Major band.

C. Na-IP: isoelectric precipitate from NaOH extraction.
DLA-IP: isoelectric precipitate from diluted NaOH extraction.

with lower MW ranging from 14-31 kDa in comparison to fractions EF. A summary of the results obtained from RP-HPLC, SDS-PAGE and ESI-MS is shown in Table 4.4.4. A major band from fraction HF2 with MW 30.6 Da could be the glutelin subunit (acidic subunits: 28-31 kDa in rice; Takaiwa et al., 1999); this subunit was not identified in cryoprecipitates and other isoprecipitated isolates. The major subunits of fraction HF4 (MW 23.4 and 19.0 kDa), and HF5 (MW 19.2 and 16.1 kDa) could represent subunits of vicilins. ESI-MS interpreted MW of fractions from the two protein isolates (Table 4.4.4) also showed differences in subunit MW. A subunit of MW 16094 Da can be found in fractions EF2, HF3 and HF4 and can be related to the MW 16.1 kDa from SDS-PAGE (HF3 and HF4). ESI-MS interpreted MW 20532 and 10266 Da from fraction HF2 could be fragments of glutelin subunits (MW 30.6 from SDS-PAGE).

(v) Citric acid extract (C. C-E) and NaOH extract (C. Na-E)

RP-HPLC of the extract C. C-E from citric acid extraction (Figure 4.4.33) gave 6 fractions: AF1 (Rt: 4.0 min), AF2 (Rt: 5.6 min), AF3 (Rt: 8.0 min), AF4 (Rt: 13.2 min), AF 5 (Rt: 14.0 min) and AF6 (Rt: 22.6 min); the extract C. Na-E from NaOH extraction (Figure 4.4.34) gave 4 fractions: DF1 (Rt: 5.8 min), DF2 (Rt: 14.3 min), DF3 (Rt: 22.2 min) and DF4 (Rt: 33.4 min). Figures 4.4.35 and Figure 4.4.36 show the Native-PAGE and SDS-PAGE of fraction from these two extracts. Native-PAGE of the fraction DF3 and AF6 showed similar bands. In SDS-PAGE, the major subunits of fraction DF3 and AF6 from both NaOH and citric acid extracts were MW 30.0 and 20.0 kDa which can be related to the acidic and basic subunit of glutelins (Takaiwa et al., 1999). Fraction DF4 with subunits MW 54.3, 41.1, 33.2 and 25.8 kDa can represent the subunits of globulins (MW from 21.5-45 kDa). Figure 4.4.37-Figure 4.4.39 showed the ESI-MS spectra of

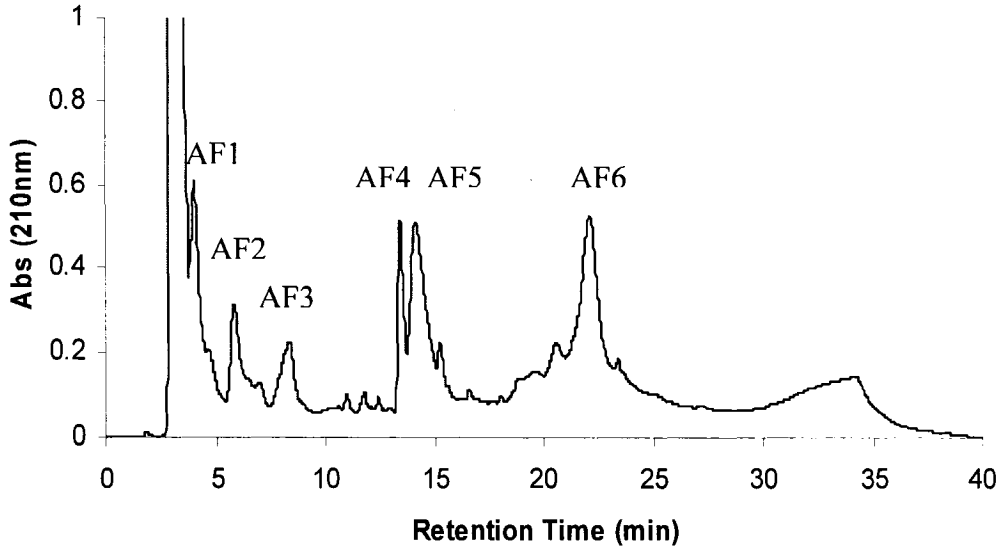

Figure 4.4.33: RP-HPLC chromatogram of chickpea extract (C. C-E) from the citric acid extraction.

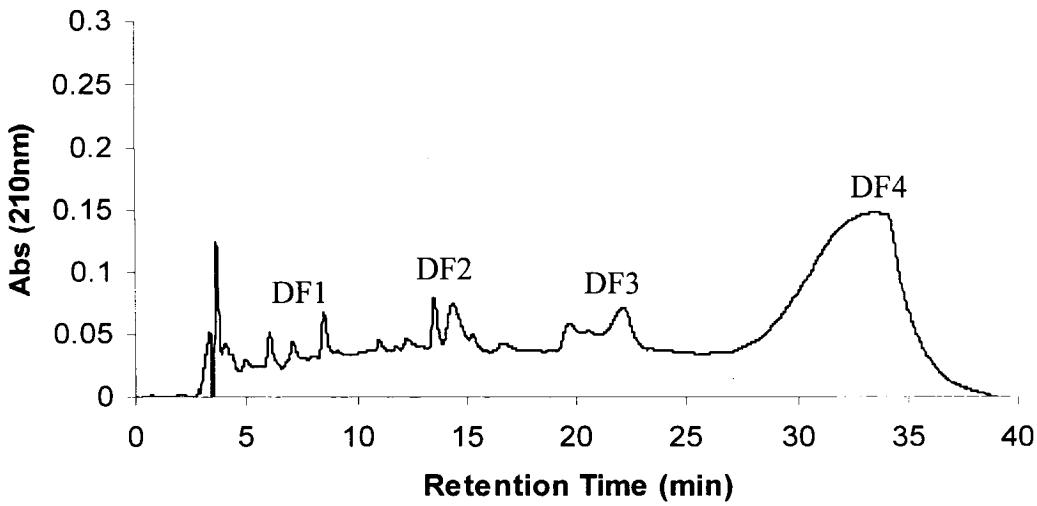

Figure 4.4.34: RP-HPLC chromatogram of chickpea extract (C. Na-E) from NaOH extraction.

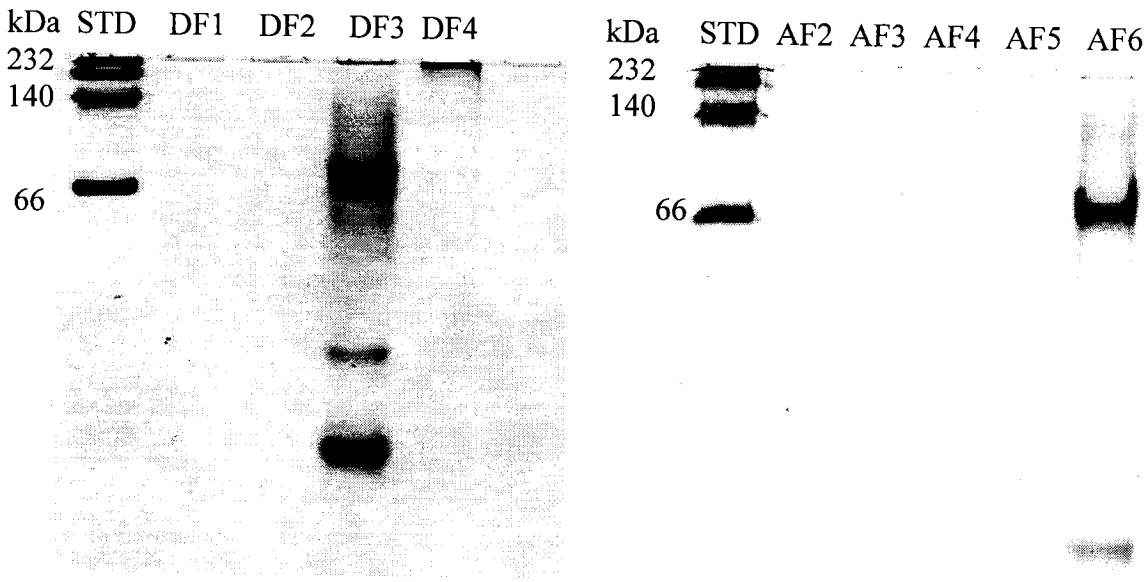

Figure 4.4.35: Native PAGE of RP-HPLC fractions (DF & AF) of extracts (C. Na-E & C. C-E) from NaOH extraction and citric acid extraction. STD = molecular weight standard markers.

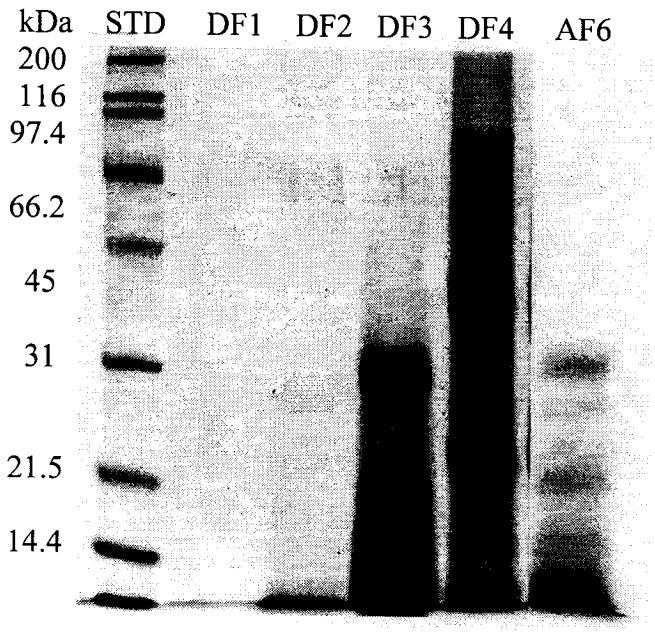

Figure 4.4.36: SDS PAGE of RP-HPLC fractions (DF & AF) of extracts (C. Na-E & C. C-E) from NaOH extraction and citric acid extraction. STD = molecular weight standard markers.

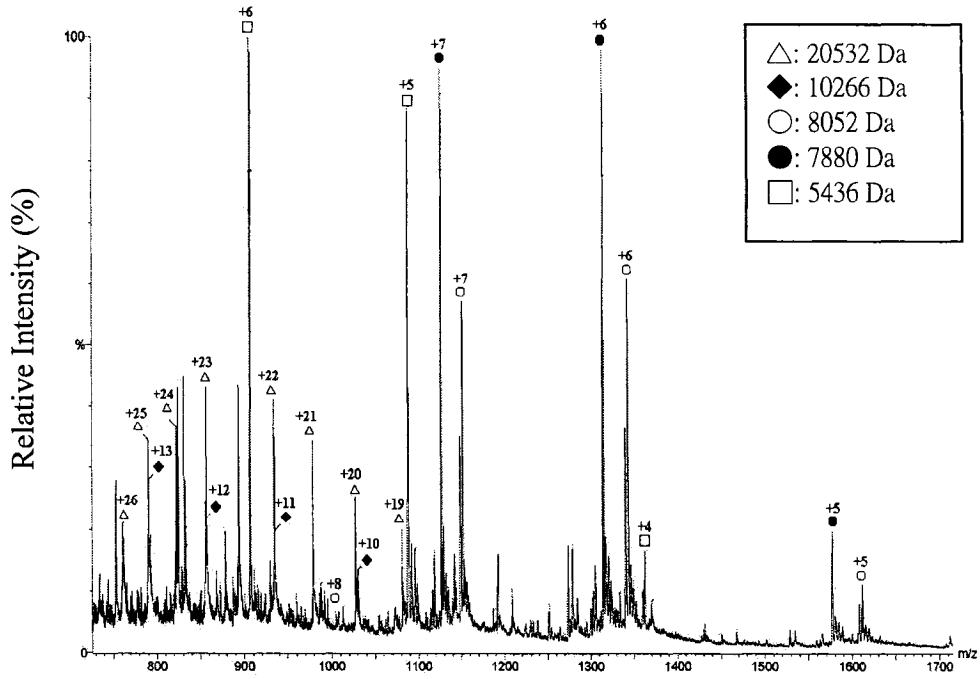

Figure 4.4.37: Interpreted ESI-MS spectrum (m/z) of fraction AF6 of extract (C. C-E) from citric acid extraction.

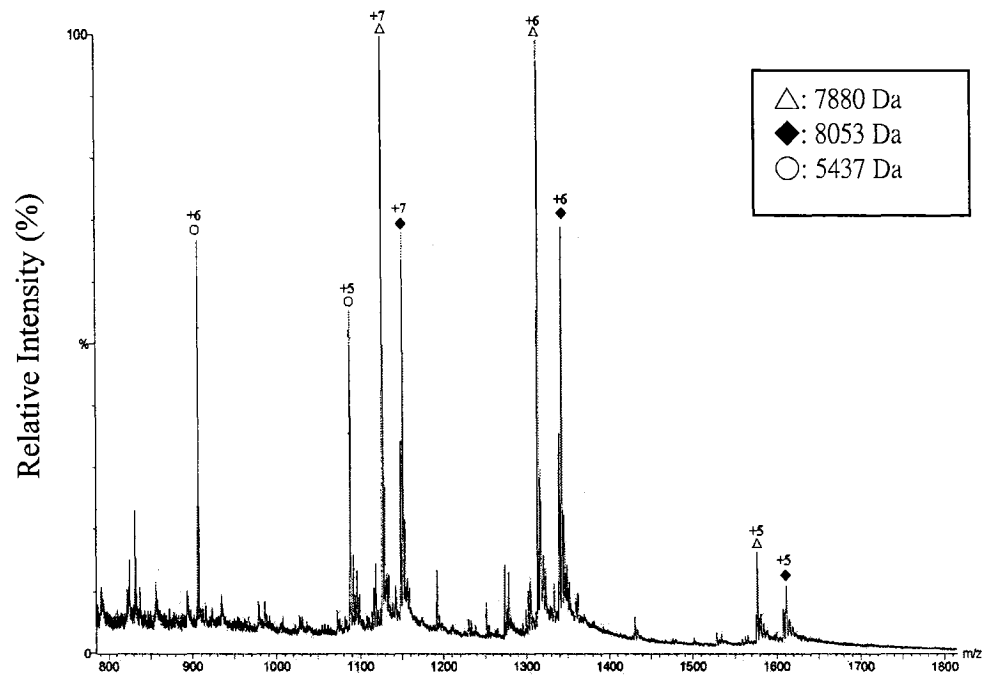

Figure 4.4.38: Interpreted ESI-MS spectrum (m/z) of fraction DF3 of extract (C. Na-E) from NaOH extraction.

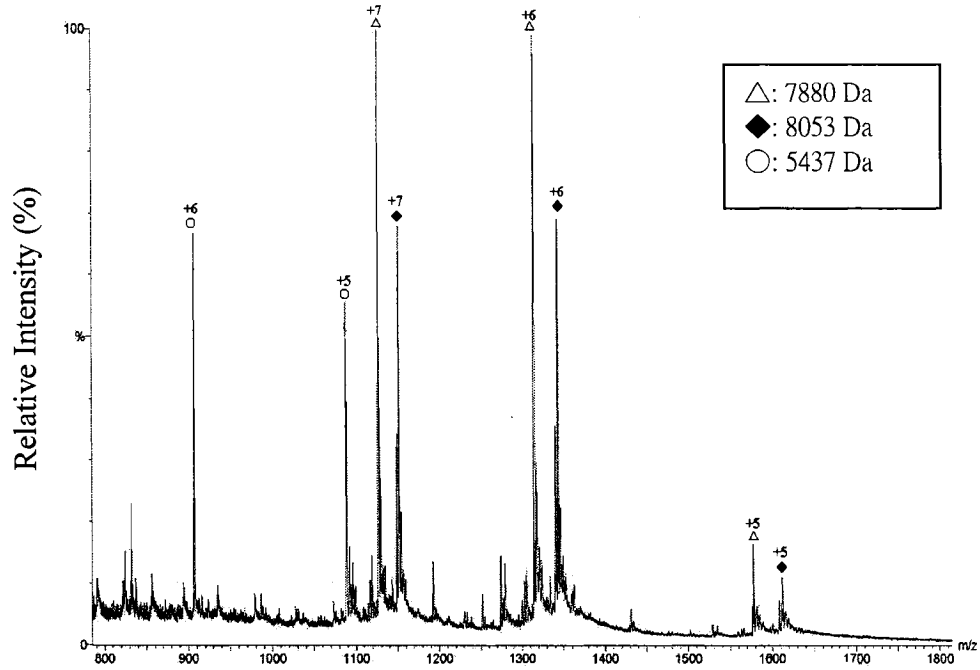

Figure 4.4.39: Interpreted ESI-MS spectrum (m/z) of fraction DF4 of extract (C. Na-E) from NaOH extraction.

Table 4.4.5: Characterization of chickpea protein isolates C. C-E and C. Na-E.

Isolate	RP-HPLC Fractions	Rt (min)	Estimated MW of subunits from SDS-PAGE (kDa)	Interpreted MW of subunits from ESI-MS (Da)	Reported subuniuts (kDa)
C. C-E	AF6	22.6	30.0[A], 26.1, 20.5[A], 15.3, 7.4	20532, 10266, 8052, 7880, 5436	Glutelin[1] 28-31[2]
C. Na-E	DF3	22.2	30.0[A], 20.0[A], 15.2	8053, 7880, 5437	20-22[3]
	DF4	33.4	77.3, 54.3[A], 41.1[A], 33.2[A], 25.8[A], 15.2, 7.4	8053, 7880, 5437	

1: Takaiwa et al., 1999.
2: Acidic subunits.
3: Basic subunuits.
A: Major band.

C. Na-E: extract from NaOH extraction.
C. C-E: extract from citric acid extraction.

fraction AF and DF. Table 4.4.5 summarizes the results obtained from RP-HPLC, SDS-PAGE and ESI-MS analysis. From ESI-MS results, fraction AF6 gave MW 20532 and 10266 Da which is similar to the MW subunits for fraction HF2 gave; fraction AF6, DF3 and HF2 showed similar Rt at 21-22 min; however the ESI-MS interpreted MW from fraction DF3 are different from those of fractions AF6 and HF2 (Table 4.4.5).

General Conclusions

The protein contents of the chickpea protein preparations ranged from 49% to 97% with yields ranged from 0.1 to 67.2 %. The protein contents and yields of isolates from sodium hydroxide extraction are generally higher than those isolates from citric acid extraction. The cryoprecipitates (C. Na-CP and C. C-CP) from NaOH and citric acid extraction showed very low yields (2.3% and 0.1%, respectively).

Scanning electron microscopy of cryoprecipitate (C. C-CP) revealed the presence of starch grains in the protein; this reflected the relatively low protein content of this protein.

The globulins (legumins and vicilins), glutelins, and albumins from both citric acid and NaOH isolates were characterized by Native-PAGE and SDS-PAGE. The cryoprecipitates contained mainly the globulin-rich proteins.

From RP-HPLC analysis, the main separated fractions at retention time 20-30 min showed MW under 140 kDa (Native-PAGE), with subunits in the range of MW 6.5-31 kDa (SDS-PAGE). At elution time 30-36 min, the fractions obtained were mainly composed of mixtures of legumin and vicilin subunits (MW 14-45 kDa, SDS-PAGE).

Fractions of citric acid cryoprecipitate (C. C-CP) and NaOH cryoprecipitate (C. Na-CP) consisted mainly of subunits from globulins. Fractions citric acid cryoprecipitate (C. C-CP) are vicilin-rich proteins based on SDS-PAGE; fractions from NaOH cryoprecipitate (C. Na-CP) are both legumin-rich (particularly in basic legumin subunits based on SDS-PAGE) and vicilin-rich proteins (based on ESI-MS analysis).

Fractions of NaOH isoelectric precipitate (C. Na-IP) and NaOH isoelectric precipitate (C. Na-CIP, after cryoprecipitation) are similar and comprise mostly the

subunits from globulins; some are legumin-rich proteins while others are vicilin-rich proteins. Fractions obtained from NaOH isoelectric precipitate at 55 °C (DLA-IP) consist largely of subunits of globulins (MW 14.4-31 kDa in SDS-PAGE). Extracts from citric acid and NaOH extraction gave subunits of glutelins based on SDS-PAGE and ESI-MS analysis.

References

Abdolgader, R. E. 2000. Isolation and characterization of a high gelling protein from soybean. Ph.D. Thesis. McGill University.

Alli, I. 1979. Isolation and characterization of crystalline and non-crystalline proteins from phaseolus beans. Ph.D. Thesis. McGill University.

Alli, I. and B. E. Baker, 1980. Constitution of leguminous seed: Ⅷ. The microscopic structure of proteins isolated from Phaseolus beans. *J. Sci. Food Agric.* 31:1316-1322.

Alli, I., B. F. Gibbs, M. K. Okoniewska, Y. Konishi and F. Dumas. 1993. Identification and characterization of phaseolin polypeptides in a crystalline protein isolated from white kidney beans *(Phaseolus vulgaris). J. Agric. Food Chem.* 41:1830-1834.

Anonymous. 2004. Chickfood Project, Web Site: *http://www.chickfood.info/*

Anson, M. L. and M. Pader. 1957. US Patent 2785155.

AOAC. 1980. Automated Kjeldahl method for total nitrogen determination. In Official Methods of Analysis of the association of Official Analytical Chemists; Horwitz, W., Ed.; AOAC: Washington, DC.

Boulter, D. 1983. Protein composition of grains of the Leguminosae. *Qual. Plant-Plant Foods Hum. Nutr.* 32:247-252.

Cai, R., B. Klamczynska and B.-K. Baik. 2001. Preparation of bean curd from protein fractions of six legumes. *J. Agri. Food Chem.* 49:3068-3073.

Casey, R., C. Domoney and A. M. Smith. 1993. Biochemistry and molecular biology of seed products. In: R. Casey and D. R. Davies (Ed.), *Peas: Genetics, Molecular Biology and Biotechnology*, CAB International: Wallingford, U.K., p.121-164.

Champell, N. F., F. F. Shih and W. E. Marshall. 1992. Enzymatic phosphorylation of soy protein isolate for improved functional properties. *J. Agric. Food Chem.* 40:403-406.

Chavan, J. K., S. S. Kadam, and D. K. Salunkhe. 1989. Chickpea. In: D. K. Salunkhe and S. S. Kadam (Ed.), *CRC Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization.* Volume I. CRC Press, Inc. Florida, USA, p.247-288.

Chominot, A. 1992. Les proteagineux dans L' economie europeenne des grandes cultures. In: *Proceedings of the first European Conference on Grain Legumes.* Anger, France. p.529-538.

Clemente, A., J. Vioque, R. Sanchez-Vioque, J. Pedroche, J. Bautista and F. Millan. 2000. Factors affecting the *in vitro* protein digestibility of chickpea albumins. *J. Sci. Food Agric.* 80:79-84.

Croy, R. R. D., M. S Hoque, J. A. Gatehouse and D. Boulter. 1984. The major albumin proteins from pea (*Pisum sativum* L.). Purification and some properties. *Biochem. J.* 218: 795-803.

Damodaran, S. 1996. Functional Properties. In: S. Nakai and H. W. Modler (Ed.), *Food Protein Properties and Characterization.* VCH Publishers, Inc. N. Y., p.167-234.

Davis, B. J. 1964. Disc electrophoresis- II . Method and application to human serum proteins. *Ann. NY. Acad. Sci.* 12:404-427.

Duke, J. A. 1981. *Handbook of legumes of world economic importance.* Food and Agriculture Organization of the United Nations, Plenum Press, N.Y., p.52-57.

Erbman, J. W., Jr. 1981. Bioavailability of trace minerals from cereals and legumes. *Cereal Chem.* 58:21-26.

Esh, G. C., T. S. De and U. P Basu. 1959. Influence of genetic strain and environment on the protein content of pulse. *Science*. 129-148.

Fan, T. Y. and F.W. Soduldki. 1974. Dispersibility and isolation of protein from legume flours. *Can. Inst. Food Sci.* 7:256-259.

FAO, 1970 *Amino acids content of food and biological data on proteins.* FAO Nutritional Studies, no. 24, Rome, Italy.

FAOSTAT, 2004. FAOSTAT Agriculture data. FAOSTAT Database collections. Food and Agriculture Organization Web Site:
http://faostat.fao.org/faostat/collections?version=ext&hasbulk=0&subset=agriculture

Friedman, M. 1996. Nutritional value of proteins from different food sources. A review. *J. Agri. Food Chem.* 44:6-29.

Geervani, P. 1991. Utilization of chickpea in India and scope for novel and alternative uses. In uses of tropical grain legumes, Proc. Consultant' Meeting, ICRISAT Center, Patancheru, Andhra Pradesh, India. p.47-54.

Gueguen, J. 1991. Pea and fababean proteins. In: B. J. F. Hudson (Ed.), *Development in Food Proteins*, Elsevier: London, U.K., p.35-78.

Hamada, J. S. 1992. Modification of food proteins by enzymatic methods. In: B. J. F. Husdon (Ed.), *Biochemistry of Food Protein* Elsevier Appl. *Sci.* Lodon, U.K., p.249-270.

Huisman, J. and A. F. B. van der Poel. 1994. Aspects of the nutritional quality and use of cool swason food legume in animal feed. In: F. J. Muehlbauer and W. J. Kaiser (Ed.), *Expanding the production and use of cool season legumes*. Kluwer Academic Publishers, Dordrecht, p. 53-76.

Hulse, J. H. 1991. Nature, composition and utilization of grain legumes. In: Uses of tropical legumes: Proceedings of a Consultants' Meeting, 27-30 March 1989, ICRISAT Center. ICRISAT, Patancheru, A.P. 502 324, India. p. 11-27.

Kinsella, L. E. 1976. Functional properties of food proteins: A review. *CRC Crit. Rev. Food Sci. Nutri.* 7:219-280.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature.* 227:680-685.

Lahl, W. J. and S. D. Braun. 1994. Enzymatic production of protein hydrolysates for food use. *Food Technol.* 10:68-95.

Li-Chan, E. 1996. Separation and purification. In: S. Nakai and H. W. Modler (Ed.), *Food Protein Properties and Characterization.* VCH Publishers, Inc., N. Y., p.429-503.

Liu, L. H., T. V. Hung, R. G. Black and M. A. Trewhella. 1994. Solubility of grain legume proteins measured by infra-red spectroscopy. *ASEAN Food J.* 9:24-29.

Liu, L. H. and T. V. Hung. 1998. Flow properties of chickpea proteins. *J. Food. Sci.* 63:229-233.

Maiti, R. K. 2001 The chickpea crop. In: R. K. Maiti and P. Wesche-Ebeling (Ed.), *Advances in Chickpea Science*. Science Publishers Inc., Enfield, NH, USA, p.1-32.

Maiti, R. K., S. Moreno-Limon, and P. Wesche-Ebeling. 2001. Seeds, Germination,and Crop Establishment. In: R. K. Maiti and P. Wesche-Ebeling (Ed.), *Advances in chickpea science*. Science Publishers Inc., Enfield, NH, USA, p.33-66.

Malhotra, R.S., R.P.S. Pundir and A.E. Slinkard. 1987. Genetic resources of chickpea. In: M.C. Saxena and K.B. Singh (Ed.), *The chickpea.* C.A.B. International Cambrian News Ltd, Aberystwyth, U.K., p.67-81.

Marconi, E., S. Ruggeri, M. Cappelloni, D. Leonardi and E. Carnovale. 2000. Physicochemical, nutritional, and microstructural characteristics of Chickpeas (*Cicer arietinum* L.) and common beans (*Phaseolus vulgaris* L.) following microwave cooking. *J. Agric. Food Chem.* 48:5986 -5994.

Marquez, M. C. and R. Alonso. 1999. Inactivation of trypsin inhibitor in chickpea. *J. Food Compost. Anal.* 12:211-217.

Melnychyn, P. 1969. Process for isolating proteins from beans in crystalline state using an alkali salt of a carboxylic acid. U.S. Patent no. 3450688.

Muehlbauer, F.J and Abebe Tullu.1997.Cicer arietinum L. *New Crops*. Fact sheet. Web Site: *http://www.hort.purdue.edu/newcrop/cropfactsheets/Chickpea.html*

Muehlbauer, F.J. and K.B. Singh, 1987. Genetics of chickpea. In: M.C. Saxena and K.B. Singh (Ed.), *The Chickpea*. CAB. International, Wallingford, Oxon, U.K., p. 99-125.

Murray, E. D., C. D. Myers, and L. D. 1978. Barker Protein product and process for preparing same. Canada Patent 1,028,552.

Murray, E. D., C. D. Myers, L. D. Barker and T. J. Maurice. 1981. Functional attributes of protein – A noncovalent approach to processing and utilizing plant proteins. In: E. D. Murray and D. W. Lee (Ed.), *Utilization of protein resources*. Food and Nutrition Press, Inc., Westport, CT. p.158-176.

O'Dell, B. L. 1969. Effect of dietary component upon zinc availability: a review with original data. *Am. J. Clin. Nutr.* 22:1315-1322.

Paredes-Lopez, O., C. Ordorica-Falomir and M. R. Olivares-Vazquez. 1991. Chickpea protein isolates: physicochemical, functional and nutritional characterization. *J. Food Sci.* 56:726-729.

Price, M. L., A. E. Hagerman and L. G. Butler. 1980. Tannins content of cowpeas, chickpea, pigeonpeas and mung beans. *J. Agric. Food Chem.* 28:459-461.

Plietz, B., G. Damaschum and K. D. Schwenke. 1980. Quaternary structure of 11S globulin from different plant seeds. *Studia Biophysica*. 79:145-146.

Rao, P. U. and Y. G. Deosthale. 1982. Tannin contents of pulses: varietal differences and effect of cooking and germination. *J. Sci. Food Agric.* 33:1013-1016.

Reddy, N. R., M. D. Pierson, S. K. Sathe and D. K. Salunkhe. 1985. Dry bean tannins: a review of nutritional implications. *J. Am. Oil Chem. Soc.* 62:541-549.

Rubio, L. A., G. Grant, C. Cavallé, A. Martinez-Aragon and A. Pusztai. 1994. High in-vivo (rat) digestibility of faba bean (*Vicia faba*), lupin (*Lupinus angustifolius*) and soya bean (*Glycine max*) soluble globulins. *J. Sci. Food Agric.* 66:289-292.

Sanchez-Vioque, R., A. Clemente, J. Vioque, J. Bautista, and F. Millán. 1999. Protein isolate from chickpea *(Cier arietinum L.)*: chemical composition, functional properties and protein characterization. *J. Agric. Food Chem.* 64:237-243.

Sathe, S. K., G. G. Lilley, A. C. Mason and C. M. Weaver. 1987. High-resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis of soybean (glycine max L.) seed proteins. *Cereal Chem.* 64:380-384.

Sathe, S. K., S. S. Deshpande and D. K. Salunkhe. 1984. Dry beans of *Phaseolus*: a review. Part 2. Chemical composition: carbohydrates, fiber, minerals, vitamins, and lipids. *CRC Crit. Rev. Food Sci. Nutr.* 21:41-93.

Schroeder, H. E. 1984. Major albumins of Pisum cotyledons. *J. Sci. Food Agri.* 35:191-198.

Shewry, P. R. and A. S. Tatham. 1990. The prolamin storage proteins of cereal seeds: structure and evolution. *Biochem.l J.* 267:1-12.

Shewry P. R., J. A. Napier and A. S. Tatham. 1995. Seed storage proteins: structures and biosynthesis. *Plant Cell.* 7:945-956.

Shewry P. R. and N. G. Halford. 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. *J. Expe. Bot.* 53:947-958.

Shotwell, M. A. 1999. Oat globulins. In: P. R. Shewry and R. Casey (Ed), *Seed proteins.* Dordrecht: Kluwer Academic Publishers, Dordrecht, p.389-400.

Singh, K.B. 1997. Chickpea *(Cier arietinum L.). Field Crops Res.* 53:161-170.

Singh, K.B. and B. Ocampo. 1997. Exploitation of wild Cicer species for yield improvement in chickpea. *Theor. Appl. Genet.* 95:418-423.

Singh, R., M. George, and G. L. Soni, 1983. Role of dietary fiber from pulses as hypocholesterolemic agent. *J. Food Sci. Technol.* 20:228-230.

Singh, U. 1984[a]. Dietary fiber and its constituents in desi and kabuli chickpea *(Cier arietinum L.)* cultivals. *Nutr. Rep. Int.* 29:419-26.

Singh, U. 1984[b]. The inhibition of digestive enzymes of polyphenols of chickpea *(Cier arietinum L.)* and pigeonpea *(Cajanus cajan (L.) Millsp). Nutr. Rep. Int.* 29:745-753.

Singh, U. and R. Jambunathan. 1982. Distribution of seed protein fractions and amino acid in different anatomical parts of chickpea *(Cier arietinum L.)* and pigeon pea *(Canajus cajan L.) Qual. Plant-Plant Food Hum. Nutr.* 31: 347-354.

Smartt, J. 1990. The other legume oilseeds. In: *Grain legumes.* Cambridge University Press, N. Y., p. 245-277.

Smith, L.H. 1984. Seed development, metabolism, and composition. In: M.B. Tesar (Ed.), *Physiological basis of crop growth and development.* Amer. Soc. Agron., Crop Sci. Soc. American, Madison, Wisconsin, USA, p.13-52.

Sosulski, F. W., L. Elkowicz, and R. D. Reichert. 1982. Oligosaccharides in eleven legumes and their airclassified protein and starch fractions, *J. Food Sci.* 47:498-502.

Sotelo, A. and R. N. Adsule 1996. Chickpea (*Cicer arietinum L.*) In: E. Nwokolo and J. Smartt (Ed.), *Food and feed from legumes and oilseeds.* Chapman, London, U.K., p.82-89.

Sotelo, A., Flores, F. and Hernandez, M. 1987. Chemical composition and nutritional value of Mexican varieties of chickpea (*Cicer arietinum L.*). *Qual. Plan-Plant Foods Hum. Nutr.* 37: 299-306.

Sumathi, S. and T. N. Pattabiraman. 1976. Natural plant enzyme inhibitors. II. Protease inhibitors of seeds. *Indian J. Biochem. Biophys.* 13:52-56.

Takaiwa F., M. Ogawa, and T. W.Okita. 1999. Rich glutelins. In: P. R. Shewry and R. Casey (Ed), Seed proteins. Dordrecht: Kluwer Academic Publishers, Dordrecht, P.401-425.

Tömösközi, S., R. Lásztity, E. Süle, J. Gaugecz and J. Varga. 1999. Functional properties of native and modified wheat germ protein isolates and fractions. *J. Nahrung Food.* 43:245-247.

Vairinhos, F. and D. R. Murray. 1982. Changs in polypeptides composition during seed development in chickpea (*Cicer arietinum L.*), *Z. Pflanzenphysiol. Bd.* 106:447-452.

Vioque, J., A. Clemente, R. Sánchez-Vioque, J. Pedroche, J. Bautista and F. Millán. 1998. Comparative study of chickpea and pea PA2 albumins. *J. Agric. Food Chem.* 46:3609-3613.

Vioque, J., R. Sánchez-Vioque, A. Clemente, J. Pedroche, J. Bautista, and F. Millán. 1999. Purification and partial characterization of chickpea 2S albumin. *J. Agric. Food Chem.* 47:1405 -1409.

Wesche-Ebeling, P., R. K. Maiti, B. Cuevas-Hernandez and J. Verde-Star 2001. Food science and feed quality, In: Maiti and P. Wesche-Ebeling (Ed.), *Advances in chickpea science*. Science Publishers Inc., Enfield, NH, USA, p.189-214.

Wolf, W. 1970. Soybean proteins: their functional, chemical, and physical properties. *J. Agric. Food Chem.* 18:969-970.

Yada, R. Y., R. B. Jackman, J. L. Smith and A. G. Marangoni. 1996. Analysis: quantitation and physical characterization. In: S. Nakai and H. W. Modler (Ed.), *Food protein properties and characterization.* VCH Publishers, Inc., N.Y., p.333-403.

Yust, M. M., J. Pedroche, J. Giron-Calle, M. Alaiz, F. Millan and J. Vioque, 2003. Production of ace inhibitory peptides by digestion of chickpea legumin with alcalase. *Food Chem.* 81:363-369.