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Centrifugal partition chromatography as a tool for preparative purification of pea albumin with enhanced yields☆

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Abstract

A new procedure including the use of centrifugal partition chromatography (CPC) is proposed to purify PA1b and its isoforms. These pea (*Pisum sativum L.*) seed proteins are toxic against weevils and can be used as an environment-friendly insecticide. CPC was applied to a whole albumin fraction prepared from pea flour. The butanol:aqueous TFA system used in CPC allowed the separation of PA1b from other albumins and a degree of purification above 95%. Compared to analytical procedures based on methanol extraction, anion exchange and then reversed-phase chromatography (RPC), CPC recovered PA1b in much better yield, which is indispensable for large-scale purification of a biodegradable insecticide.

Keywords: Pea albumin; Insecticide; Centrifugal partition chromatography

1. Introduction

The use of chemical insecticides is the main answer to the damage caused by stored product pests. However, increased consumer concern over the toxicity of the residues of insecticides applied to stored grain and the occurrence of insecticide-resistant insect strains have highlighted the need for new approaches to control stored product insect pests.

The recent isolation from pea of polypeptides that are toxic for stored product insects has enlarged the possibilities for cereal grain protection [1]. These polypeptides are related to pea albumins of the PA1b type [2] and belong to the cystine-knot family of toxins [3]. PA1b binds with high affinity to the microsomal fraction of susceptible, but not resistant, *Sitophilus* spp. [4].

Pea seeds contain two major classes of proteins extractable in water and salt solution, albumin and globulin, respectively. Albumin accounts for approximately 30% of the total protein [5]. When the total albumin fraction is analysed by SDS-PAGE in reducing conditions, two major components are observed: PA2, Mr ∼ 26000 and PA1, Mr ∼ 6000 [2,5]. PA2 contributes approximately 5% of the total pea seed protein [5]. In mature PA2 albumin, subunits PA2a and PA2b are supposed to associate non-covalently and form homodimers of Mr 53000 and 48000 [6]. In contrast to the pea globulins, PA2 is localised in the cytosol rather than in the protein storage vacuole [7]. The physiological function of PA2 is unknown, but *in vitro* PA2 was characterised as a surface-active component involved in foaming and emulsifying properties of pea albumins [8].

In the mature grain, PA1 consists of two components, PA1a Mr 6000 and PA1b Mr 4000 [2]. PA1b comprises about 4% of the total pea seed protein and contributes about 23% of the seed's sulphur amino acids [2]. It induces short-term mortality in several pests, including the cereal weevils *Sitophilus* spp. (*S. oryzae*, *S. granarius*, *S. zeamais*) which constitute a major nuisance for stored cereals all over the world [1].

Ion exchange and reversed-phase chromatographies can be used to separate PA1b and PA2, due to their different characteristics, i.e. pI (7.82 and 5.16) and hydropathicity (+0.376 and −0.425) [2,5,9].

PA1b was purified from a total pea albumin fraction (TPA), obtained according Crévieu et al. [10], using 60% methanol solubilisation followed by anion exchange chromatography (PA1b not retained thanks to its alkaline pI) and finally RPC. This procedure gave sufficiently purified PA1b and separated the different

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PA1b isoforms, but unfortunately the recovery yield of PA1b was very low and any extrapolation impossible.

Among the different modern chromatographic techniques, centrifugal partition chromatography is known to be suitable for large-scale purifications.

Countercurrent chromatography (CCC, inventor: Y. Ito [11]) and centrifugal partition chromatography (CPC, inventor: K. Nunogaki [12]) are close chromatographic techniques which use a liquid–liquid biphasic system without solid support to maintain the stationary phase. Basically, a CPC instrument is a series of channels linked in cascade by ducts and aligned in cartridges or disks in a circle around a rotor. When the rotor is set in motion, the assembly is subject to a constant centrifugal field. Two immiscible liquids, prepared by mixing two or more solvents or solutions, are used as mobile and stationary phases, the mobile phase being percolated through the stationary phase thanks to a pump and the centrifugal field. Due to their liquid nature, the upper and lower phases of the biphasic system can be selected as mobile or stationary (ascending and descending modes).

Selection of a two-phase solvent system for CCC and CPC is similar to choosing a column and an eluant for HPLC. Important criteria include the polarity of the sample and its solubility, charge state, and ability to form complexes. A tremendous variety of biphasic solvent systems can be found in the literature, but only a few of them are now widely used. The most popular is the quaternary system comprising heptane (or hexane)/ethyl acetate/methanol (or acetonitrile)/water. By judicious variations in the relative amounts of each of these four components, it is possible to produce a wide range of systems, from nonpolar biphasic heptane/methanol to polar biphasic ethyl acetate/water. For more polar applications, systems combining butanol/methanol or acetonitrile or acetic acid/water (or buffered solution) are commonly used. A rational approach to the choice of medium polar solvent systems has been published recently [13]. Applications of CCC and CPC are numerous, and two excellent reviews are devoted to the developments in their application to plant analysis [14], and to the recent advances in peptide separation by CCC [15]. M. Knight has been a pioneer in peptide purification by CCC [12], routinely using the solvent systems *n*-butanol/acetic acid/water (4:1:5, v/v) and *n*-butanol/aqueous ammonium acetate (1:1). Introduction of displacement chromatography (also called pH-zone-refining) in CCC [16] has enhanced the preparative capabilities of the technique for obtaining pure peptides, using solvent systems like methyl *tert*-butyl ether/acetonitrile/water in combination with acids and bases [16].

The aim of this project was to apply phase partitioning then CPC to the purification of PA1b in order to develop a procedure applicable on an industrial scale.

2. Experimental

2.1. Analytical techniques

Reversed-phase high-performance liquid chromatography was carried out on a Gilson system controlled by 715 software (Gilson). Samples were diluted to 1 mg/ml in a mix of buffers (A) aqueous 0.06% trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.04% TFA in a 80/20 vol. ratio. One hundred microlitres were loaded on a C18 Nucleosil column (4.6 mm × 250 mm, 4.1 ml, Nucleosil) equilibrated in the same mix. Proteins were eluted by a gradient of acetonitrile (20–56% in 45 min) and detected at 220 and 280 nm.

2.2. Extraction of total pea albumin

The extraction was carried out on a pilot scale as described by [10]. The pea flour was obtained by grinding dehulled seeds (c.v. Frilene) with an industrial roller mill. The crude protein extract was prepared by stirring pea flour in 50 mM sodium acetate, pH 4.9, for 3 h at 4 °C. The flour/buffer ratio was 100 g/L. The suspension was clarified with a continuous centrifuge separator (Westfalia SA-14). Supernatant fraction was concentrated by ultrafiltration then desalted by diafiltration on a loop equipped with Novasep Carbosep (Miribel, France) M5 membranes (nominal molecular weight cut-off 10 kg/mol, 1.6 m^2 area, transmembrane pressure 0.3 MPa). Total pea albumin was obtained after freeze-drying.

2.3. Fractionation of total albumin on an analytical scale

TPA (20 g) was stirred with 200 ml 60% methanol for 1 h at 4 °C, then centrifuged for 30 min at 16000 × *g*, 4 °C. The clear supernatant was dialysed against distilled water for 72 h at 4 °C (dialysis bag with molecular cut-off of 3500) and then lyophilised. This fraction was loaded on a preparative DEAE Sepharose Fast Flow column for anion exchange chromatography (AEC). The non-adsorbed proteins were lyophilised and purified on a reversed-phase HPLC column (Si-C18).

2.4. Phase partitioning

The biphasic systems studied were *n*-butanol–water, *n*-butanol–aqueous 20 mM trifluoroacetic acid (TFA), and heptane–*n*-butanol–aqueous 20 mM TFA.

TPA powder was solubilised in aqueous phase and the organic solution was then added until the final volume was reached by vortexing for 35 s. The system was centrifuged at 105 or 525 × *g* for 10 min, and the volume of each phase was measured. Upper and lower phases were recovered, evaporated under vacuum and diluted with RP-HPLC buffers (A) aqueous 0.06% TFA and (B) acetonitrile 0.04% TFA in an 80/20 ratio (v/v).

The partition coefficient K was determined from the RP-HPLC peak areas, as $K = A_{up}/A_{lo}$ with A_{up} and A_{lo} being the areas of the peaks corresponding to a given protein found in upper and lower phase, respectively.

2.5. Protein purification by CPC

A semi-preparative fast centrifugal partition chromatography Kromaton model 200 ml (Angers, France) was used as CPC apparatus. It was composed of a column containing 1300 cells for a 200 ml total volume. This apparatus was able to rotate from 200 to 2000 rpm, to support up to 8 MPa pressure and a maximal

flow rate equal to 20 ml/min. Its injection loop had a volume of 10 ml.

The complete chromatographic chain was completed by a Gilson 307 isocratic pump and several devices from General Electric Healthcare: a UV-Vis detector UV-1/214 with a 3 mm cell, a conductimeter–pH-meter pH/C-900, a fraction collector and a paper recorder.

Each phase was prepared separately, then the two phases were mixed vigorously to be sure that each phase was saturated with the other one, and the mix was left to settle.

The column was filled with stationary phase (butanol) at 2.5 ml/min and 400 rpm in a descending mode. The rotation speed was increased up to 500 or 1200 rpm. The elution was carried out at 5 ml/min. In these conditions, the volumes of stationary and mobile phases were measured as 144 and 56 ml, respectively.

The sample was diluted with the aqueous mobile phase saturated in stationary phase and pumped into the column at 2.5 ml/min. After complete injection, proteins were eluted first by the mobile phase in descending mode, then by the butanol phase in ascending mode.

3. Results

3.1. Total pea albumin characterisation

TPA contained hydrophilic and more hydrophobic proteins as determined by RPC (Fig. 1). Hydrophilic proteins were composed mainly of trypsin inhibitors. Hydrophobic ones comprised PA1b and PA2.

3.2. Purification without CPC

PA2 was not completely solubilised by 60% methanol/water unlike the hydrophilic proteins and PA1b. AEC completely separated PA1b, which was not retained, and PA2, most of which was retained by the DEAE column.

Finally, RPC purified PA1b from hydrophilic proteins (Fig. 2). PA1b gave four peaks, in accordance with the findings of Delobel et al. [1]. All four are toxic against weevils and differ only by a few amino acids.

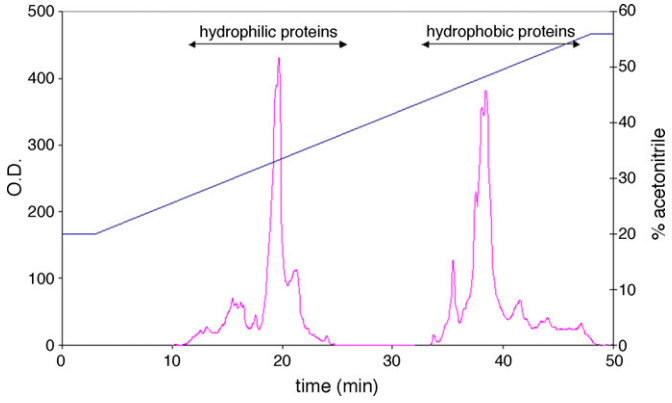

Fig. 1. RPC of total pea albumin on C18 Nucleosil column. Elution from 20 to 56% acetonitrile.

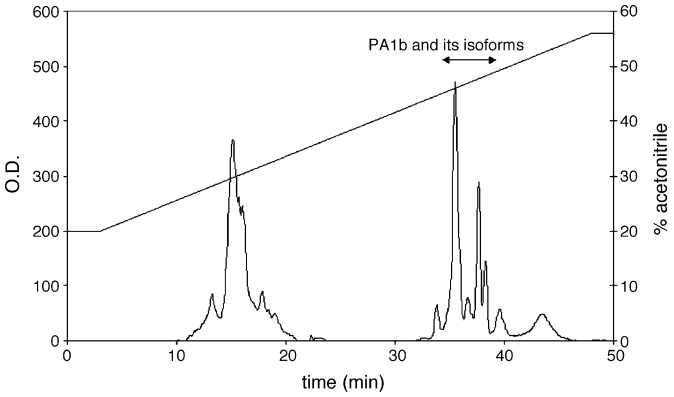

Fig. 2. RPC on C18 Nucleosil column of the fraction not retained on DEAE Sepharose column. Elution from 20 to 56% acetonitrile.

The whole purification procedure gave PA1b of 95% purity. From 40 g of TPA, 64 mg of PA1b were recovered.

3.3. Phase partitioning

PA2 elution by RPC did not differ to a great extent from that of PA1b (Fig. 3): it merged with the two most retained PA1b peaks, but not with the two less retained. Consequently, we were obliged to calculate the amounts of PA1b and PA2 assuming that the proportion of each isoform of PA1b does not change whatever the extraction conditions used.

The published procedure used a 60% methanol extraction as first step in the purification of PA1b [1]. Consequently, we studied the extraction of the proteins of TPA in 60% methanol, methanol and butanol because butanol/water is the first biphasic system usable, in the hope that PA1b would be partitioned in the solvent because it is the most hydrophobic of the proteins to be purified.

The solubility of PA1b was 14% in 60% methanol/water, 4% in methanol and only 2% in butanol, proving that this protein is not so hydrophobic.

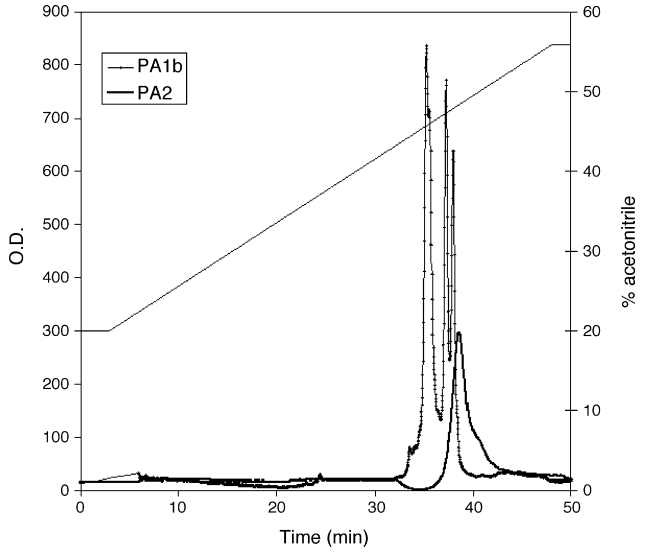

Fig. 3. RPC on C18 Nucleosil column of PA1b and PA2.

Table 1
Partitioning of PA1b (K values) in several biphasic systems (K: $C_{upper\ phase}/C_{lower\ phase}$)

Biphasic systems	K
Butanol:water 1:1 vol.	0.3
Butanol:20 mM aqueous TFA 1:1 vol. (pH 2.2)	1.7
Butanol:2 mM aqueous TFA 1:1 vol. (pH 4.0)	0.6
Butanol:acetic acid:water 4:1:5 vol.	0.2
Ethyl acetate:butanol:water 1.1:4:4.9 vol.	0.7
Ethyl acetate:butanol:TFA 20 mM in water 1.1:4:4.9 vol.	2.6
Propanol:3 M NaCl in water 1:1 vol.	1.7

Owing to this poor solubilisation of PA1b in solvents, we solubilised PA1b in water after which butanol was added to a final butanol:water ratio of 1:1 (v/v). The low partition of PA1b in butanol for a butanol:water system was greatly increased in the butanol:water system containing 20 mM TFA (Table 1). This behaviour can be explained by the high isoelectric point of PA1b (9.8): due to ion pairing, PA1b is more partitioned in the butanol phase and more hydrophobic in the presence of acid in water. These conditions were the best obtained, either because the partition of PA1b in the organic phase decreased in other biphasic systems or because the biphasic system was more difficult to apply in an industrial unit (ethyl acetate:butanol:aqueous TFA).

In butanol:20 mM aqueous TFA, the behaviours of the proteins differed sufficiently to hope for a good application of phase partitioning. K was 0.3 for PA2, 0.5 for most of hydrophilic proteins and 1.7 for PA1b.

For a convenient recovery of PA1b after partitioning in the butanol phase, it is possible to add a more apolar solvent in order to partition the protein in the water phase. This is possible with the use of heptane (Table 2).

3.4. Protein purification by CPC

PA1b was purified by CPC on a preparative scale, at 1200 and 500 rpm.

TPA was diluted in 20 mM aqueous TFA saturated in butanol, then 112 ml of the solution were injected into the CPC column. At the end of the injection, a biphasic fraction F1 containing

Table 2
Influence of heptane on the partitioning of PA1b from butanol phase

Heptane (%)	Proportion of PA1b partitioned in the water phase (%)
0	27
10	81
20	100

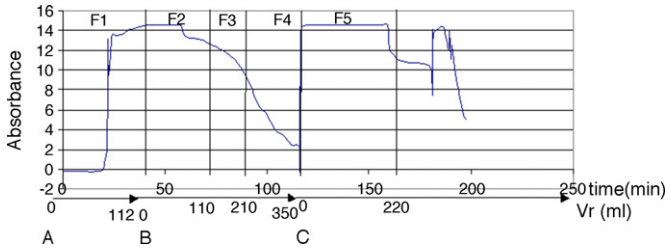

Fig. 4. CPC Chromatogramm of total pea albumins (TPA) at 1200 rpm. A: Injection of 560 mg TPA diluted in 112 ml butanol:20 mM aqueous TFA 0.1:0.9 vol. at 2.5 ml/min. B: Elution at 5 ml/min by 20 mM aqueous TFA in descending mode. C: Elution at 5 ml/min by butanol in ascending mode.

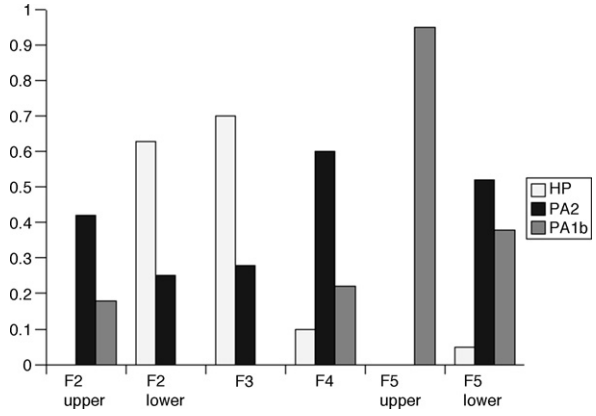

Fig. 5. Composition of the CPC fractions of total pea albumin (HP: hydrophilic proteins).

mainly hydrophilic proteins appeared (Fig. 4). Proteins were first eluted by 20 mM aqueous TFA, giving fractions F2 to F4, and then by butanol in a dual mode, giving fraction F5.

F1 contained only hydrophilic proteins. F2 fraction was biphasic (Table 3). Its upper phase contained PA2 and PA1b (Fig. 5) but the protein content of this upper phase was negligible (Fig. 6). The lower phase of F2 and F3 (monophasic) contained a great proportion of hydrophilic proteins and PA2 (Figs. 5 and 6). Although it was monophasic, F4 contained mainly PA2, but also PA1b. The protein yield of F4 was low. By elution with butanol in dual mode, a biphasic fraction F5 was obtained. Its upper phase contained only PA1b, whereas its lower phase contained PA2 and PA1b in low yield.

Because this new procedure consisted of aqueous solubilisation of TPA, more complete than the solubilisation in 60% methanol, and because it comprised only one CPC step, the yield of PA1b was much higher than that of the usual procedure. From 560 mg of TPA, 50 mg of PA1b with a purity greater than 95% was obtained versus 64 mg of identical purity from 40 g, which means that the recovery yield was increased more than 50-fold.

Table 3
Characteristics of the CPC fractions (1200 rpm). Vr: retention volume

Fraction	F1	F2	F3	F4	F5
Elution by	Injection	20 mM aqueous TFA	20 mM aqueous TFA	20 mM aqueous TFA	Butanol
Butanol:water vol.	0.2:1	1.2:1	0:1	0:1	1.9:1
Vr (ml)	110	0 to 110	110 to 210	210 to 350	0 to 220

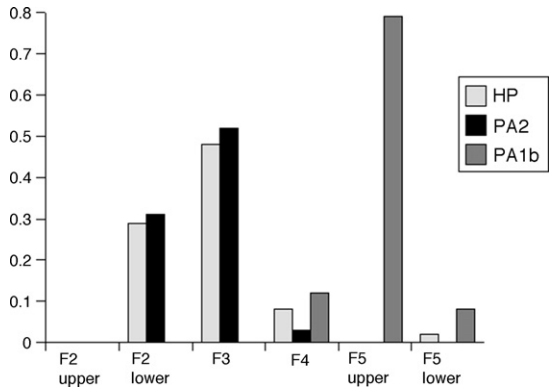

Fig. 6. Yield of the CPC fractions of total pea albumin (HP: hydrophilic proteins).

There was no change in purification results when the rotation speed of CPC was reduced to 500 rpm. This low speed generated a low pressure (0.7 Mpa versus 2.0 Mpa at 1200 rpm).

Although butanol:water CPC was based on the same criteria as RPC-C18, i.e. hydrophobicity, the behaviours of PA2 and PA1b differed completely depending on the technique used. PA2 was eluted mainly in the aqueous fractions in CPC, far from the elution of PA1b (Fig. 6). Conversely, in RPC, PA2 was eluted in conditions very close to those of PA1b elution (Fig. 3). This was quite surprising because PA2 is rather a hydrophilic protein as proved by its amino acid sequence and its insolubility in solvents. We can conclude that PA2 was denatured in RPC by adsorption onto the stationary phase, whereas this was not the case in CPC because the stationary phase was liquid.

4. Conclusion

Our new procedure including centrifugal partition chromatography is particularly suitable for the purification of PA1b. This pea albumin, which is toxic to cereal weevils, was obtained from pea flour by a very limited series of techniques: sodium acetate extraction, concentration by membrane techniques, giving a total pea albumin fraction, and then drying and extraction of albumins in water and CPC (butanol:water system). PA1b behaviour in CPC was sufficiently different from that of the other pea albumins to give a good purity and a very enhanced yield. These conditions allow scaling-up of this procedure to industrial production of a biodegradable insecticide.

References

[1] B. Delobel, A. Grenier, J. Gueguen, E. Ferrasson, M. Mbailao, PCT patent, WO 99/58695 (1999).
[2] T.J.V. Higgins, P.M. Chandler, P.J. Randall, D. Spencer, L.R. Beach, R.J. Blagrove, A.A. Kortt, A.S. Inglis, J. Biol. Chem. 261 (1986) 11124.
[3] L. Jouvensal, L. Quillien, E. Ferrasson, Y. Rahbe, J. Gueguen, F. Vovelle, Biochemistry 42 (2003) 11915.
[4] F. Gressent, I. Rahioui, Y. Rahbe, Eur. J. Biochem. 270 (2003) 2429.
[5] T.J.V. Higgins, L.R. Beach, D. Spencer, P.M. Chandler, P.J. Randall, R.J. Blagrove, A.A. Kortt, R.E. Guthrie, Plant Mol. Biol. 8 (1987) 37.
[6] R.R.D. Croy, M.S. Hoque, J.A. Gatehouse, D. Boulter, Biochem. J. 218 (1984) 795.
[7] N. Harris, R.R.D. Croy, Planta 165 (1985) 522.
[8] B.Y. Lu, L. Quillien, Y. Popineau, J. Sci. Food Agric. 80 (2000) 1964.
[9] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M.R. Wilkins, R.D. Appel, A. Bairoch, in: J.M. Walker (Ed.), The Proteomics Protocols Handbook, Humana Press, 2005, p. 571.
[10] I. Crévieu, S. Bérot, J. Gueguen, Nahrung 40 (1996) 237.
[11] N.B. Mandava, Y. Ito (Eds.), Countercurrent Chromatography-Theory and Practice, Chromatographic Science Series, vol. 44, Marcel Dekker, New York, 1988.
[12] A.P. Foucault (Ed.), Centrifugal Partition Chromatography, Chromatographic Science Series, vol. 68, Marcel Dekker, New York, 1994.
[13] J.B. Friesen, G.F. Pauli, J. Liquid Chromatogr. Relat. Technol. 28 (2005) 2777.
[14] A. Marston, K. Hostettmann, J. Chromatogr. A 1112 (2006) 181.
[15] Y. Ma, Y. Ito, Anal. Chim. Acta 352 (1997) 411.
[16] A. Weisz, A.L. Scher, K. Shinomiya, H.M. Fales, Y. Ito, J. Am. Chem. Soc. 116 (1994) 704.