Comparative Study of Chickpea and Pea Pa2 Albumins

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PA2 albumins from five different cultivars of kabuli-type chickpea and from one pea cultivar have been purified and characterized. Selective extraction of albumins was followed by two gel filtration purification steps. In all chickpea cultivars, PA2 is made up of one type of protein while pea PA2 is made up of two isoforms as observed by nondenaturating PAGE. PA2 has so far no defined function in pea while in chickpea a similar protein to PA2 has been described as a lectin. The present study confirms that PA2 from pea and chickpea agglutinates papainized human erythrocytes. Also it is reported that chickpea and pea PA2 are allergens for individuals sensitive to chickpea intake.

Keywords: *PA2 albumin; pea; chickpea; allergen; lectin*

INTRODUCTION

Legume seed proteins are usually classified in two classical solubility fractions, the majority being globulins or proteins soluble in dilute salt and albumins or water-soluble proteins (Osborne and Campbell, 1897). Globulins function as storage proteins representing a source of energy, carbon, and reduced nitrogen for germination and seedling growth. On the other hand, the albumin fraction contains a large number of minor proteins including enzymes, protease inhibitors, amylase inhibitors, or lectins. Also, some albumins are major allergens such as the 2S albumin from the seeds of yellow mustard (Menéndez-Arias et al., 1988).

In pea (*Pisum sativum* L.) the main albumin protein is PA2 (Schroeder, 1984). Pea PA2 has been purified and partially characterized (Croy et al., 1984; Harris and Croy, 1985; Gruen et al., 1987; Higgins et al., 1987). It consists of two distinct polypeptides (PA2a and PA2b) of similar molecular weights (24−25 kDa), the relative proportions of which can vary significantly between genotypes (Rao et al., 1989). When purified to homogeneity, the native protein contains a larger isomer (M_r ∼53 kDa) which consists of two 25 kDa PA2b subunits and a smaller isomer (M_r ∼48 kDa) consisting of two 24 kDa PA2a subunits. Although the resistance to breakdown during germination and the cytoplasmic location suggest that they are not storage proteins (Casey et al., 1993), pea PA2 function is unkown.

In chickpea, a protein with similar physicochemical characteristics to pea PA2 has been purified and characterized (Kolberg et al., 1983). SDS−PAGE showed one polypeptide band corresponding to a molecular mass of 26 kDa. The native protein has a molecular mass of 44 kDa as determined by ultracentrifugation and gel filtration. In addition it has a high degree of homology in the N-terminal amino acid sequence to pea PA2. Chickpea PA2 has been described as a lectin that is able to agglutinate papainized human erythrocytes of the different ABO groups. Lectins are proteins that bind carbohydrates with considerable specificity. These proteins can participate in numerous cellular processes such as host−pathogen interactions, targeting of proteins, or cell−cell communications (Elgavish and Shaanan, 1997). Other functions such as carbohydrate transporters, recognition agents, storage proteins, or growth regulators have been suggested (Pusztai, 1991). In plants, lectins may also function as defensive molecules that bind to glycan receptors of the predator intestinal mucosa and change the cellular membrane permeability generating nausea, vomiting, and diarrhea (Peumans and Van Damme, 1996). In legumes, known lectins are made of four identical peptides with molecular masses between 25 and 30 kDa or formed by two light (M_r ∼5 kDa) and two heavy chains (M_r ∼18 kDa) (Liener, 1989). In pea, the presence of the second type of lectins has been reported (Trowbridge, 1974; Higgins et al., 1983a,b).

Because a similar protein to pea PA2 has been described in chickpea as a lectin and to establish the function of PA2 albumins, we have purified and functionally characterized these proteins from pea and chickpea.

MATERIALS AND METHODS

Material. Pea seeds (*Pisum sativum* L.), commercial variety Jumbo, were obtained locally; seeds of chickpea (*Cicer arietinum* L.) kabuli-type cultivars athenas, chamao, mejicano, CA-71-21-7-8M, and CA-20, were a gift from Koipesol Semillas S.A. (Sevilla, Spain). Sephadex G-100, Sephacryl S-300 HR, and standard proteins were from Pharmacia (Pharmacia/LKB, Uppsala, Sweden). Sodium dodecyl sulfate, Tween 20, papain, concanavalin A, alkaline phosphatase-conjugate mouse anti-human IgE, and nitrocellulose membranes were purchased from Sigma Chemical Co., St. Louis, MO. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were supplied by Boehringer Mannheim GmbH (Mannheim, Germany).

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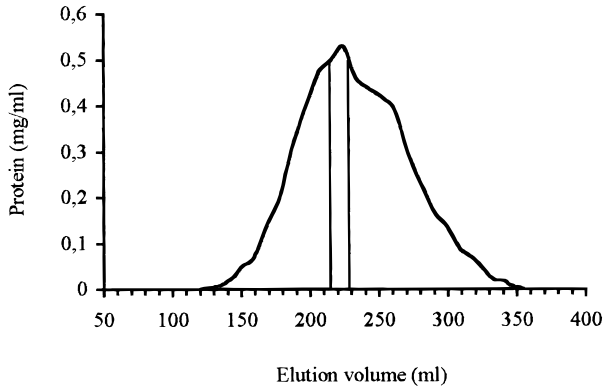

Figure 1. Gel filtration elution profile of chickpea albumins on a Sephacryl S-300 HR column. Fractions enriched in PA2 (between lines) were pooled.

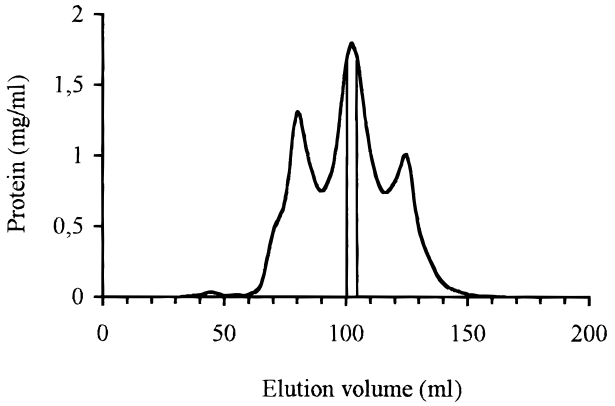

Figure 2. Gel filtration elution profile of enriched chickpea PA2 albumin on a Sephadex G-100 column. Fractions pooled are between lines.

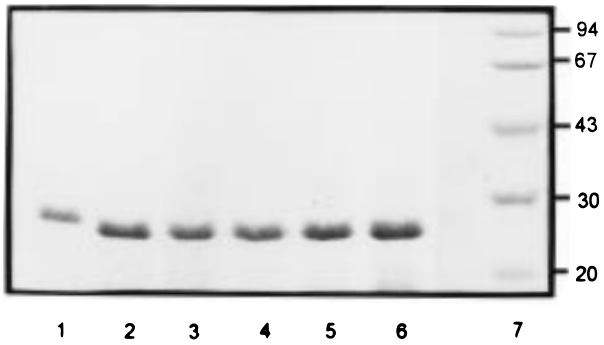

Figure 3. SDS−PAGE analysis of PA2 purified from pea and different chickpea cultivar varieties: (lane 1) pea, (lane 2) chickpea cv. athenas, (lane 3) chickpea cv. chamao, (lane 4) chickpea cv. mejicano, (lane 5) chickpea cv. CA-71-21-7-8M, (lane 6) chickpea cv. CA-20, and (lane 7) protein standards.

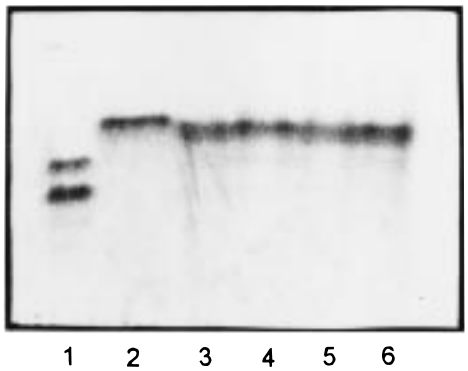

Figure 4. Native PAGE analysis of PA2 purified from pea and different chickpea cultivar varieties: (lane 1) pea, (lane 2) chickpea cv. athenas, (lane 3) chickpea cv. chamao, (lane 4) chickpea cv. mejicano, (lane 5) chickpea cv. CA-71-21-7-8M, and (lane 6) chickpea cv. CA-20.

Acrylamide and bis-acrylamide was from Serva (Heidelberg, Germany). Mercaptoethanol was supplied by Merck (Darmstadt, Germany). Coomassie G-250 was purchased from BioRad (Richmond, CA). All other reagents were of analytical grade.

Purification of PA2 Albumins. For the obtention of flour, seeds were ground with a domestic blender. Fractionation of chickpea proteins was performed according to Singh et al. (1988). For this, meal was extracted three times with 0.1 M borate buffer pH 8.3 (1:10 w/v) during 1 h. Extracts were centrifuged at 8000*g* and the supernatants pooled and dialyzed against 25 mM sodium citrate buffer pH 4.6 (1:10 v/v) for 48 h at 4 °C. Dialyzed extracts were centrifuged as above. The resulting supernatants and pellets were the albumin and globulin fractions, respectively. For purification of PA2, after concentration by lyophilization, albumins were loaded on a Sephacryl S-300 HR gel filtration column (75 × 2.6 cm) at a flow rate of 40 mL/h using as eluent 20 mM phosphate buffer, 0.5 M NaCl, pH 7. Fractions enriched in PA2, as monitored by SDS−PAGE, were pooled, concentrated by liophylization and chromatographed on a Sephadex G-100 gel filtration column (100 × 2 cm) at a flow rate of 30 mL/h using the same buffer. Fractions in which PA2 was pure, as observed by SDS−PAGE, were pooled.

M_r Determination. For native molecular weights determination the Sephadex G-100 gel filtration column was used. The column was calibrated with the following standard proteins: blue dextrane, lipoxygenase (108 kDa), bovine serum albumin (67 kDa), ovoalbumin (43 kDa), ribonuclease (13.7 kDa), and cytocrome *c* (12.2 kDa).

Protein Measurements. Protein concentrations were determined by the method of Bradford (1976). Bovine serum albumin (BSA) was used as standard.

Electrophoresis. SDS−PAGE was performed by the method of Laemmli (1970). The gel system, containing 0.2% (w/v) SDS consisted of a 15% polyacrylamide resolving gel (pH 8.8) and a 3% stacking gel (pH 6.8). The length of the resolving and stacking gels were 10 and 2 cm, respectively, with a gel thickness of 0.75 mm. Electrophoresis was performed at a constant current of 25 mA. Protein bands were stained by immersion of the gels in a 0.05% (w/v) Coomasie G-250 solution, in 45% methanol and 9% acetic acid solution. Molecular masses were determined using the low molecular weight standard kit from Pharmacia. Native electrophoresis was carried out in 8.5% (w/v) acrylamide gels as described (Laemmli, 1970) but omitting SDS and mercaptoethanol from all buffers.

Amino Acid Sequence Analyses. Automatic N-terminal sequence analysis was performed on a Procise Protein Sequencing System Sequenator (model 494, Applied Biosystems, Foster City, CA) according to the method described by Edman and Begg (1967). The sample was analyzed in a flow-through reactor directly applied to a glass-fiber filter and the phenylthiohydantoin derivatives were identified with an on-line HPLC analyzer.

Carbohydrate Analysis. PA2 albumins were precipitated and washed extensively with 10% (w/v) trichloroacetic acid, and their sugar contents were esteemed colorimetrically by the phenol−sulfuric acid method using a standard of glucose (Dubois et al., 1956).

Immunochemical Assay. For dot blotting, PA2 samples were blotted on 0.45 μm nitrocellulose membranes. Membranes were dried, and the remaining active sites were blocked by incubation with 5% deffated milk in TBS (Tris-HCl 20 mM, NaCl 0.05 M, pH 7.5), for 2 h. The nitrocellulose membranes were then washed three times with TTBS (0.05% Tween 20 in TBS) for 15 min and incubated with the serum of sensitive individuals (1:1000 v/v dilution) overnight at 4 °C. The nitrocellulose blot was then washed three times with TTBS

Pea[a]	**T** K **T G Y I N A A F R S S Q N N E A Y L F I N** D **K**
Chickpea[b]	**T** N **F G Y I N A A F R S S** X **N N E A Y L F I N** G **K**
Chickpea cv. athenas	**T** X **F G Y I N A** L **F R S S**

a Higgins et al., 1987.
b Kolberg et al., 1983.

Figure 5. N-terminal amino acid sequence of PA2 albumins from pea, chickpea, and chickpea cv. athenas. Identical amino acids are in bold.

for 15 min each, alkaline phosphatase-conjugate mouse anti-human IgE (1:1000 v/v in TTBS) was then added, and the blot was incubated at room temperature for 2 h. The nitrocellulose membrane was then washed two times with TTBS and once with 1 M borate buffer pH 8.3 for 15 min each. The blot was developed with 50 μL of nitroblue tetrazolium and 37.5 μL of 5-bromo-4-chloro-3-indolyl phosphate in 10 mL of 1 M borate buffer at room temperature for 10 min. The reaction was stopped by placing in distilled water for 15 min, and the membrane was air-dried. A protein extract of chickpea (10 μg) and BSA (3 μg) were used as positive and negative control, respectively. Membranes were loaded with 1 μg of the samples.

Enzyme Treatment of Human Erythrocytes. Packed erythrocytes (0.2 mL) were suspended in 1 mL of 50 mM phosphate, 0.15 M NaCl buffer, pH 7.5 containing 0.1 mg of papain (15 U/mg). Cells were incubated with the protease at 37 °C for 30 min and stored at 4 °C until used.

Haemagglutination Assay. The haemagglutination assay was performed in Eppendorf tubes at 4 °C. Protein samples were serially diluted (2-fold) in phosphate buffered saline (pH 7.5). An equal volume of 1% papainized human erythrocytes was added to each dilution. After 1 h incubation at 4 °C, the tubes were centrifuged at 2000*g* for 1 min. Titer value was determined visually. This was the highest dilution still exhibiting a residue of agluttinated cells along the wall of the tube that is absent from the blank. Concanavalin A and BSA were used as positive and negative controls, respectively.

RESULTS AND DISCUSSION

PA2 Purification. Albumins were extracted according to Singh et al. (1988), as described in Materials and Methods. This procedure was more effective to bulk purify albumins than the classical water extraction system used by Osborne and Campbell (1897). With the second one a contamination of albumins with globulins have been observed (data not shown), probably because a fraction of globulins are soluble in water and extracted together with albumins.

Extracted albumins were applied to a Sephacryl S-300 HR gel filtration column and fractions enriched in PA2, as monitored by SDS−PAGE, were pooled (Figure 1). These fractions were concentrated by lyophilization and loaded on a Sephadex G-100 gel filtration column, and fractions in which PA2 was pure, as monitored by SDS−PAGE, were pooled (Figure 2).

Chickpea PA2 has been purified from different cultivars (Figure 3). The PA2 albumin preparations of all chickpea varieties showed a similar R_f in SDS−PAGE with an apparent M_r of 23 kDa. This M_r is slightly smaller than the previously reported value of 26 kDa for chickpea (Kolberg et al., 1983). Pea PA2 purified by us showed a higher M_r (26 kDa) under denaturing conditions than the observed in chickpea. This M_r is similar to that previously reported by Croy et al. (1984) for the two polypeptides isoforms of pea PA2 (24 and 25 kDa).

In pea, PA2 has been described to be formed by two similar proteins. The larger protein consists of two 25 kDa subunits, whereas the smaller one contains two 24 kDa subunits (Croy et al., 1984). On the other hand, in chickpea only one type of PA2 has been described (Kolberg et al., 1983). When pea and chickpea PA2 albumins were subjected by us to native electrophoresis, pea PA2 albumin resolved in two bands corresponding to the reported small and large PA2 proteins, whereas chickpea PA2 albumin migrated as one band with a similar mobility in all cultivars analyzed (Figure 4).

Figure 6. Dot blot of chickpea cv. athenas PA2 (1 μg) and pea PA2 (1 μg) against sera of a person sensitive to chickpeas. Chickpea total proteins (10 μg) were used as positive control. BSA (3 μg) was used as negative control.

Purified chickpea PA2 was eluted from the Sephadex G-100 gel filtration column as a single symmetrical peak, corresponding to a M_r of 46.4 ± 2.9 kDa. This value is in good agreement with the previously reported value of 44 kDa for chickpea PA2 (Kolberg et al., 1983).

N-Terminal Amino Acid Sequence. Chickpea cv. athenas was N-terminally sequenced (Figure 5). Amino acid sequencing revealed one residue in each position. This denotes that the protein was pure, since the sample used for sequencing was taken directly from an aqueous solution of the fractions pooled from the last purification step. Also, this confirms that chickpea PA2 albumin is made of a single isoform. The alignment of the N-terminal amino acid sequence, obtained by us, with that previously reported for chickpea (Kolberg et al., 1983) shows a high degree of similarity in the first 13 amino acids, differing in one residue (leucine instead of alanine in position 9). Also, the homology between pea and chickpea sequences is very high with 88% identity in the first 25 residues.

Allergenic Characterization of Chickpea and Pea PA2. Dot blotting using sera from 10 chickpea-sensitive individuals gave positive reaction against chickpea and pea PA2 (Figure 6).

It has been reported that prosthetic glucide groups are responsible for the positive reaction of most protein allergens (Cheftel et al., 1985). In this sense, carbohydrate analysis of chickpea PA2 shows that it is not a glycoprotein, with less than one sugar residue per protein molecule. A similar sugar content has been reported for pea PA2 (Croy et al., 1984). Nevertheless, other albumins without sugar residues, such as the 2S albumin from *Sinapis alba* L. seeds, also have been

described as allergenic (Menéndez-Arias et al., 1988). To our knowledge, this is the first report on the allergenic nature of pea and chickpea PA2 albumins.

Functional Characterization. Pea PA2 has been partially characterized in the past but no function has been described for this albumin (Schroeder, 1984; Croy et al., 1984; Harris and Croy, 1985; Gruen et al., 1987; Higgins et al., 1987; Rao et al., 1989). It was analyzed for haemagglutinating activity with negative results using untreated rabbit erythrocytes (Croy et al., 1984). A protein homologous to pea PA2 has been purified and functionally characterized in chickpea (Kolberg et al., 1983). It has been described as a lectin capable of agglutinating papainized human erythrocytes. We have assayed several cultivars of chickpea and pea for the lectin activity of PA2 albumin. When untreated human red blood cells were used in the experiment there was no positive agglutination reaction, even with a protein concentration of 1 mg/mL. On the other hand, using papainized human erythrocytes, agglutination was observed with a titer concentration of 12 μg of protein/mL. It seems that this class of lectins requires erythrocytes with the membrane partially digested by proteases so that certain membrane sugars residues are accessible to the protein. This requirement has been observed in another lectin purified from *Psophocarpus tetragonolobus* (L.) DC (Pueppke, 1979). This protein is also a homodimer with a M_r of 46 $\pm$ 2 kDa and a subunit M_r of 29 $\pm$ 3 kDa, which agglutinated trypsinized human red blood cells. Thus, the partial disruption of the membrane cell appears as a general requirement for the activity of these proteins.

Some lectins agglutinate only erythrocytes from an unique animal species (Liener, 1989). This may be another reason for the negative agglutination results obtained previously with pea PA2, since it was tested with rabbit red blood cells. Although, because of the close taxonomical relationship between pea and chickpea, this seems unlikely.

In contrast to most lectins that are glycoproteins with 4−10% carbohydrates (Liener, 1989), PA2 does not contain sugar residues. Another difference to common lectins is that it does not require Mn^{2+} or Ca^{2+} ions for agglutination activity. Also, in contrast to described lectins, the agglutination is unstable at room temperature.

The molecular weight range, quaternary structure, absence of sugars, and physicochemical requirements for agglutination make PA2 albumins an unusual group of lectins clearly separated from those commonly found in legumes.

Further studies on the functional characteristics of PA2 are needed. Although legume lectins has been considered in general as defensive molecules (Peumans and Van Damne, 1996), no direct evidence for the function of PA2 has been obtained. The possible role of lectins in host−pathogen interactions, cell−cell communications, and the sequence homology of PA2 albumin with structural proteins such as vitronectin (Jenne, 1991) makes a deeper study of the real function that PA2 may play in legume seeds of interest.

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