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Analytical Methods

Comparison of the phytohaemagglutinin from red kidney bean (*Phaseolus vulgaris*) purified by different affinity chromatography

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Abstract

Affinity chromatography (AC) on Affi-Gel blue gel column and thyroglobulin (Tg)-Sepharose 4B column, respectively, were compared for their efficiency in purifying phytohaemagglutinin (PHA) from red kidney beans (*Phaseolus vulgaris*). Considering the purity and haemagglutinating activity of the obtained samples, Affi-Gel blue gel exhibited less affinity for PHA than Tg-Sepharose matrix. Affi-Gel blue purified sample showed multiple bands in SDS-PAGE gel, which further confirmed that Affi-Gel blue bound non-PHA proteins as well as PHA. PHA purified by one-step Tg-Sepharose column gave significantly ($p < 0.05$) higher purity (0.75 ± 0.13 mg PHA/mg lyophilized powder) than the sample purified by two-step (Affi-Gel blue first and then Tg-Sepharose) purification (0.62 ± 0.20 mg PHA/mg lyophilized powder). Circular dichroism (CD) spectra showed that the sample purified by one-step Tg-Sepharose column had similar secondary structures with the sample purified by two-step purification. Thus, one-step Tg-Sepharose purification was effective and time-saving for the preparation of PHA and a promising substitute for the two-step purification method.

Keywords: Affinity chromatography; Circular dichroism; Haemagglutinating; Phytohaemagglutinin; Red kidney bean

1. Introduction

Lectin is a major part of our daily food intake. Among 88 common foods including fruits, nuts, and cereals, 29 of them are found to possess significant lectin-like activities (Etzler, 1985). Members of the leguminous plants and seeds are especially rich in lectins (Nachbar & Oppenheim, 1980). Kidney bean contains $0.41\% \pm 1.15\%$ lectin, which is higher than other beans (Rudiger & Gabius, 2001). The lectin extracted from red kidney beans (*Phaseolus vulgaris*) is called phytohaemagglutinin (PHA). PHA consists of five tetrameric isoforms viz. L_4, L_3E_1, L_2E_2, L_1E_3, and E_4, which are formed by the combination of lymphocyte-specific (L) and erythrocyte-specific (E) subunits (Richard, Ronald, & Nicholas, 1976). PHA has been demonstrated to directly inhibit HIV-1 reverse transcriptase, an enzyme crucial for HIV replication, and β-glucosidase which has a role in the HIV-1 envelope protein gp 120 processing (Ye, Ng, Tsang, & Wang, 2001). PHA has also been reported to be able to inhibit the growth of lymphoid tumor *in vitro* and *in vivo* (D'Costa & Hurwitz, 2003). Medically, it can be used as a mitogen to trigger the synthesis of T-lymphocytes and used for the separa-

Abbreviations: AC, affinity chromatography; Tg, thyroglobulin; CD, circular dichroism; PHA, phytohaemagglutinin; PHA-L, erythroagglutinin; PHA-E, leucoagglutinin; PHA-P, the protein form of PHA prior to separation of erythroagglutinin and leucoagglutinin.

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tion of leukocytes from whole blood (Theodor, Heikki, & Clas, 1972). Due to its remarkable bioactivities, PHA has long been and remains an object of intensive studies.

Affinity chromatography (AC) has been extensively used to purify lectins from beans due to its high specific selectivity (Ma, Guan, & Liu, 2006). During the purification by AC, lectins bind to the immobilized ligands while the remaining molecules flow through the column. By disrupting the interactions with the ligands, the bound lectins can be eluted (Reynoso-Camacho, Gaozales de Mejia, & Loarca-Pina, 2003). There are various AC matrices that are available for the preparation of lectins. For example, 3-*O*-methylglucosamine–CH–Sepharose, galactosamine–CH–Sepharose and phenyl–Sepharose have been used to purify lectins from broad beans (*Vicia faba*), soy beans (*Glycine max*) and endophytic fungus (*Fusarium solani*), respectively (Allen, Desai, & Neuberger, 1976; Khan, Ahmad, & Khan, 2007). In terms of the purification of PHA from kidney beans, AC on Affi-Gel blue gel (Ye et al., 2001), fetuin–Sepharose (Herzig et al., 1997), CM-Sepharose (Ye et al., 2001) and Thyroglobulin (Tg)-Sepharose columns (Richard et al., 1976) have been reported before. Among them, Affi-Gel blue and Tg–Sepharose matrices are most popularly used.

Affi-Gel blue affinity gel is a beaded, cross-linked agarose gel with covalently attached sulphonated triazine dye (Cibacron Blue F3GA dye), which functions as an ionic, hydrophobic, aromatic, or sterically active binding site in various applications (Bio-Rad Laboratories, 2000). With respect to Tg, it is a glycoprotein (Mr = 669 kDa) that contains 8–10% total carbohydrates. The native Tg is composed of two equal sized subunits (around 330 kDa each) (Yves et al., 1989). So far, the mechanism by which PHA binds to Affi-Gel blue gel or Tg-Sepharose is still not clear. The lack of understanding how PHA binds to affinity matrices makes it difficult in choosing an effective AC column for the purification of PHA.

Generally, more than one-step purifications on different AC columns are used in order to get high purity of PHA (Ye et al., 2001). However, the purification cycle is really time-consuming due to the complicated procedures including adsorption and elution of the protein, recharging of the gel, dialysis and freeze-drying. Accordingly, more time is needed when two or more AC operations are applied. Furthermore, the addition of purification steps leads to an unavoidable loss of PHA during the processing. The objective of this work was to assess the affinities of the two commonly used AC matrices, Affi-Gel blue and Tg-Sepharose, for PHA. AC on Affi-Gel blue column, Tg-Sepharose column, and two-step purification by both Affi-Gel blue and Tg-Sepharsoe columns were investigated. Also, this work examined whether one-step purification could be a substituent of two-step purification by considering both purity and bioactivity of the obtained PHA. The results would contribute to a better understanding of the affinities of these two AC matrices for PHA, which would be helpful for selecting proper AC matrices to purify PHA.

2. Materials and methods

2.1. Materials

Red kidney beans (*P. vulgaris*) were supplied by Heinz Co., Limington, Ontario, Canada. Affi-Gel blue gel and Bio-Rad protein assay reagent were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Tg from porcine thyroid gland, Cyanogen bromide-activated-Sepharose® 4B, red blood cells (Human group B, trypsinized and glutaraldehyde treated), and PHA-P standard (*P. vulgaris*, the protein form of PHA prior to separation and purification of erythroagglutinin and leucoagglutinin) were all obtained from Sigma, Oakville, Ontario, Canada.

2.2. Extraction and purification of PHA

Dry red kidney beans were ground to fine powder, mixed with ten volumes of distilled water and then stirred overnight at 4 °C. The mixture was centrifuged at 5000*g* for 1 h at 4 °C. The supernatant was transferred to a fritted glass Büchner Funnel and the filtrate was collected as crude extraction. The extraction was divided into three aliquots for further purification.

2.2.1. One-step purification by Affi-Gel blue gel

AC on an Affi-Gel blue column was applied according to the method reported by Ye et al. (2001) with some modification. One of the three extraction solutions was mixed with Affi-Gel blue gel, shaken for at least 4 h at 4 °C and then transferred to a fritted glass Büchner Funnel. The gel was washed with 10 mM Tris–HCl buffer (pH 7.2) and the unbound proteins were removed. The adsorbed proteins were then eluted off with 1.4 M NaCl dissolved in 10 mM Tris–HCl buffer (pH 7.2). The eluted fraction was dialyzed using cellulose tubular membrane (Molecule cut-off = 3500 Da, Fisher Scientific, Ottawa, Ontario, Canada) against 10 mM Tris–HCl buffer (7.2) and lyophilized.

2.2.2. One-step purification by Tg-Sepharose

2.2.2.1. The preparation of Tg-Sepharose gel. This was performed based on the modification of the method described by Felsted, Leavitt, and Bachur (1975). Tg (500 mg) was dissolved in 20 mL buffer (pH 8.3) of 0.1 M $NaHCO_3$ containing 0.5 M NaCl. Cyanogen-bromide activated resin (2.5 g) was swelled in 500 ml cold (4 °C) HCl (1 mM) for 1 h. The swollen resin mixture was gently transferred to a Büchner funnel and washed first with 500 mL distilled water and then 25 mL $NaHCO_3$/NaCl coupling buffer (pH 8.3). The resin was immediately transferred to the ligand (Tg) solution and shaken overnight at 4 °C. The unreacted ligand was washed away using $NaHCO_3$/NaCl coupling buffer and the gel was mixed with 1 M ethanolamine (the blocking solution) for 2 h at room temperature. The blocking solution was removed by extensive washing using $NaHCO_3$/NaCl coupling buffer first and then acetate buffer (0.1 M, pH 4) containing NaCl (0.5 M). This cycle of

high and low pH buffer solutions was repeated four times. The obtained Tg-Sepharose gel was extensively washed with 6.7 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl.

2.2.2.2. The purification of PHA by Tg-Sepharose. One of the three extraction solutions was mixed with Tg-Sepharose and shaken overnight at 4 °C. The mixture was washed with 1 mM phosphate (pH 7.2) with 1 M NaCl until no protein was detected by Bradford assay. The gel was then eluted using 0.05 M glycine–HCl (pH 3.0) containing 0.5 M NaCl. The collected PHA fraction was desalted using cellulose tubular membrane (Molecule cut-off = 3500 Da, Fisher Scientific, Canada) against 6.7 mM potassium phosphate buffer (pH 7.4) and freeze-dried.

2.2.3. Two-step purification by Affi-Gel blue first and then Tg-Sepharose

One of the three extraction solutions was mixed with Affi-Gel blue and purified according to the procedure introduced in Section 2.2.1. The freeze-dried powder was then dissolved in a buffer of 6.7 mM potassium phosphate buffer containing 0.15 M NaCl (pH 7.4) and mixed with Tg-Sepharose for the second step purification by following the protocol described in Section 2.2.2.

2.3. Protein concentration

Protein concentration was determined by Bradford assay in 96-well microtiter plate (Bio-Rad Protein Assay). The dye reagent was diluted with four volumes of distilled water and filtered. PHA-P standard was dissolved in 6.7 mM potassium phosphate buffer containing 0.15 M NaCl (pH 7.4) to obtain serial concentration solutions: 5.0, 2.5, 1.25, 0.625 and 0.3125 mg/mL, respectively. Fifty microlitre of each standard solution or extracted sample solution was thoroughly mixed with 200 μL of the diluted dye reagent in separate well and incubated at room temperature for 15 min. The potassium phosphate buffer (pH 7.4) was used as blank and the absorbance was measured at 595 nm. The plot of PHA-P standard concentration against A_{595} was constructed and the PHA concentrations of the extracted samples were then calculated according to the standard plot.

2.4. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were subjected to SDS–PAGE for molecular mass determination using Phast System (Pharmacia Biotech, Baie d'Urfé, Quebec, Canada). PHA-P standard and the purified PHA samples were respectively dissolved in distilled water to obtain a concentration of 2 mg/mL. Twenty microlitre of each solution was mixed with 80 μL SDS and β-mercaptoethanol. The denaturation was performed in boiling water for 30 min. The denatured solutions were then centrifuged at 10,000*g* for 3 min. Five microlitre of the supernatant was loaded onto a 20% homogeneous Phastgel (Pharmacia LKB-Phast system) and tested according to the method of Marcone and Yada (1997). Gels were stained using Coommassie Brilliant Blue.

2.5. Haemagglutination assay

The haemagglutination assay was performed on a 2% suspension of red blood cells type B on a 96-well microtiter plate. PHA-P standard and the purified PHA samples were dissolved in 6.7 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl to obtain a concentration of 1 mg/mL, respectively. A 25 μL aliquot of each sample was twofold serially diluted, and 25 μL of blood were added to each of the wells. The agglutination was examined visually after incubation for 30 min. Phosphate buffer without PHA was used as negative control. The titer (*U*) was expressed as the reciprocal of the maximal dilution of the sample that gave visible agglutination. The haemagglutination activity (U/mg) was defined as the number of titer per milligram of the lyophilized powder (Khan et al., 2007).

2.6. Circular dichroism (CD) spectroscopy

CD measurements were made on a Jasco J-810 spectrometer (Jasco International Co., Ltd., Tokyo, Japan) purged with nitrogen gas and equipped with a constant thermostat. The measurements were done at 25 °C using cuvette with 1 mm path length. The sample concentration was 0.125 mg/mL for measurements in the far-UV region (250–190 nm) and 2.5 mg/mL for measurements in the near-UV region (300–250 nm). The spectra were recorded with data collection at 0.2 nm intervals using a scan speed of 100 nm/ min, and averaged five scans to eliminate signal noise. The phosphate buffer without PHA was used as blank. The data obtained were normalized by subtracting the baseline recorded for the blank. Secondary structure estimates (α-helix, β-sheet and β-turn) were analyzed by the CDSSTR program using the routines available at DICHROWEB (Compton & Johnson, 1986; Lobley & Wallace, 2001; Lobley, Whitmore, & Wallace, 2002).

2.7. Statistical analyses

Statistical analysis of the obtained data was performed by SPSS 14.0 software. ANOVA and two-tailed paired samples T tests were performed to analyze the secondary structure data detected by CD and to compare the statistical difference between the PHA samples extracted by different methods.

3. Results

3.1. Purification of PHA from red kidney beans

AC on Affi-Gel blue column and Tg-Sepharose column were applied, respectively, for the purification of PHA from red kidney beans (*P. vulgaris*). The yield and concentration

Table 1
The yield and concentration of the PHA purified by different AC

Sample	PHA Purified by AC[A]		
	Purified by Affi-Gel blue	Purified by Tg-Sepharose	Purified by both Affi-Gel blue and Tg-Sepharose
Yield (mg protein/g bean meal)	11.3 ± 1.2^{a}	3.4 ± 1.3^{b}	0.4 ± 0.1^{c}
PHA concentration (mg PHA/mg lyophilized powder)	0.20 ± 0.01^{a}	0.75 ± 0.13^{b}	0.62 ± 0.20^{c}

[A] Expressed as means ± S.D. of triplicates. The difference between the means with different superscript in the rows was significant at 95% confidence interval.

of the purified PHA are shown in Table 1. The yields (mg protein/g bean meal) were 11.3 ± 0.12 and 3.4 ± 0.13 for one-step Affil-Gel blue and one-step Tg-Sepharose purified samples, respectively, while the PHA concentration (mg PHA/mg lyophilized powder) of Tg-Sepharose purified sample (0.75 ± 0.13) was much higher than that of the Affi-Gel blue purified sample (0.20 ± 0.01). Apparently, Affi-Gel blue bound non-PHA proteins as well as PHA and thus exhibited significantly higher yield but lower PHA concentration ($p < 0.05$).

The two-step AC purified sample gave lower PHA yield (0.4 ± 0.1 mg protein/g bean meal) than one-step AC purified samples, which was probably due to two reasons: (i) multiple purification steps caused more chances to lose PHA, since there was almost always less than 100% yield from each step; and (ii) the first step purification by Affi-Gel blue allowed the competitive binding of non-PHA protein to the gel, therefore, when the eluted fraction was further supplied to Tg-Sepharose column, the maximum PHA that Tg-Sepharose could bind was restricted. Accordingly,

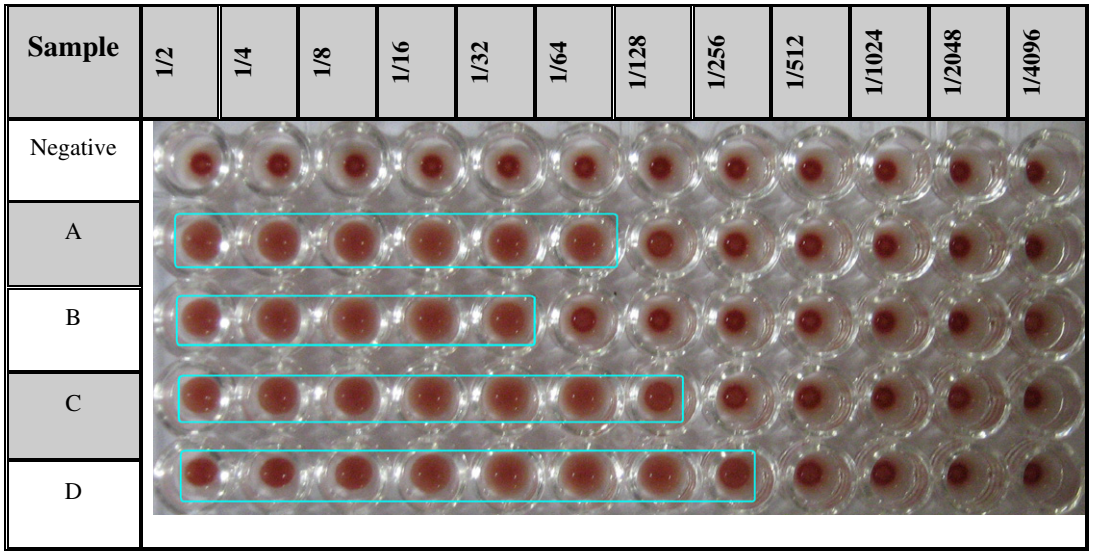

Fig. 1. Haemagglutination activities of PHA-P standard (A), PHA purified by Affi-Gel blue (B), PHA purified by Tg-Sepharose column (C) and PHA purified by Affi-Gel blue first and then Tg-Sepharose (D). Phosphate buffer was used as negative control. Each well of the microtiter plate contained 20 μL of sample or its dilution and 20 μL of 2% suspension of human erythrocytes. The visible agglutinations were shown in the highlighted wells. Picture was taken after incubation for 30 min.

Table 2
The haemagglutination activities of PHA samples

Sample	PHA-P standard	PHA Purified by AC		
		Purified by Affi-Gel blue	Purified by Tg-Sepharose	Purified by both Affi-Gel blue and Tg-Sepharose
Titer (U)[a]	64	32	128	256
Haemagglutination activity ($\times 10^3$ U/mg)[b]	204.8	51.2	819.2	3276.8

[a] Titer (U) was the reciprocal of the maximal dilution of the sample that gave visible agglutination.
[b] Haemagglutination activity (U/mg) was the number of titer per milligram of lyophilized powder.

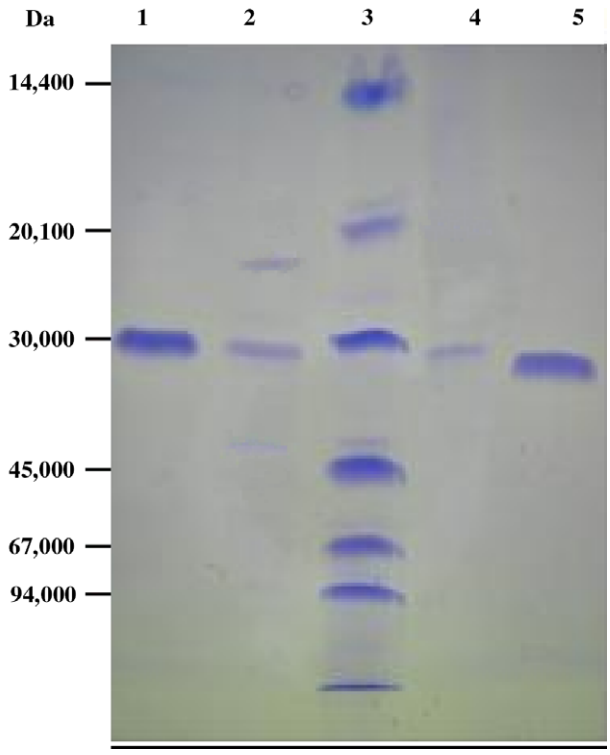

Fig. 2. SDS-PAGE analysis of PHA samples. Lane 1 was the PHA purified by Tg-Sepharose; Lane 2 was the PHA purified by Affi-Gel blue; Lane 3 was the standard ladder for electrophoresis; Lane 4 was the PHA purified by Affi-Gel blue first and then Tg-Sepharose; and Lane 5 was the PHA-P standard. The sample concentrations used were all 2 mg/mL.

the PHA concentration (0.62 ± 0.20 mg/mg lyophilized powder) of the two-step AC purified sample was higher than that of the Affi-Gel blue sample and lower than that of the Tg-Sepharose sample.

3.2. Haemagglutinating assay

The haemagglutinating activities of the purified samples were tested using human erythrocytes type B. PHA-E (erythrocyte-specific subunit) was presumed to bind to erythrocyte by localizing on the extracellular portion of a membrane-spanning sialoglycoprotein, glycophorin A, which was considered to be the major receptor (MacKenzie, Prestegard, & Engelman, 1997). Another assumption was that PHA-E may combine with oligosaccharides such as Gal α1 → 4Gal or Gal β1 → 4Glc, which were frequent occurring sequences of many glycosphingolipids located at the mammalian erythrocyte membranes for ligand binding (Wu, Chin, Hartmut, Uwe, & Anthony, 1992). The formation of PHA-erythrocyte-PHA matrix prevented erythrocytes from sinking into the bottom of the well when haemagglutination occurred (Fig. 1). As shown in Table 2, the titers for PHA-P standard, Affi-Gel blue purified sample, Tg-Sepharose purified sample and two-step AC purified sample were 64, 32, 128 and 256 U, respectively. Affi-Gel blue purified sample gave the lowest haemagglutinating activity (51.2×10^3 U/mg) due to the poor affinity

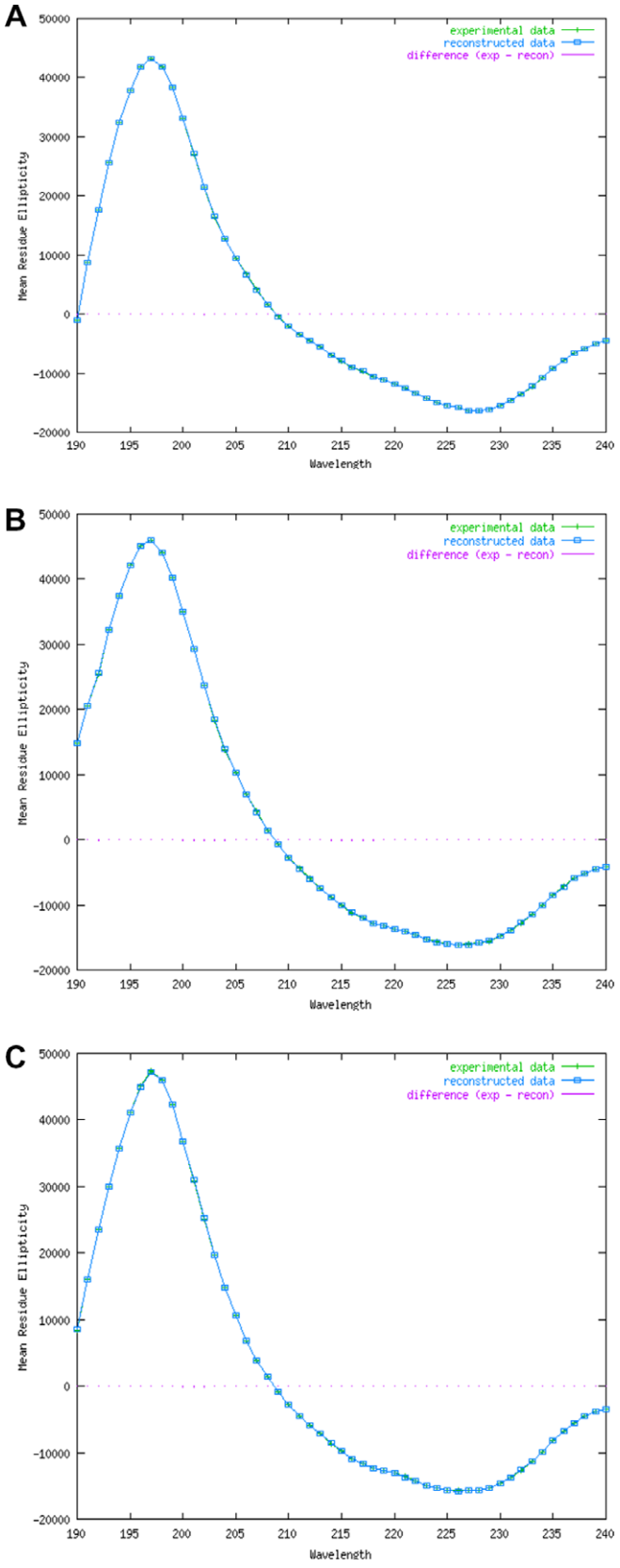

Fig. 3. The Far-UV (250–190 nm) CD spectra of PHA samples: (A) PHA-P standard; (B) PHA purified by Tg-Sepharose and (C) PHA purified by Affi-Gel blue first and then Tg-Sepharose. The CD measurements were all performed in a 1 mm cuvette at a sample concentration of 0.125 mg/mL.

Table 3
Secondary structures of the PHA samples analyzed by CD[A]

	PHA-P Standard	PHA purified by Tg-Sepharose	PHA purified by Affi-Gel blue first and then Tg-Sepharose
NRMSD	0.001	0.002	0.002
α-Helix (%)	51.6 ± 1.1^{a}	53.8 ± 2.7^{a}	52.8 ± 2.2^{a}
β-Sheet (%)	21.6 ± 2.1^{a}	20.2 ± 2.6^{a}	20.6 ± 2.1^{a}
Turns (%)	10.8 ± 1.5^{a}	11.6 ± 0.4^{a}	11.6 ± 0.8^{a}
Unordered (%)	17.0 ± 1.0^{a}	14.4 ± 1.5^{ab}	15.0 ± 1.1^{b}

[A] Expressed as means ± S.D. of triplicates. Means sharing the same superscript in the rows were not significantly different at $p < 0.05$.

of the gel for PHA. The two-step AC purified samples exhibited higher activity (3276.8×10^3 U/mg) than Tg-Sepharose purified sample (819.2×10^3 U/mg), which was likely to be that Affi-Gel blue had stronger affinity for PHA-E than PHA-L. It signified that Affi-Gel blue and Tg-Sepharose had different selectivity for PHA-E and -L isoforms. Both one-step Tg-Sepharose purified sample and two-step AC purified sample exhibited higher activities than PHA-P standard (204.8×10^3 U/mg), which was probably due to the higher ratio of PHA-E to PHA-L subunits in those samples than in PHA-P standard.

3.3. Molecule weight distribution assay by SDS-PAGE

The molecule weight distribution of the purified samples was analyzed using SDS-PAGE. As Fig. 2 has shown, Tg-Sepharose could fully purify PHA and gave one clear band (Lane 1) which was similar to the PHA-P standard located at around 32 kDa (Lane 5). However, multiple bands appeared in Affi-Gel blue purified sample (Lane 2). PHA with higher purity was obtained when Affi-Gel blue purified sample was further loaded to Tg-Sepharose, since the two-step purified sample only showed one band (Lane 4). Besides, Tg-Sepharose purified sample (Lane 1) gave a more concentrated band than Affi-Gel blue purified sample (Lane 2). All these results further confirmed that Affi-Gel blue had weaker affinity for PHA than Tg-Sepharose. Due to the restriction of Affi-Gel blue in the first step purification, the two-step purified sample showed a lighter color band (Lane 4) than one-step Tg-Sepharose purified sample (Lane 1). Therefore, the conclusion could be made that PHA with high purity could be prepared by one-step AC on Tg-Sepharose column, which was more economical and time-saving than the two-step purification method.

3.4. CD Spectroscopy and secondary structure

Far-UV CD ellipticity is highly sensitive to the protein secondary structures and each secondary structure type has specific bands with characteristic wavelengths and intensities (Raussens, Ruysschaert, & Goormaghtigh, 2003). Thus, CD assay was performed for further comparison of the efficiency of the one-step Tg-Sepharose purification method and the two-step purification method. The purified PHA samples were analyzed by four different methods, K2D, SELCON3, CDSSTR and CONTINLL which were available at http://public-1.cryst.bbk.ac.uk/cdweb/html/ (Compton & Johnson, 1986; Lobley & Wallace, 2001; Lobley et al., 2002; Whitmore & Wallace, 2004), to derive more quantitative information regarding secondary structural elements. A basis set containing 43 proteins was used as a reference for fitting the experimental spectrum. Reference set 4 in CDSSTR program was found to yield the best fit values. As Fig. 3(A–C) has shown, the experimental and reconstructed spectra overlapped (NRMSD = 0.001, 0.002 and 0.002, respectively), which indicated that the experimental data were in excellent agreement with the calculated data based on the reference database of CDSSTR program.

The values obtained for different types of secondary structures are shown in Table 3. α-Helix was found in biggest amounts in all samples tested. There was insignificant difference in secondary structures between the samples purified by one-step Tg-Sepharose method and by two-step AC method ($p > 0.05$). This confirmed the above conclusion that one-step Tg-Sepharose could be a good substitute of the two-step purification method.

Generally, most of the legume lectins contain predominantly β-sheet structures and have negligible or no α-helical content (Sharon & Lis, 2003; Loris, Hamelryck, Bouckaert, & Wyns, 1998). Although the high α-helix content presented in this study was quite unusual for a legume lectin, the spectra were in accord with the report of Shyamasri Biswas et al. (Biswas & Kayastha, 2004), which examined the unfolding and refolding of PHA-L originated from kidney beans (*P. vulgaris*) by CD. Quite similar secondary structures were also found in a galactose-specific seed lectin from *Dolichos lablab* with 57% α-helix, 21% β-sheet, 7% β-turns and 15% unordered structures (Mohammed, Rao, Nadimpalli, & Swamy, 2006).

The near-UV CD spectra of the extracted samples were also detected and shown in Fig. 4(A–C). All the figures were characterized by the presence of the maximum mean residue ellipticity at around 285 nm. This feature was likely due to the side chains of tryptophan and tyrosine residues, which had maximum absorbance in the 270–300 nm regions (Mohammed et al., 2006).

4. Discussion

AC on Affi-Gel blue and Tg-Sepharose columns were compared for the purification of PHA from red kidney beans (*P. vulgaris*). The sample purified by Affi-Gel blue gave significantly higher protein yield but lower PHA

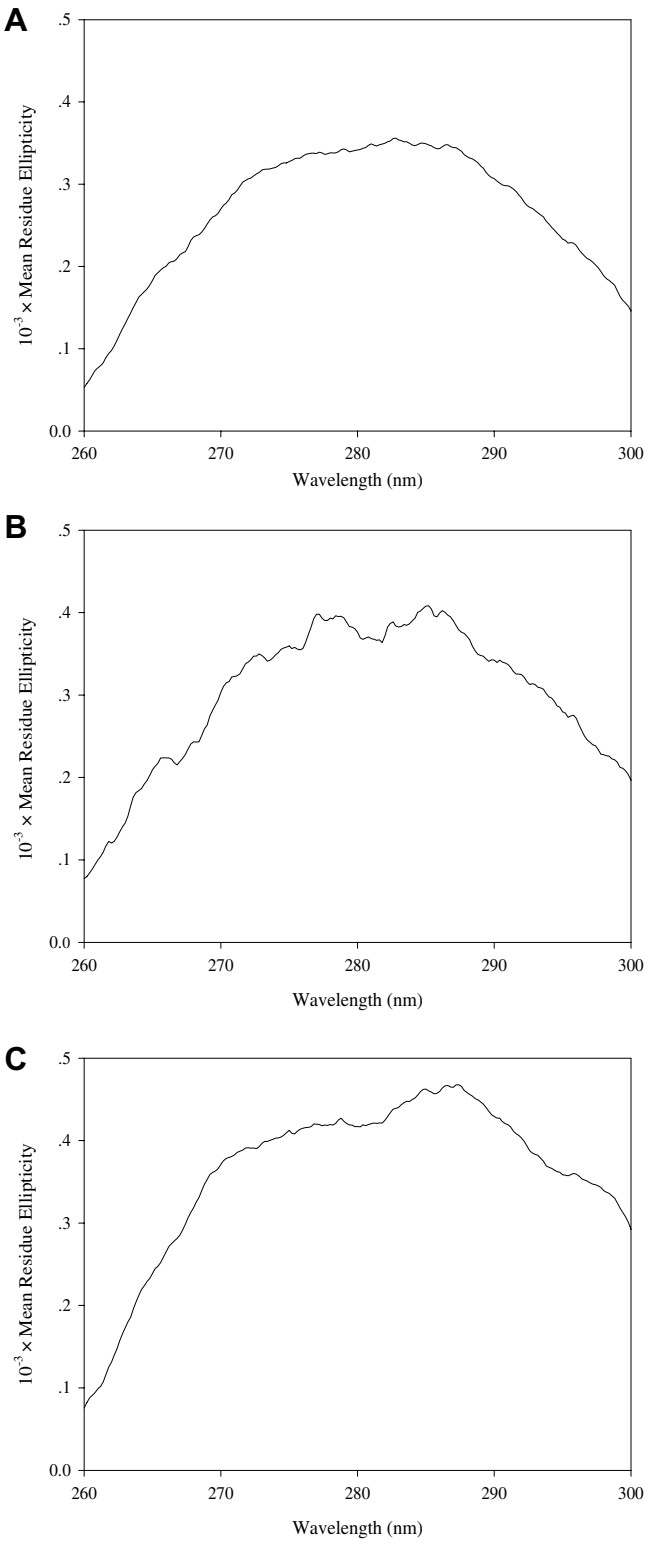

Fig. 4. The Near-UV (300–250 nm) CD spectra of PHA samples: (A) PHA-P standard; (B) PHA purified by Tg-Sepharose; and (C) PHA purified by Affi-Gel blue first and then Tg-Sepharose. The CD measurements were all performed in a 1 mm cuvette at a sample concentration of 2.5 mg/mL.

concentration compared with Tg-Sepharose purified sample ($p < 0.05$), which demonstrated that Affi-Gel blue bound non-PHA proteins as well as PHA. Besides, Affi-Gel blue purified sample exhibited lower haemagglutinating activity in contrast with Tg-Sepharose purified sample. SDS-PAGE also proved that Affi-Gel blue could not fully purify PHA since multiple bands appeared in the gel, while Tg-Sepharose had higher affinity for PHA and displayed one clear band. Therefore, different mechanisms were expected for how these two matrices bound to PHA.

In terms of Affi-Gel blue gel, the functional group lies in a sulphonated dye of the triazine class, Cibacron Blue F3GA, which is covalently coupled to cross-linked agarose gel (Bio-Rad Laboratories, 2000). The matrix is considered to interact with proteins based on the following rules: ion-exchange interaction, hydrophobic interaction, exclusion–diffusion and affinity binding (Gianazza & Arnaud, 1982). Among them, exclusion–diffusion is involved only when the bound proteins are eluted at basic pH and at low ionic strength; and affinity binding is only related to the binding of some particular enzymes to the gel (Gianazza & Arnaud, 1982). In terms of the present study, ionic forces formed between PHA and sulphonic groups of the dye were considered to drive the binding. Consequently, chances are that some non-PHA protein bound to the gel by binding to sulphonic groups. As a result, non-PHA proteins as well as PHA were both eluted because high ionic strength saline solution (1 M NaCl at pH 7.0) could elute almost all proteins except small amounts of lipoproteins (Gianazza & Arnaud, 1982). It was also possible that hydrophobic interactions played an accessory role to the dominant one played by ionic forces due to the presence of polycyclic aromatic rings in the dye (Gianazza & Arnaud, 1982).

With respect to Tg, it is a glycoprotein contains 8–10% total carbohydrate with *N*-acetyl glucosamine, mannose, galactose, fucose and sialic acid residues (Yves et al., 1989). Among them, *N*-acetyl glucosamine and mannose are also presented in PHA (Theodor et al., 1972). The affinity of Tg for lectin may be attributed to the following reasons: (i) PHA has more than one binding sites specific for saccharides (Lis & Sharon, 1998), therefore, it can bind to *N*-acetyl glucosamine or mannose of Tg by hydrogen bonds and Van Der Waals interactions; (ii) Several loops are arranged in the 3D structure of Tg due to the interacting turns (Guncar, Pungercic, Klemencic, Turk, & Turk, 1999), and thus the resulting wedge shape structure can be anchored into the large channel formed in the 3D structure center of PHA molecule (Thomas et al., 1996); (iii) The sialic acid in Tg is considered to be essential for the binding of other proteins (Tarutani, Kondo, & Shulman, 1977; Tarutani & Shulman, 1971), so PHA may bind to sialic acid since similar binding has already been proved for some other plant lectins (Gao et al., 2006).

5. Conclusions

The results in this study confirmed that Tg-Sepharose had stronger affinity for PHA in contrast to Affi-Gel blue. Besides, CD spectra showed that there was not significant difference ($p > 0.05$) in secondary structures between the

sample purified by one-step Tg-Sepharose method and two-step purification method. Therefore, it was suggested that the purification of PHA from red kidney beans could be efficiently fulfilled by applying one-step AC on Tg-Sepharose column instead of the time-consuming two-step purification approach.

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