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Separation and purification of puerarin using β-cyclodextrin-coupled agarose gel media

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Abstract

The isoflavonoid puerarin, a well-known traditional Chinese drug, has been purified in one step from an extract of *Radix puerariae* (root of the plant *Pueraria lobata*) by adsorption chromatography on an epichlorohydrin polymerized β-cyclodextrin ligand coupled to brominated allyl-group substituted Sepharose HP. Acetic acid (10%) was used as the mobile phase and the optimum loading capacity was around 1.2 mg crude extract/ml packed gel. The purity of the collected puerarin was about 98% with a recovery of about 62%.

Keywords: Stationary phases, LC; Pharmaceutical analysis; Puerarin; Cyclodextrin; Agarose; Isoflavonoids

1. Introduction

Radix puerariae, also called kudzu root, is the root of *Pueraria lobata* (Willd.) Ohwi which is a perennial leguminous plant native to eastern Asia. Extracts of *Radix puerariae* are rich in isoflavones and have been widely used as antipyretic, antidiarrhoetic, diaphoretic, and antiemetic agents in traditional Chinese medicine [1], recently also as a possible remedy against alcoholism [2]. Puerarin (daidzein 8-*C*-glucoside), daidzin (daidzein 7-*O*-glucoside), and daidzein are the major isoflavonoids of *Radix puerariae* [3]. The common practise in traditional Chinese medicine is to use the whole herb or the whole herb extract and this is why the active components have not been widely used in pure form. In recent years there has been an increasing interest in studying the individual active herb components. However, this approach has been restricted primarily because the traditional separation methods used so far were limited to simple techniques such as extraction and precipitation leading to low recoveries. Thus the recovery of the main component of *Radix puerarie*, puerarin, was reported to be very low, about 1%, although with a purity as high as 97% [4,5]. Traditionally, the chromatographic procedures that have been used are based on resin adsorbents with low purity grade, in combination with other unit operations such as filtration and extraction [6–8]. This is why a more efficient and simple purification method for puerarin is required.

β-Cyclodextrin (cyclomaltoheptaose, M_w 1135) is composed of seven cyclic-arranged α-1,4-linked glucose units. The outside of the cyclic molecule is hydrophilic and the central cavity hydrophobic. β-Cyclodextrin is widely used in drug formulations for the molecular encapsulation of a variety of compounds that are only sparingly soluble in water to enhance the aqueous availability of the encapsulated compound. It is also widely used as a ligand in liquid chromatography [9], often coupled to silica gels for HPLC applications. Thus, Armstrong [10] synthesized a β-cyclodextrin-based chiral solid phase for the separation of various optical isomers. Agarose-based media have so far not been used as carriers for β-cyclodextrin ligands, even if these gel media have certain advantages such as neutral hydrophilicity and adequate porosity. This paper will show that puerarin can be isolated with 98% purity in one step from extracts of *Radix puerariae* with 62% recovery by adsorption chromatography on an epichlorohydrin-polymerized β-cyclodextrin ligand coupled to Sepharose HP base matrix with 10% acetic acid as the mobile phase.

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2. Experimental

2.1. Equipment

HPLC CR-10A (Shimadzu, Japan), Pump (Beijing Xingda Company), UV detector 8823-A (Beijing New Technology Application Institute), chromatography control unit N2000 (Zhejiang University) and motor valve MV-7 (Amersham Biosciences, Uppsala, Sweden) were used.

2.2. Reagents

Ethanol, methanol, acetic acid, acetone, acetonitrile, sodium hydroxide, nitric acid and ammonia solution were of analytical grade and obtained from Beijing Chemicals Factory, Beijing, China. Epichlorohydrin and 1,4-butanediol diglycidyl ether were of synthesis grade and gifts from Amersham Biosciences in Uppsala, Sweden. β-Cyclodextrin (β-CD) was obtained from the Nankai University Fine Chemicals Factory, Tianjin, China. A crude extract powder of *Radix puerariae* called "*Radix puerariae* flavone", and reference puerarin with a purity of >98% were bought from Luye Biology, Huainan, China. Allyl-group substituted Sepharose HP base matrix (34 μm average particle diameter) was a gift from Amersham Biosciences. The following agarose gel media derivatives were synthesized and used in this study:

(1) Mono-β-CD coupled to Superose 12 pg using bisepoxide (1,4-butanediol diglycidyl ether) (β-CD content about 6.6 mg/ml).
(2) Poly-β-CD coupled to allyl-substituted Sepharose HP base matrix (β-CD content about 31 mg/ml).

Gel 1 was packed in a 110 mm × 16 mm i.d. column (total volume 22 ml), and gel 2 was packed in a 420 mm × 10 mm i.d. column (total volume 33 ml).

2.3. Synthesis of poly-β-cyclodextrin

A mixture of 95 ml 50% NaOH, 95 ml water and 0.2 g $NaBH_4$ were added to a 500 ml round bottom glass flask equipped with a U-shaped stainless steel stirrer, a reflux cooler and placed in a controlled temperature water bath. The stirring was adjusted to 500 rpm and 90 g β-cyclodextrin was added. The dissolution was allowed to proceed overnight at room temperature under constant stirring. In the morning, the temperature was increased to 30 °C, 30 ml distilled water was added to reduce the viscosity followed by the rapid addition of 62.5 ml epichlorohydrin. After 4 h reaction time, 100 ml acetone was added to stop the reaction. Stirring was allowed to continue for another hour at 500 rpm followed by the addition of 6 M HCl until the pH of the mixture decreased to around 12. When the HCl was added a top layer of non-reacted epichlorohydrin was formed, around 50 ml of which was removed. The temperature of the water bath was increased to 50 °C and the stirring was continued at 350 rpm for another 6 h. The reaction was stopped by adjusting the pH to around 7 with 1 M HCl. The reaction mixture was allowed to stand overnight at room temperature.

The neutralized β-cyclodextrin polymerisation mixture (450 ml) was diluted five times with water and 450 ml aliquots were loaded to a 1.7 l Sephadex G-25 Fine column. Fractions of 100 ml were collected through the whole chromatography. The void fraction between elution volumes 380 and 780 ml (i.e. 400 ml) from each individual run were pooled (total volume 2000 ml) and concentrated by rotatory evaporation at 80 °C to a final volume of 250 ml.

2.4. Synthesis of the β-cyclodextrin agarose gel derivatives

2.4.1. Coupling of β-CD to Superose 12 pg using 1,4-butanediol diglycidyl ether

An amount of 250 ml Superose 12 pg was washed on a sintered glass funnel with 2 l distilled water. After draining, the moist gel was transferred to a 1 l round bottomed glass reaction vessel equipped with a U-shaped stainless steel stirrer and submerged in a temperature controlled water bath. Distilled water (100 ml) was added and the stirring started. After addition of 40 g 50% NaOH and 0.1 g $NaBH_4$, 45 ml 1,4-butanediol diglycidylether were added over a period of 2.5 h (3 ml/10 min). The stirring was allowed to proceed for another 2 h at room temperature. After pH-adjustment to 6–7 using 60% acetic acid, the gel was washed on a sintered glass funnel with 10 l distilled water.

The epoxy-group-substituted Superose 12 pg was transferred back into the reaction vessel and 90 ml distilled water was added followed by 20 g β-cyclodextrin. The temperature was adjusted to 30 °C and stirring was allowed to proceed for 1 h followed by the addition of 20 g 50% NaOH and 0.2 g $NaBH_4$ under continuous stirring. The stirring continued at 30 °C for another 18 h when the reaction was interrupted by the adjustment of pH to 6–7 using 60% acetic acid. The coupled gel was washed with 10 l distilled water to remove non-coupled β-cyclodextrin. The gel was stored in 20% ethanol at room temperature until used.

Coupling efficiency was estimated indirectly by measuring the difference in β-cyclodextrin amount in the reaction liquids before and after coupling. For the concentration measurements size exclusion chromatography on Superdex Peptide HR 10/30 column was applied. A calibration curve was prepared by plotting peak heights from refractomonitor recordings from runs of pure β-cyclodextrin at concentrations 1.7, 1.2, 0.9, 0.6, 0.3 and 0.1%. To the 1,4-butanediol diglycidylether activated gel, a coupling yield of 1.66 g of β-cyclodextrin was obtained.

2.4.2. Coupling of poly-β-CD to a brominated allyl-derivative of Sepharose HP base matrix

An amount of 100 g wet weight drained and moist allyl-substituted Sepharose HP (0.224 mmol allyl-groups

per ml gel) was transferred to a 500 ml beaker. An amount of 400 ml distilled water and sodium acetate to 100 mM were added followed by dropwise addition of bromine during stirring at 300 rpm until a yellow colour remained. The bromine-activated gel was washed with 5 l distilled water on a sintered glass funnel and drained. The drained, moist gel was transferred to a 1 l round bottomed glass reaction vessel equipped with a U-shaped stainless steel stirrer and submerged in a temperature controlled water bath. An amount of 80 ml of the poly-β-cyclodextrin solution (see Section 2.3) was added and the stirring started at 100 rpm. After the addition of 12.5 g NaOH and 0.2 g $NaBH_4$, the stirring was continued overnight at 30 °C. After pH-adjustment to 6–7 using 60% acetic acid, the gel was washed on a sintered glass funnel with 10 l distilled water. The coupled gel was stored in 20% ethanol at room temperature.

2.5. Preparation of the mobile phases

Volumes of 5, 10, 20 and 50 ml ethanol were diluted with distilled water to a final volume of 100 ml. Likewise 1, 5 and 80 ml methanol were diluted with distilled water to a final volume of 100 ml and 0.1, 0.5, 5, 10, 20, 30 and 50 ml acetic acid were diluted with distilled water to a final volume of 100 ml. All mobile phases were prefiltrated to remove possible dust before use.

2.6. Sample preparation

Varying quantities (10–400 mg) of crude *Radix puerariae* flavone were dissolved in 10 ml of different concentrations of ethanol and acetic acid. All samples were prefiltrated to remove possible dust before injection to the columns. The injected sample volume was invariably 2 ml.

2.7. Separation of Radix puerariae flavone by chromatography

The isoflavonoids in *Radix puerariae* were separated on the different media listed in Section 2.2 equilibrated in different mobile phases at a linear flow velocity of 1 cm/min. The eluent was monitored using a UV detector at 280 nm.

2.8. Analysis of puerarin by HPLC

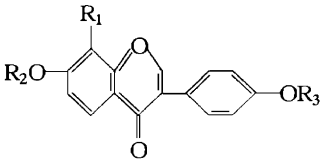
The following conditions were applied: column C_{18} reversed phase silica, 250 mm × 4 mm i.d.; mobile phase: methanol–0.5% acetic acid (27:73 (v/v)); detection: UV at 280 nm; flow-rate: 0.5 ml/min; loading volume: 20 μl, calculation: standard line method.

2.9. Column cleaning

Mono-β-CD-substituted Superose 12 pg was cleaned-in-place using four column volumes of 0.35 M NaOH and poly-β-CD-substituted Sepharose HP using four column volumes of 50% acetic acid. Both at a flow velocity of 1 cm/min. The columns were stored in 20% (v/v) ethanol.

3. Results and discussion

The isoflavone content of *Radix puerariae* flavone is 10–14%, mainly composed of daidzein, daidzin, puerarin, and daidzein-4′,7-diglucoside, whose structures are shown in Fig. 1. The content of puerarin is approximately 4%. Two chromatographic gel media were synthesized by coupling monomeric β-CD to the agarose gel Superose 12 pg and polymeric β-CD to allyl-substituted Sepharose HP, respectively. The highest selectivity was obtained with the



Compound	Group		
	R_1	R_2	R_3
Daidzein	H	H	H
Daidzin	H	Glucose	H
Puerarin	Glucose	H	H
Daidzein-4',7-diglucoside	H	Glucose	Glucose

Fig. 1. The molecular structures of *Radix puerariae* flavonoides.

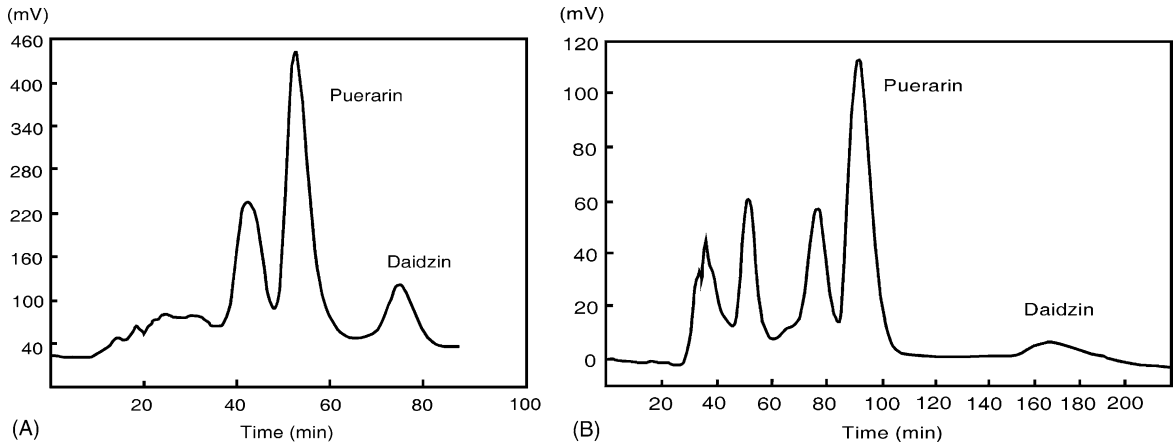


Fig. 2. Chromatogrrarns of *Radix puerariae* flavone crude extract on (A) mono-β-CD-substituted Superose 12 pg equilibrated in 0.5% HAc (sample 0.8 mg in 2 ml); (B) poly-β-CD-substituted Sepharose HP in 10% HAc (sample 4 mg in 2 ml). Linear flow velocity: 1 cm/mm.

poly-β-CD-substituted Sepharose HP as shown in Fig. 2. Tentatively, the explanation for this is the considerably higher local concentration of the β-CD group in this gel because of the large soluble molecular aggregates formed by the polymerization of the monomer before coupling. A contributing factor might also be the larger pores of the Sepharose HP compared to those of the Superose 12 pg facilitating the diffusional transport of the solutes to the polymer ligand inside the gel matrix.

The composition of the mobile phase is an important factor affecting the separation of the isoflavones on the β-CD-substituted agarose gel media. Thus, the acetic acid concentration could be used to optimize the degree of separation of puerarin from the neighbouring peaks. Except for the target component puerarin, we only identified the location of the daidzin, the other isoflavone mono-glucoside, that eluted well behind the puerarin in the chromatogram. The structures of the other two major isoflavonoid components, the non-glucoside daidzein and the daidzein-4′,7-diglucoside were regarded as being different enough from that of the target compound to force their elution position even further away from the puerarin. For the mono-β-CD-substituted Superose 12 pg, 0.5% HAc gave the best separation whereas 10% HAc was optimal for the poly-β-CD-substituted Sepharose HP. Obviously, the optimal concentration of acetic acid in the mobile phase is correlated to the concentration of the of β-CD groups in the agarose gel media. The delocalised electrons of the carboxylic group of acetic acid impose a quenching effect on the adsorption of aromatic group containing solutes on tightly cross-linked polysaccharide gel media. That this effect can be used to advantage for the separation of low molecular weight substances, is not well-known and seldom utilized but was published already during the 1960s [11,12]. It is well-known that the hydrophobic inner core of β-CD has got the ability to adsorb hydrophobic solutes and it is reasonable to assume that the adsorption properties of the β-CD-substituted agarose gel media is based on this property. In fact, neither neat Superose 12 pg, nor neat Sepharose HP base matrix (nor the allyl-derivative of the latter gel) possess the ability to separate puerarin from the other components of *Radix puerariae* flavone crude extract as efficiently as do the β-CD-substituted gel media (data not shown). The separation obtained was tentatively interpreted as primarily being based on the molecular size of the sample components. Attempts to use other solvent–water mixtures than acetic acid, such as ethanol–water (5:95, 10:90, 20:80, 50:50 (v/v)), methanol–water (80:20, 5:95, 1:99 (v/v)) as well as ammonium nitrate (0.05 mol/l), phosphoric acid (0.4%), and pure distilled water did not lead to better separations (data not shown).

Different solvent mixtures were tried to dissolve the crude *Radix puerariae* flavone powder. The best result was obtained with 20% ethanol in distilled water. This solvent mixture gave the deepest colour of the sample solution and also the largest peak height of the puerarin in the chromatograms.

Sample loading capacity studies were performed with 0.7–40 mg *Radix puerariae* flavone powder dissolved in 2 ml solvent mixture. Fig. 3 shows the result of a sample load of 18 mg (0.8 mg/ml gel) to the mono-β-CD-substituted Superose 12 pg in 0.5% HAc and of 40 mg (1.2 mg/ml gel) to the poly-β-CD-substituted Sepharose HP in 10% HAc.

As expected, the separation efficiency decreased due to gel fouling with increasing number of sample cycles. After 15 cycles, the top of the column became brown-yellowish in colour and the separation capability for the puerarin declined considerably for the mono-β-CD-substituted Superose 12 pg. Different solvent and reagent mixtures were investigated for the cleaning and regeneration of the gel, including acetone, acetonitrile, methanol-distilled water, ethanol-distilled water, and NaOH solutions. The best result was obtained with 0.35 M NaOH. After regeneration, the separating efficiency was restored to the original. The poly-β-CD-substituted Sepharose HP showed less fouling tendency and the best regenerating solvent was 50% acetic acid.

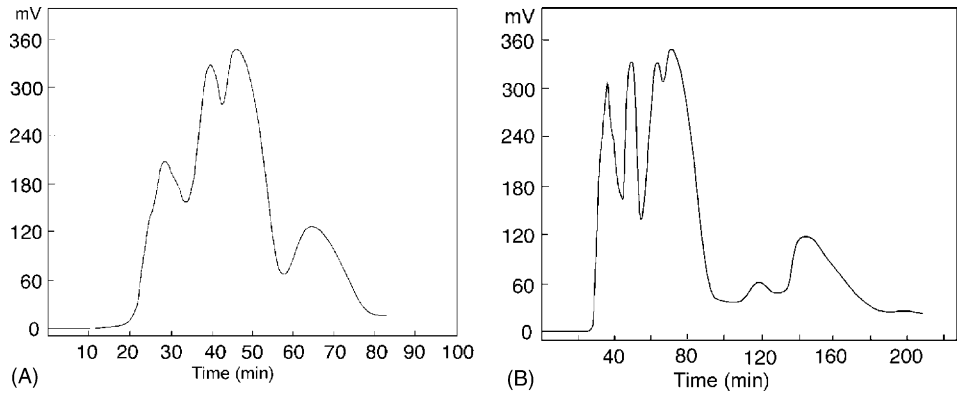


Fig. 3. Effect of higher loading of *Radix puerariae* flavone crude extract on the resolution. (A) Mono-β-CD-substituted Superose 12 pg equilibrated in 0.5% HAc (sample 18 mg in 2 ml); (B) poly-β-CD-substituted Sepharose HP in 10% HAc (sample 40 mg in 2 ml). Linear flow velocity: 1 cm/mm.

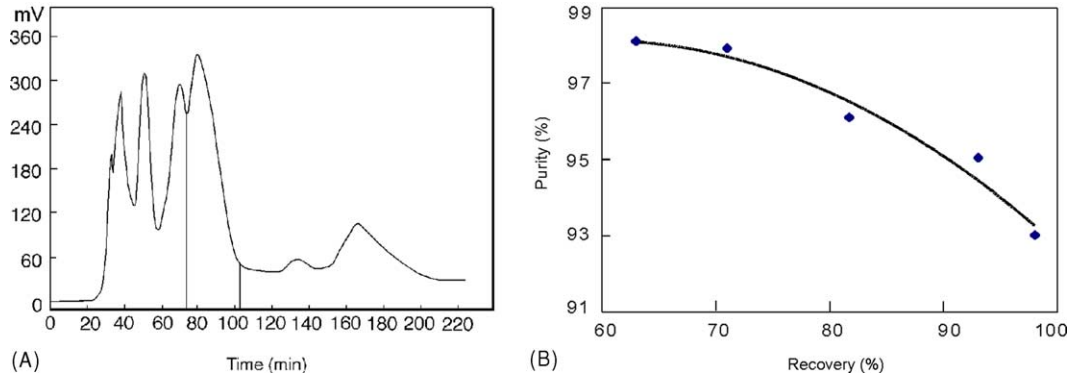


Fig. 4. Degree of purification and recovery of puerarin using poly-β-CD-substituted Sepharose HP in 10% HAc (sample load: 40 mg; linear flow velocity: 1 cm/min. The curve in (B) is based on HPLC analyses for purity and recovery of fractions collected between the time intervals 79–94, 79–102, 77–104, 75–104 and 73–104 min, respectively. The vertical lines in (A) represent time interval 73–104 min.

Fig. 4 shows how the degree of purity is related to the amount of recovered puerarin after peak cutting in a preparative run on the poly-β-CD-substituted Sepharose HP column. Different peak cuts, as indicated, were collected and analyzed for purity and recovery by HPLC. As expected, the more narrow cuts gave higher purity and lower recovery. Thus, a purity of about 98% was obtained with a recovery of about 62%. When applying a somewhat broader cut, a purity of about 93% was obtained with a recovery of about 98%.

4. Conclusions

Poly-β-CD coupled to allyl-substituted Sepharose HP base matrix can be used as ligand for the preparative separation and purification of puerarin, a Chinese traditional medicine. The conditions for separation were optimized for a sample dissolved in 20% (v/v) ethanol. The optimal mobile phase was 10% acetic acid at a linear flow velocity of 1 cm/min. The optimal sample load was 1.2 mg/ml adsorbent with a degree of purity and recovery of the target compound ranging from 93–98 to 62–98%, respectively, depending on the extent of peak cutting.

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