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ISOLATION OF A GLYCININ-RICH PROTEIN FROM DEFATTED SOYBEAN

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ABSTRACT

Defatted soybean flour was subjected to isolation procedures as follows: NaOH extraction / acid precipitation, water extraction / acid precipitation, NaOH extraction / cryoprecipitation and citric acid extraction / cryoprecipitation.

NaOH extraction / cryoprecipitation and citric acid extraction / cryoprecipitation caused precipitation of one protein mainly consisting 11 S (glycinin) and 7 S (β-conglycinin) respectively. In contrast, NaOH extraction / acid precipitation and water extraction / acid precipitation precipitated the two major soybean proteins as judged by PAGE and SDS-PAGE.

Using differential scanning calorimetry (DSC), NaOH extraction / acid precipitation showed two thermal transition peaks at 72.8^{o}C and 86.4^{o}C, where as both NaOH extraction / cryoprecipitation and citric acid extraction / cryoprecipitation observed only one endothermic peak at 85.3 ^{o}C and minor peak at 75.3^{o}C, which may corresponding to 11S (glycinin) and 7 S (β-conglycinin) respectively. Second recycling of the thermal properties of all soy isolates exhibited irreversible denaturation behavior.

INTRODUCTION

For more than 2000 years people throughout East Asia have consumed soybeans in the form of traditional soy foods, sush as Nimame (cooked whole soy), Edamame (green fresh soy), soy milk and tofu (Fukushima, 2004). In the West, soybean is still best known for its oil and protein content. In comparison to most other legumes, soybean contains from 35 to 40% protein, 15 to 20% oil and 20 to 25% CHO (Rhee, 1994). The increased acceptance of soy protein is due to its good functional properties in food application, high nutritional value, availability and low cost, and more recently, its suggested health benefits (Fuller 1988). Further more, the FDA confirmed the 'Soy Protein Health Claim` on 26 October, 1999, that 25 grams of soy protein a day may reduce the risk of heart disease (Fukushima, 2004)

Structurally, soy protein is classified based on sedimentation characteristics, the conventional nomenclature for soy protein fraction is the 2-S, 7-S, 11-S and 15-S. The predominant proteins are the two major globulin's species, β-conglycinin (7-S) and glycinin (11-S) (Kinsella 1979; Kilara and Sharkasi 1985 and Sathe *et al* 1987). Glycinin has an estimated molecular weight of about 350000 dalton and is composed of at least six nonidentical subunits. Each of these subunits contains an acidic polypeptide linked to a basic polypeptide by a single disulfide bond. Molecular weight of the acidic subunits range between 37000 and 42000 and these of the basic subunits range between 17000 and 20000 (Moreira *et al* 1979; Utsumi *et al* 1997; Sathe 1991). β-conglycinin is a trimeric glycoprotein with molecular weight of

140000-170000, which consists of three types of subunits α (83000-57000), α(76000-57000) and β (53000-42000) (Arrese *et al* 1991; Bazinet *et al* 1997).

There are several procedures for producing protein isolates from soybeans. The principle commercial procedure consists of extraction from defatted soy bean with water or dilute NaOH, followed by centrifugation of the slurry to separate the insoluble materials and obtain a dispersion containing soluble protein and some non-protein solutes (mother liquor). The mother liquor is acidified (pH 4.5) to precipitate the protein; the crude isolate is washed to remove as much of the non-protein material as possible (Fan and Sosulski 1974; Johnson and Kikuchi 1988; Kolar *et al* 1985; Wolf and Cowan 1975). The disadvantages of this method includes denaturation of protein upon exposure to alkali and acid precipitation, high ash content and alteration of protein solubility after rehydration, in addition imparting off-flavors. These disadvantages limit the use of acid precipitation of soy protein in foods (Anderson 1974; Anderson and Warner 1976; Bazinet *et al* 1997; Fisher *et al* 1986; Kilara and Sharkasi 1985; Kilara and Harwalkar 1996; Kinsella *et al* 1985; Nash *et al* 1967; Wolf 1978).

A simple method can be used to fractionation 11 S (glycinin) and 7 S (β-conglycinin) by dissolving defatted soybean flour in dilute alkaline (0.02%, pH ~ 11), removing the insoluble residues by centrifugation. By cryoprecipitation, glycinin rich protein will be precipitated and then collected by centrifugation. After that, the 7 S (β-conglycinin) recovered by dilute HCl to pH4.5. This simple method can be scaled up and might be used in commercial scaled for fractionation of the two major soybean proteins. This research was devoted to the extraction and isolation of soybean protein by acid, alkali, and water extraction followed by acid precipitation and cryoprecipitation. The major protein components in the extracts, isolates and supernatant were identified.

MATERIALS AND METHODS

Materials

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

Preparation of Proteins

Sodium Hydroxide and Water Extraction / Acid Precipitation.

Proteins were extracted using the procedure of Fan and Sosulski (1974) as modified by Alli and Baker (1980). Defatted soybean (100g) was mixed with distilled H_2O (1 L) or dilute NaOH (1L, 0.02%, pH 10.7) and allowed to stand for 1h with intermittent stirring. The mixture was centrifuged (12000 x g) for 10 min, the extract filtered through glass wool and the residue discarded. The pH of the filtrate was adjusted to 4.5 by the dropwise addition of HCl (2N) with continuous stirring. The precipitated proteins were recovered by centrifugation (12000 x g) for 10 min and then lyophilized. These isolates were designated as H_2O isoelectric isolate (H_2O-IE) and NaOH isoelectric isolate (NaOH-IE). Samples from the NaOH and H_2O

extracts and supernatants were dialyzed against deionized water (Spectra / Pormembrane, molecular weight cut off 8,000) and lyophilized.

Citric Acid Extraction / Cryoprecipitation.

The procedures described Melnychyn (1969) and modified by Alli and Baker (1980) were used for the extraction of proteins with citric acid solution. Defatted soybean (100g) was mixed with citric acid solution (1L, 0.2N, pH 4) and allowed to stand for 1 h with intermittent stirring; the mixture was centrifuged (12000 x g) for 10 min. The extract was filtered through glass wool and the residue discarded. The filtrate was refrigerated (4 ^{o}C) for 18 h, the proteins, which precipitated, were recovered by centrifugation (12000 x g) for 10 min followed by lyophilization. This isolate was designated as citric acid cryoprecipitate (CA-CP). Samples from the citric acid extracts and citric acid supernatants were dialyzed against deionized water (Spectra / Pormembrane, molecular weight cut off 8,000) and lyophilized to use for further analysis.

Sodium Hydroxide Extraction / Cryoprecipitation

Proteins were extracted using the procedure of Davidson *et al*. (1979) with some modifications. The defatted soybean meal (100g) was mixed with dilute NaOH (1L, 0.02 %, pH 10.7) and allowed to stand for 1 h at room temperature with intermittent stirring. The mixture was centrifuged (12000 x g) for 10 min. The extract was refrigerated (4 ^{o}C) for 18 h and the proteins which precipitated were recovered by centrifugation (12000 x g) for 10 min followed by lyophilization. This isolate was designated as sodium hydroxide cryoprecipitate (NaOH-CP). Samples from the NaOH extract and NaOH-CP supernatant were dialyzed against deionized water (Spectra / Pormembrane, molecular weight cut off 8,000) and lyophilized.

Protein Contents and Yields of Isolates

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

Protein Characterization

Electrophoresis on Polyacrylamide Gels (PAGE)

PAGE was performed according to the method of Davis (1964) using a Mini-Protean II Electrophoresis Cell unit (Bio-Rad, Hercules, CA). A 4 % acrylamide stacking gel and 6 % separation gel were used. Sample solutions (15 μl), prepared from 1 mg of freeze-dried protein extracts, isolates, and supernatants dissolved in 1 ml sample buffer (0.3M tris-HCl pH 8.8, 1 % glyecrol and 0.05 % bromophenol blue), was applied to each sample well. Electrophoresis was carried out for 3 h at constant current (6 mA / gel) using tris glycine buffer (pH 8.3). Gels were stained with Coomassie Brilliant Blue R-250 (0.1 % w/v) in water / methanol / acetic acid and destained with the same solvent system but without dye.

SDS-PAGE electrophoresis was carried out on slab gels (4 % stacking and 12 % separation gels) using the technique described by Laemmli (1970). The protein samples (15 μ) prepared from 1 mg of extract, isolates and supernatants and dissolved in 1 ml sample buffer (3 % SDS, 0.7M 2-mercaptoethanol, 25 mM tris-HCl pH 6.8, 1 % glycerol and 0.05 % bromophenol blue) were heated at 95 ^{o}C for 5 min. Electrophoresis was performed at constant current (30 mA / gel) for 1 h. The gels were stained with 0.1 % Coomassie Brilliant Blue R-250 in water / methanol / acetic acid, and destained in the same solvent system but with out dye. A SDS-PAGE broad range molecular weight standard, (Bio-Rad Hercules, CA) was subjected to the same procedure as described above.

Fractionation of Proteins by Size-Exclusion Chromatography

A glass column (34 cm long, 2.6 cm internal diameter) was packed with Sepharose-CL 6B gel (Pharmacia, Sweden) previously washed with distilled water, followed by a phosphate buffer (0.0325 M K_2HPO_4, 0.0026 M KH_2PO_4, 0.4M NaCl, 0.01 M 2-ME, 0.02 % NaN_3, pH 7.6), and equilibrated (0.8 ml / min) for three days with the same buffer. A quantity (100 mg) of the lyophilized soy protein extracts, isolates, and supernatants was dissolved in 7 ml of the phosphate buffer and the solution was filtered through a membrane filter (0.45 μm, Millipore). A quantity 5 ml of the filtrate was loaded to the column using a constant flow rate of 0.8 ml / min. The eluted protein was collected with an automatic fraction collector. Detection of the protein fraction was done by measurement of UV absorbancy at 280 nm. The collected fractions were dialyzed against deionized water (Spectra / Pormembrane, molecular weight cut off 8,000) and lyophilized. Fraction 2 obtained from the protein isolates (Figure 3) was subjected to SDS-PAGE using the procedure described above.

Differential Scanning Calorimetry (DSC)

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

RESULTS AND DISCUSSIONS

Protein Contents and Yields of Isolates

The protein contents of the isolates (Table 1) were similar (84 – 86 % protein) except for the NaOH-CP (91 % protein), these values are similar to protein contents of soy protein isolates reported by other researchers (Johnson and Kikuchi 1988, Dilollo *et al* 1991). The yield of proteins which were obtained from deffated soybean with the various solutions, are shown in

Table 1. The highest protein yield (78 %) was obtained for NaOH-IE while the yield of NaOH-CP was 22 %. By extraction soy protein with a salt solution at pH 8, Howard *et al*. (1983) obtained 22.8 % glycinin. The lowest yield (2.5 %) was obtained from CA-CP; Alli and Baker (1980) reported that a limitation of organic acid extraction, was the relatively low yield of protein.

Polyacrylamide Gel Electrophoresis
Native Conditions

The acid precipitated proteins (NaOH-IE, H_2O-IE) and the citric acid cryoprecipitated (CA-CP) gave two distinct bands designated as I and II (Figure 1) the cryoprecipitate NaOH-CP showed a predominance of band I and only trace of band II. Sathe (1991) reported that soy protein contains two major proteins with a molecular weight of 350 kDa for glycinin and 180 kDa for β-conglycinin.

Table 1: Protein contents and protein yields of soy protein isolates

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

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

SDS-PAGE

The molecular weights of the subunits of the proteins in soybean have been reported by several researchers. Sathe *et al* (1987) reported the MW of lipoxygenase was 93330 Da, β -conglycinin subunits ά , ά □□and β were 82220, 70630, 48420 Da respectively, acidic and basic subunits of glycinin were 33570 and 20650 Da respectively. MW similar to these have been reported by Petruccelli and Anon (1995) Sathe (1991), Iwabuchi and Yamauchi (1987) and Fontes *et al* (1984). Brooks and Morr (1985) reported the MW of β -conglycinin ά subunits ranged from 83000 - 57000 □□ ά subunit ranged from 76000- 57000□□□and□□ subunits ranged from 53000 - 42000, these values was confirmed by Arrese *et al* (1991) who also reported that glycinin acidic subunits and basic subunits showed MW, ranging from 37 to 42 kDa and 17 to 20 kDa respectively. Koshiyama *et al* (1981) reported that some of the 2-S globulins precipitated between pH 5.8 and pH 4.5 were identical with Kunitz trypsin inhibitor (MW 21500 Da).

In the presence of SDS, the NaOH-IE and the extract from which this isolate was prepared gave ~15 subunits (Figures 2Aa, 2Ba); the bands with MW 85, 75 and 50 kDa correspond to β conglycinin subunits while those of MW 42, 38, 37, 35, 22 and 12 kDa correspond to glycinin subunits. The upernatant obtained after precipitation of the NaOH-IE showed differences in the intensity of the bands which represent β □conglycinin and glycinin, this suggest that both β □conglycinin and glycinin were mainly removed from the extract during isoelectric precipitation.

The results from the H_2O extract, H_2O-IE and H_2O supernatant were similar to those from the NaOH extraction (Figures 2Ab, 2Bb, 2Cb).

The SDS-PAGE results of extract from the NaOH-CP and the NaOH-IE are identical (Figure 2Aa, 2Ac); this is expected since the extraction procedures are identical. However, the SDS-PAGE of the NaOH-CP was substantially different from that of NaOH-IE (Figure 2Ba, 2Bc). The major bands in NaOH-CP were 42, 38, 22 and 12 kDa; these represent glycinin subunits; while the bands 85, 75 and 50 kDa which represent β conglycinin subunits were minor bands. This is consistent with the results from the native PAGE which suggest that glycinin was the major component in the NaOH-CP. β -conglycinin subunits, (85, 75 and 50 kDa) which were minor bands in the gel of NaOH-CP, were major bands in of the supernatant remaining after precipitation of this NaOH cryoprecipitate (Figure 2Cc). This confirms that there was minimal precipitation of β -conglycinin during cryoprecipitation of the NaOH-CP. The bands representing glycinin subunits were minor components in the supernatant suggesting that the glycinin in the extract did not precipitate completely during cryoprecipitation.

The bands detected in the citric acid extract were comparable to those bands in the NaOH extract (Figures 2Ad, 2Aa); however, the bands obtained from the citric acid extract are less intense. The CA-CP showed bands with MW from 97 to 12 kDa (Figure 2Bd); bands with molecular weights of 85, 75 and 50 kDa which represent conglycinin subunits were the major bands in the CA-CP while glycinin subunits 42, 38, 22 and 12 kDa were minor bands. This suggests that major component in the CA-CP was the β conglycinin, with glycinin as a minor component.

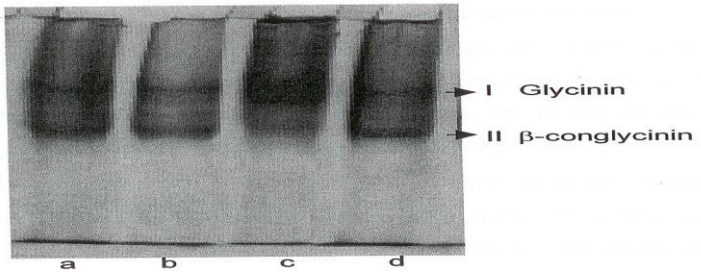

Figure 1:- Electrophoresis (native PAGE) of a- NaOH-IE, b- H2O-IE, c- NaOH-CP, d- CA-CP

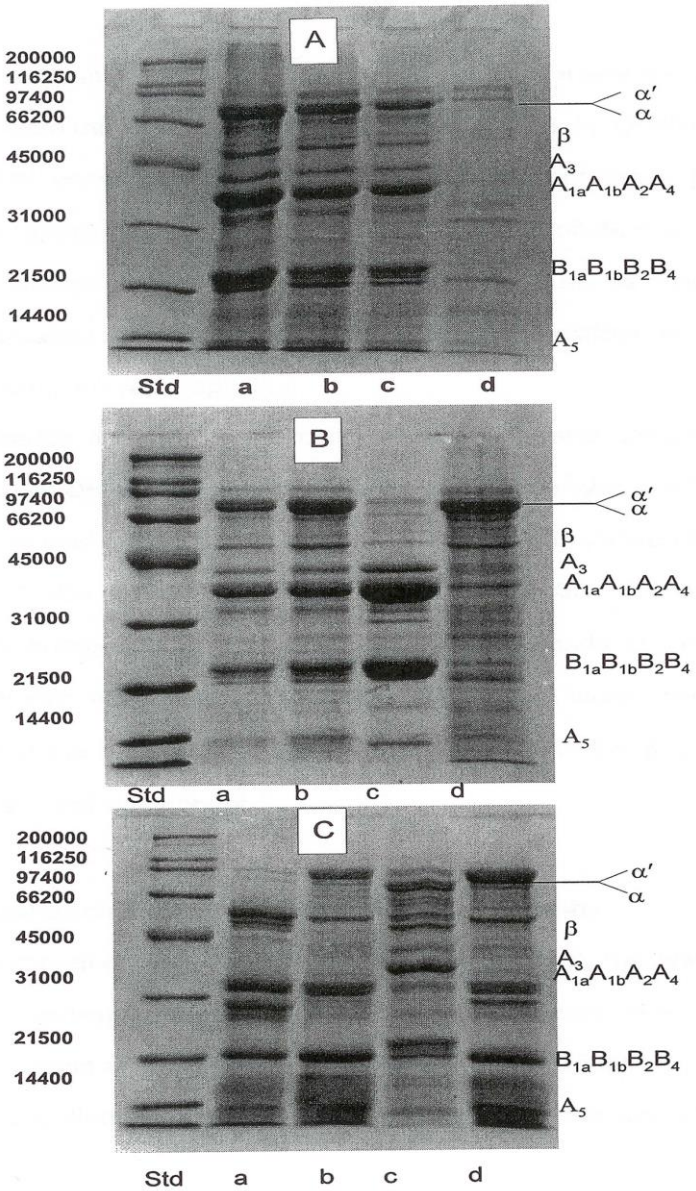

Figure 2:- Electrophoresis (presence of SDS) of extract (A), isolate (B), and supernatant (C). a- NaOH-IE, b- H2O-IE, c- NaOH-CP, d- CA-CP

Fractionation by Size Exclusion Chromatography

SE chromatograms (SEC) from the NaOH extract, the NaOH-IE and the supernatant remaining after precipitation of NaOH-IE are shown in Figure 3. The NaOH extract and the NaOH-IE contained four fractions (F_1, F_2, F_3, and F_4) with F_2 representing the major protein fraction. The F_2 was absent from the SEC of the supernatant, suggesting that during isoelectric precipitation of the NaOH-IE primarily, F_2 was recovered from the extract. SDS-PAGE of F_2 from the NaOH-IE (Figure 4a) gave bands with MW 85, 75 and 55 kDa represent subunits of β conglycinin and bands with MW 42, 38, 22 and 12 kDa which represent subunits of glycinin. The SEC results indicate that F_2 from NaOH-IE is a mixture of glycinin and β -conglycinin.

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

Three major fractions (F_1, F_2, F_4) were separated from NaOH-CP while four fractions (F_1, F_2, F_3, F_4) were found in the extract. Fraction F_3 (in the extract) was absent for NaOH-CP; in addition F_1 and F_4 were relatively minor fractions. SEC suggested that the NaOH-CP was a relatively homogenous protein. SDS-PAGE of F_2 obtained from NaOH-CP showed three major bands with MW of 38, 22 and 12 kDa and four minor bands with MW of 50, 42, 30 and 17 kDa (Figure 4c). These results suggest that F_2 of NaOH-CP containing of subunits similar to those identified in the NaOH-CP, the MW of the major subunits are similar to those of glycinin.

Figure (4d) shows the SEC of the citric acid extract, CA-CP and the supernatant obtained after the cryoprecipitate. The citric acid extract and the CA-CP contained four fractions (F_1, F_2, F_3, and F_4) with F_2 representing the major protein fraction CA-CP. SDS-PAGE of F_2 of CA-CP (Figure 4d) gave major bands with MW of 85, 75, 50 and 30 kDa; also two minor bands with MW of 22 and 12 kDa can be detected in the gel.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) thermograms of NaOH-IE, CA-CP and NaOH-CP are shown in Figure 5a. Two thermal transition peaks at 72.8 and 86.4 ^oC were obtained with the NaOH-IE corresponding to the denaturation temperature (Td) of β conglycinin (low endothermic temperature) and the thermal denaturation temperature (Td) of glycinin (high endothermic temperature); these results are in good agreement with these reported by other workers (Sheard *et al* 1986; Damodaran 1988; Arrese *et al* 1991; Wagner *et al* 1996).

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

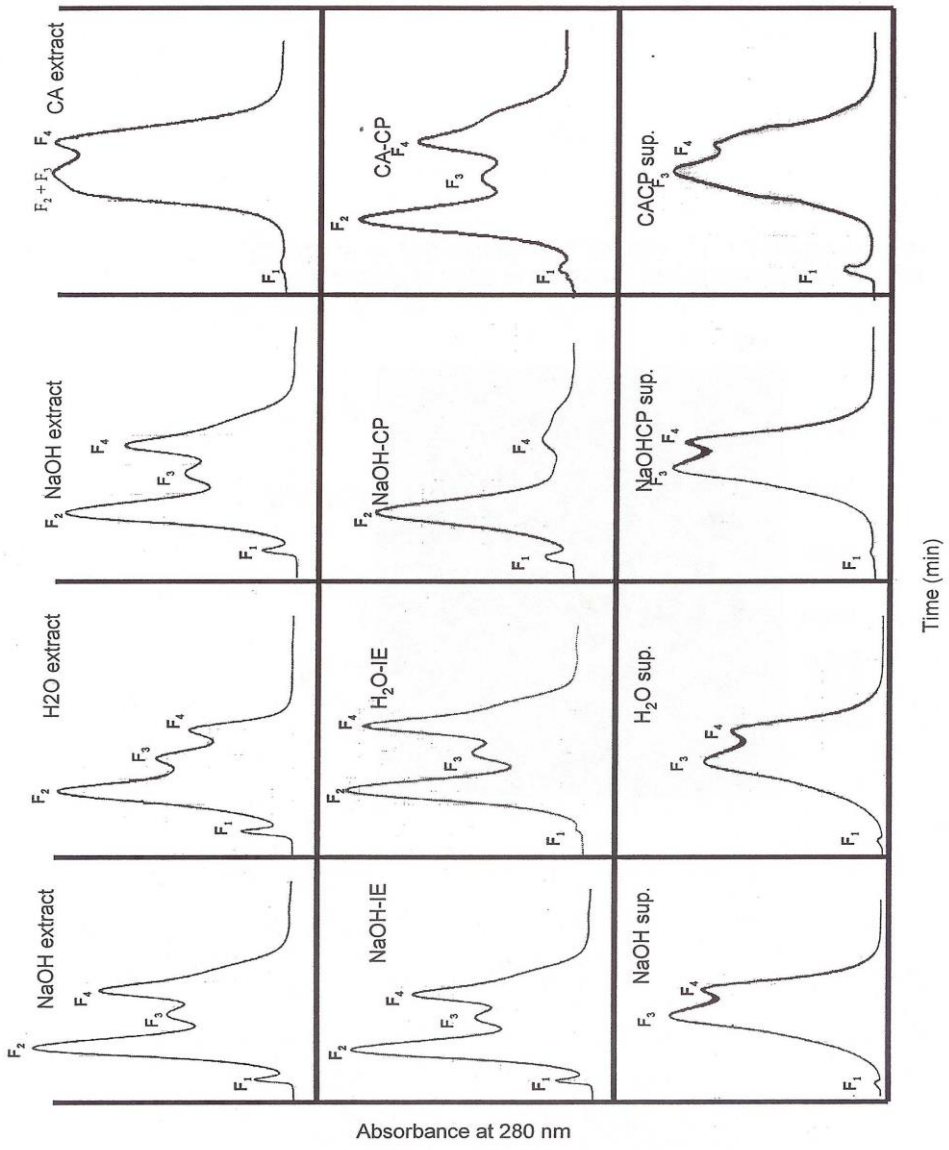

Figure3:- SEC of soy protein extracts, isolates, and supernatant

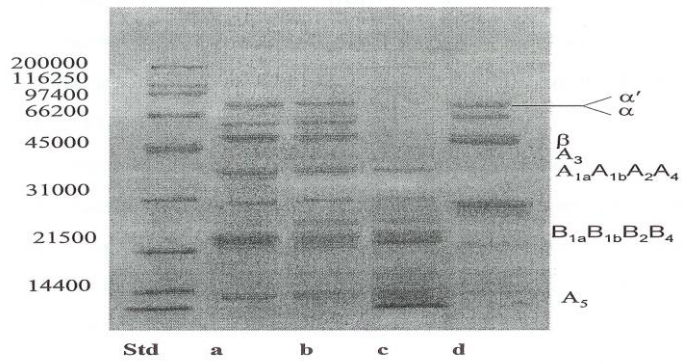

Figure 4:- Electrophoresis (presence of SDS) of F2 from SEC. a- F2(NaOH-IE), b- F2(H2O-IE), c- F2 (NaOH-CP), d- (CA-CP)

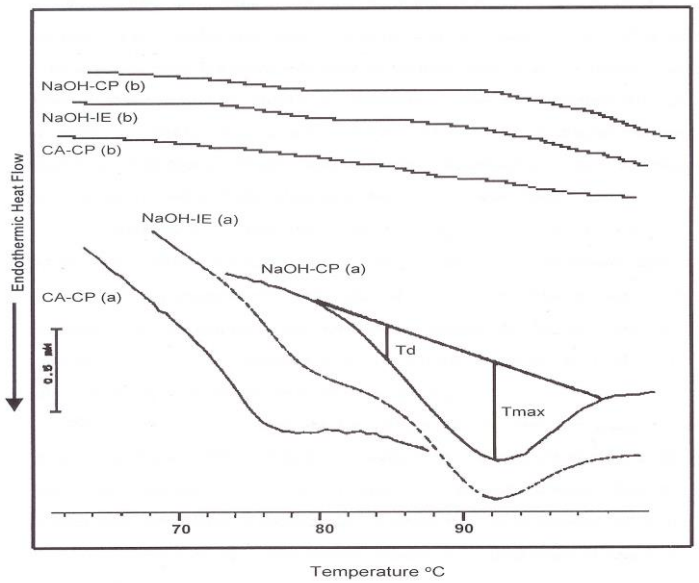

Figure 5:- DSC thermograms of soy protein isolates. a- First heating cycle, b- Second heating cycle

The much higher thermal stability of the glycinin rich protein (NaOH-CP) compared the β conglycinin-rich protein (CA-CP), might be attributed to the difference in the conformational structures of both globulin. The glycinin

contains 21 disulfide bonds of which six disulfide bonds are between the acidic and basic subunits of glycinin (intersubunit), and fifteen disulfide bonds are intra subunits; on the other hand, β □conglycinin does not have any inter or intra subunits linkages which make it less a stable structure to thermal treatment (Badley *et al* 1975 and Lin 1991). The extensive intra- and intersubunit disulfide bonds in the glycinin may provide greater stability against thermal denaturation, which is reflected in its Td and enthalpy of denaturation (Damodaran 1988).

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

No transition peak were obtained (Figure 5b) when the proteins were cooled after the first DSC run, then subjected to a second thermal analysis; this suggests that glycinin and □□conglycinin shows irreversible denaturation, regardless of the conditions used for preparation of the proteins.

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فصل بروتين الجلايسنين من دقيق فول الصويا المنزوع الدسم

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دقيق فول الصويا المنزوع الدسم تم عزله بعدة طرق تتمثل في الاستخلاص بالقلوي أو الماء مع الترسيب بالحامض بالإضافة إلى الاستخلاص القلوي أو الحامض العضوي مع الترسيب على البارد.

بينت نتائج الدراسة إن الاستخلاص بالقلوي أو الحامض العضوي مع الترسيب على البارد أدت إلى ترسيب بروتين الجلايسنين (**S**١١) بصورة رئيسية مع كمية قليلة من بروتين بيتاكونجلايسنين (**7S**) بينما الاستخلاص بالقلوي أو الماء والترسيب باستخدام الحامض أدى إلى ترسيب بروتينات فول الصويا بصورة غير متجانسة بحيث احتوى على خليط من بروتين الجلايسنين والبيتاكونجلايسنين وذلك عن طريق التعرف عليهما باستخدام كل من **PAGE** و **SDS-PAGE**.

باستخدام الماسح الحراري ألتفريقي **DSC** تبين أن بروتينات فول الصويا المرسبة بالحامض لها نقطتي انتقال حراري عند درجتي حرارة **72.8** و **86.4** درجة مئوية على الجانب الأخر فأن بروتينات فول الصويا المرسبة على البارد لها نقطة

انتقال حراري رئيسية عند **85.3** درجة مئوية والمتمثلة في الجلايسنين ونقطة انتقال حراري ثانوية عند درجة حرارة **75.3** درجة مئوية والمتمثلة في البيتاكونجلايسينين.