Chromatographic Separation and Physico-chemical Study of Vicilin from Chickpea (Cicer arietinum L) Seed.

SAEEDUL HASAN SIDDIQUI
N.W.F.P. Agricultural University
Peshawar, Pakistan

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Summary: Total salt-soluble protein was extracted from chickpea Cotyledens and was precipitated with different concentrations of ammonium sulphate into fractions 0.60% and 60-100%. Albumin was removed from fraction 60-100 by dialysis at 3-5°C against H_2O, pH 4.1, and globulin of the fraction was chromatographed on DEAE-cellulose column for separation of vicilin from protein complex.

Isolated Vicilin was tested for freedom from contaminants. Chromato-electrophoretically it was pure. Sedimentation curve also witnessed about its hemogeneity. Maximum elution on celite column was with 76% ammonium sulphate concentration. Elution constants on hydroxylapatite and DEAE-cellulose columns were 0.40 M and 0.26 µ phosphate buffers respectively. Sedimentation coefficient was 6.72 S.Vicilin was found rich in the isoleucine and poor in arginine in comparison with the results obtained by Boulter and Derbyshire (1971) for the amino acid composition of vicilin of chickpea seed.

Introduction

The chickpea seed, an important legume grain and valuable source of plant proteins, has considerably substituted the animal proteins and contributed substantially to the qualitative improvement of human diet. The chickpea seed contains between 12-31% protein, composed of reserve proteins (globulins of primary and secondary type) and albumins, whereas about 80% of the total protein of seed is composed of globulins fraction (Koinov, 1968) [1]. The vicilin and legumin are the primary globulins they possess independent genetic control system for their biosyntheses and their quantitative distribution in the seed is determined by the type of plant (Boulter et al, 1976 [2]). Klimenko (1978) [3] claimed that the testa was poor in protein content, while embryo contained albumin and low molecular weight globulins associated with nucleic acids and carbohydrates. The cotyledon is, therefore, only the morphological part of seed enriched with vicilin and legumin.

The chickpea seed contains a highly complicated heterogeneous system of individual proteins (Grigorcha, 1971) [4], and the separation of individual proteins free from contaminats is a complex problem of protein chemistry. The chemical fractionation procedures used so for often resulted in the isolation of many proteins as reported by Squire and Li (1959) [5]; and Leonov (1959) [6], but the native state and homogeneity of these remained doubtful. Grigorcha (1971) [4] and Lapteva (1967) [7] empolyed combinations of various techniques including chromatography on DEAE-cellulose and

hydroxylapatite columns and isolated the vicilins from the chickpea and lentil seeds respectively. Alekseeva (1970) [8] indicated that the dominant globulin components of total salt-soluble protein extract of chickpea seed were precipitable between 60-100% concentration of ammonium sulphate. Total salt soluble proteins of chickpea seed were fractionated and when fraction 60-100 was subjected to chromatography, it was found that the vicilin and legumin of DEAE-cellulose column had their elution constants at 0.26μ and 0.33 μ buffers respectively (Siddiqui, 1981) [9], while on hydroxylapatite and celite columns they were practically inseparable (Grigercha, 1971) [4]. It has also been observed that the elution constants of certain albumins of legume grains on celite and DEAE-cellulose columns coincided with the elution constants of primary globulins (Sayanova et al., 1971) [14]. Boulter and Derbyshire (1971) determined values of sixteen amino acids for the vicilin of chickpea seed.

The present investigation was initiated with the object to devise a simplest possible method for the isolation of chromatographically pure vicilin from chickpea seed and to study the physico-chemical properties including amino acid composition of the isolated protein component.

Experimental

The experiments were conducted at the laboratory of protein chemistry, Kishinev State University, USSR. The seeds of chickpea (*Cicer arietinum* L.) variety, Sowkhoz-14 were obtained from the Biological Station, Kishinev State University, Kishinev. The testa and embryo were removed. The cotyledons were ground into flour. Total salt-soluble protein was extracted from the defatted flour with 1 M Na Cl, pH 7.0 phosphate buffer. The total salt-soluble protein extract was fractionated by gradually adding desired amount of crystalline ammonium sulphate (Keil and Sormova, 1959). Fraction 0-60% and 60-100% were precipitated. The globulins of fraction 60-100% were freed from albumin contamination by dialysis. Cellophane membrane tubes of 10-12 mm diameter were prepared by pasting the membrane sides with zinc chloride solution. The tubes were later on washed thoroughly with distilled water for removal of Cl^- ions. The tubes were then filled with the proteins of fraction 60-100% and were tied with the thread so as to close the tubes. The tubes were placed in a jar; and distilled water acidified with CH_3 COOH to pH 4.1, corresponding to the isoelectric point of the globulins of chickpea seed, was dropped gradually in the jar [13]. The process of dialysis was carried out in a cold room at 3-5°C for 48-72 hours [14]. After dialysis the globulin precipitate and albumin solution of the cellophane tube were transferred to a centrifuge tube and centrifuged for 10 minutes at 8000 rpm. The pH of the tube solution was determined; and the full precipitation of the globulins was confirmed by carefully dropping 2% acetic acid in the albumin solution, removed from the centrifuge tube. If minimal quantity of globulin precipitate appeared in the solution, it was once again collected by centrifugation and there was no need for further dialysis [10]. The globulin components of fraction 60-100 thus obtained were subjected to chromatographic separation.

The globulins were chromatographed on DEAE-cellulose, hydroxylapatite ($Ca\ 5\ (PO_4)_3$ OH) and

celite-545 analytical and preparative columns as reported elsewhere (Anacker and Stoy, 1958; [15] and Siddiqui, 1981 [16]). The salt concentration in the eluate was determined graphically, while the concentration of ammonium sulphate in each tube was determined refractrometerically as reported by Whitker and Hughes (1949) [17]. The concentration of protein was determined spectrophotometrically in each tube based on absorption at 278 nm and was plotted on the chromatogram, while the nature of chromatographic fraction was determined on the basis of extinction correlation E-260/E-278 (nucleic acid: protein concentration ratio) at 260 nm and 278 respectively (Siddiqui, 1982) [18]. The protein of the chromatographic fraction was analysed by gel electrophoresis using 7.5% polyacrylamide gel and tris-buffer pH 8.3 (Maurer, 1971) [19].

Protein of 0.7% concentration in phosphate buffer of 0.3 pH 7.2 was ultracentrifuged (Ultracentrifuge, MOM-G-120 Hungary) at 20 °C and 42830-45640 r.p.m. (Grigorcha, 1971) [4], while the sedimentation coefficient was determined as reported by Shpikiter (1964) [20]. For amino acid analysis, 10 mg protein was hydrolysed with 6 N HCl at 132°C for 4 hours. The hydrolysate, after removing the acid, was dissolved in 5 ml citrate buffer of pH 2.2; was exposed to an amino acid analyser (amino acid analyser, Hd-1200-E, Chekoslavakia) and the amino acid values were determined as indicated by VIR (1973) [21].

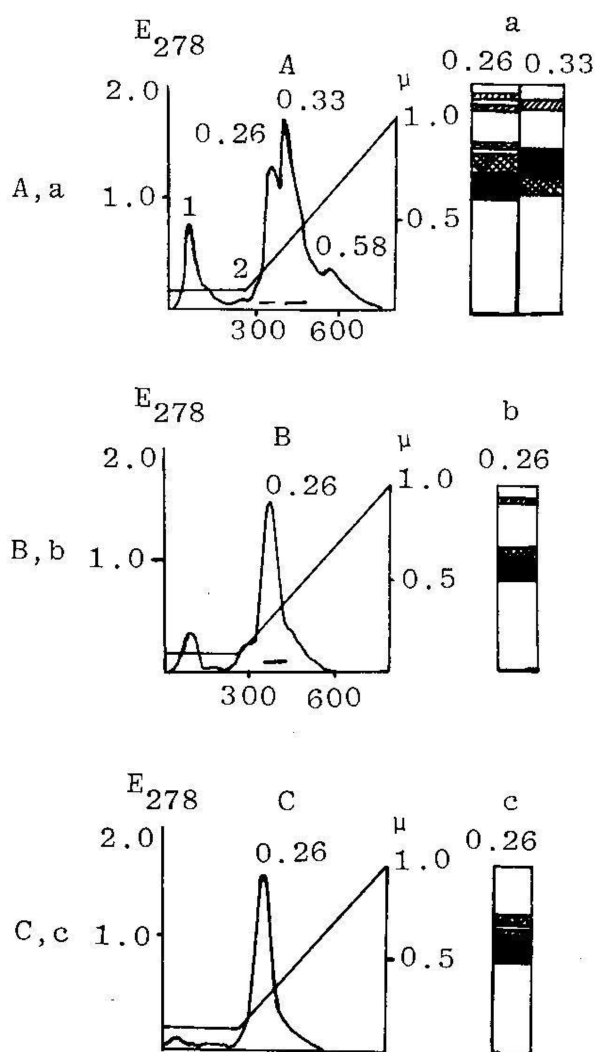

Figure 1. Chromatography on DEAE-cellulose and electrophoresis in polyacrylamide gel of protein fractions from chickpea seed.
A,a) Chromatography of fraction 60-100 and electrophoresis of Subfraction.0.26 and 0.33
B,b) Rechromatography of Subfraction 0.26 and electrophoresis of subfraction 0.26 pooled after rechromatography.
C,c) Rechromatography of Subfraction 0.26 and electrophoresis of Subfraction 0.26, pooled after rechromatography.

Results and Discussion

The results of chromatographic separation of vicilin, from total salt -soluble protein extract of chickpea seed, and its homogeneity test are presented in figures 1 and 2 respectively, while the scheme for the separation of vicilin and its amino acid composition is given in table 1 and 2 respectively.

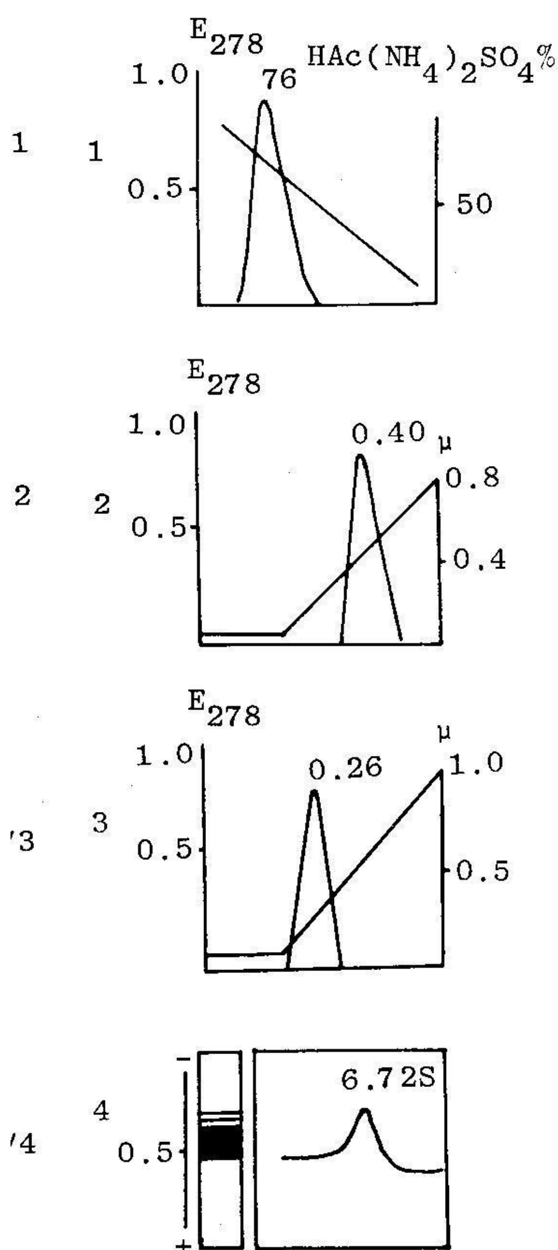

Fig.2 Homogeneity evaluation of vicilin from chickpea seed.

1) Gradient extraction on celite column (2/1),
2) Chromatography on hydroxylapatite (2/2),
3) Chromatography on DEAE-cellulose (2/3),
4) Electrophoresis in polyacrylamide gel and ultracentrifugation (2/4).

Table-1: Schematic isolation of the vicilin from chickpea seed

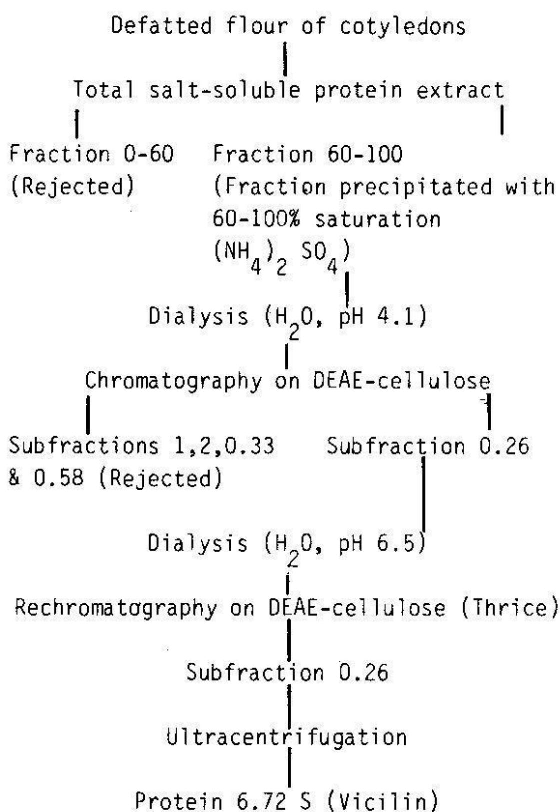

Table 2: Amino acid composition of the vicilin of chickpea seed (gm/100 gm of vicilin)

Amino acid	%	Amino acid	%
Lysine	8.32	Glycine	4.42
Histidine	3.28	Alanine	4.72
Arginine	1.26	Valine	4.47
Aspartic acid	12.19	Isoleucine	6.96
Threonine	2.45	Leucine	8.02
Serine	6.79	Tyrosine	3.44
Glutamic acid	14.78	Phenylalanine	8.28
Proline	5.57		

It was demonstrated in an earlier work (Grigorcha, 1971 and Alekseeva, 1970) [4,8] that the fraction, precipitated from total salt-soluble protein extract of chickpea seed between 60-100% ammonium sulphate concentration, was dominated by primary globulin and also contained

a considerable amount of secondary globulin and albumin. Fraction 60-100 in the initial stage was, therefore, freed from albumin contamination by dialysis and the globulins thus left in the fraction were subjected to the charomatographic separation.

Fraction 60-100 was separated by chromatography on DEAE-cellulose column in five subfractions constituting peaks 1,2, 0.26, 0.33 and 0.58 (Fig. 1-A). Subfraction 1 and 2 were enriched with secondary globulins and subfraction 0.58 with nucleic acids, whereas subfractions constituting peaks 0.26 and 0.33 were dominated by vicilin and legumin components respectively. Similar chromatographic behaviour of vicilin and legumin on DEAE-cellulose column was also observed by Grigorcha (1971) [4]. As such subfraction 0.26, enriched with vicilin, was pooled together for further analysis. Electrophoresis in polyacrylamide gel indicated that subfraction 0.26 was hihgly heterogeneous and possessed six electrophoretic zones. Principle electrophoretic zone showed a medium-fast mobility in the electrophoregram. When the electrophoretic analytical results of subfraction 0.26 were compared with that of subfraction 0.33, it was found that subfraction 0.26 was contaminated by the proteins of subfraction 0.33 (Fig.1-a). Rechromatography of subfraction 0.26 on DEAE-cellulose revealed a smaller contamination by the proteins of other subfractions (Fig.1-B). Subfraction 0.26 was pooled together and subjected to gel electrophoresis. The results indicated that the principle component of subfraction 0.26 was contaminated by three other electrophoretic zones (Fig.1-b). Therefore, subfraction 0.26 was further chromatographed and the subfraction, pooled together after second rechromatography, was analysed by gel electrophoresis. The chromato-electrophoretic results indicated an appreciable minimization of contaminants (Fig. 1-C,c), nevertheless requiring a further purification. Consequently, subfraction 0.26 was once again chromatographed on DEAE-cellulose column. Third rechromatography gave a single symmetrical peak on the chromatogram, having maximum elution with 0.26µbuffer and witnessing that subfraction 0.26 was chromatographically (on DEAE-cellulose column) pure.

However the protein of subfraction 0.26 after purification was tested for contaminants by gradient extraction on celite column, chromatography on hydroxylapatite and DEAE-cellulose columns, electrophoresis in polyacrylamide gel and ultracentrifugation (Fig.2). The isolated protein component was chromatographically pure on celite (Fig. 2/1) hydroxylapatite (Fig. 2/2) and DEAE-cellulose (Fig. 2/3) columns. The gel electrophoresis indicated presence of second minor zone, while sedimentation curve also showed the presence of admixture (Fig. 2/4), although the isolated protein was chromatographically homogeneous. The appearance of admixture on electropherogram and sedimentation curve was the aggregate of the isolated protein component formed during the isolation process. Formation of such admixtures of high molecular weight proteins which were the aggregates of the isolated protein components and appeared due to presence of proteins for a longer period in the buffers during isolation process, were also reported by Grigorcha (1971) [4].

The chromatographically pure protein component had maximum elutions with 76% ammonium sulphate concentration, 0.40 M and 0.26µphosphate buffers on celite, hydroxyla-

patite and DEAE-cellulose columns respectively. The sedimentation coefficient of the isolated protein component was 6.72 S and in general, was in agreement with the sedimentation coefficients of the vicilins isolated by Grigorcha (1971) [4] and Lapteva (1971) [7], from chickpea and lentil seeds respectively. Moreover the sedimentation coefficients of vicilin components of legume grains were reported to be, as a rule, between 6.5-7.8 S (Vaintraub and Shutov, 1968) [22]. The isolated protein component was, therefore, vicilin or 7S component of chickpea seed. The schedule developed in the present study for the isolation of vicilin from chickpea seed is simple (Table-1) as compared to the more complicated purification schemes of the former.

The amino acid analysis of vicilin revealed that the isolated component was rich in glutamic acid, aspartic acid, phenylalanine, leucine and lysine, while poor in arginine and threonine. The values for histidine, tyrosine, glycine, alanine, proline, isoleucine and serine were found between 3.28-6.97% (Table-2). The values obtained for amino acids of vicilin from chickpea seed in this study, in general, were in conformity to the results of amino acid composition of vicilin of chickpea seed,

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