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Preparation, optimization and application of affinity absorbent with a polysaccharide YCP as the ligand

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ABSTRACT

YCP, an α-glucan from the mycelium of marine filamentous fungus *Phoma herbarum* YS4108, has great antitumor potential via enhancement of host immune through Toll-like receptor (TLR) 2 and TLR4 signaling. In the current study, YCP was coupled to EAH Sepharose 4B agarose beads to prepare the YCP-Sepharose affinity absorbent using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) as the activating agent. An orthogonal experiment L_9 $(3)^4$ was applied to optimize the coupling procedure, giving the optimal parameters as follows: molar ratio of CDAP to YCP of 1:2, CDAP-activation time of 5 min, gel volume of 0.1 mL, and gel-incubation time of 72 h, respectively. Scanning electron microscopy analysis indicated successfully preparation of YCP immobilized sepharose beads, while these beads essentially maintained biological properties of free YCP since they can interact with TLR2 and TLR4 specifically at comparable level. Collectively, our findings provide an alternative approach to immobilize carbohydrate-based molecules for studying the carbohydrate–protein interaction.

1. Introduction

In recent years, the technologies of carbohydrate immobilization have been gained much attention because of its importance in the study of carbohydrate-protein interactions (Aniulyte, Liesiene, & Niemeyer, 2006; Kohn & Wilchek, 1984; Mattern & Deen, 2007). The presentation of carbohydrates on gel beads can provide an efficient way to monitor the binding events between immobilized carbohydrates and soluble proteins including receptors or antibodies (Wen & Niemeyer, 2007). In some cases, the carbohydrate-immobilized beads with good selectivity and low limits of detection have been further employed in the purification of receptors of carbohydrate-based molecules (Wen & Niemeyer, 2011). However, due to the complicated structure of carbohydrates, it is difficult to find a robust, general, and controlled strategy for immobilization of carbohydrates.

To date, few approaches have been developed for the fabrication of carbohydrate microarrays on slide absorbents (Zhou & Zhou, 2006). The method that carbohydrates coupled to epoxy-activated or bromoethanol-activated beads has been put into commercial use such as WorkBeads™40 ACT Product DataSheet and Epoxy-activated Sepharose™ 6B. People have identified the immune receptors for polysaccharides from *Ganoderma lucidum* by this method (Shao, Dai, Xu, Lin, & Gao, 2004). The reaction requires an extreme high pH (above 11) at which the polysaccharides were probably suffered to hydrolysis. Besides, the method that *N*-carbohydrate chain of carbohydrates coupled to activated beads has been developed (Mattern & Deen, 2007).

CDAP can be used as an organic cyanylating reagent to activate water soluble polysaccharides over a broad range of alkaline pH and form functional groups that can be used to couple to substrate such as resin or protein. The strategy is that couples an amino group on the resin to an activated site on the carbohydrate (Emon, 2006; Hage & Cazes, 2005; Larsen, Thygesen, Guillaumie, Willats, & Jensen, 2006). The activation has advantages that are less modification of the polysaccharide, the ability to couple polysaccharide to the substrate directly and ease of use (Carpenter & Purdy, 1992; Kohn & Wilchek, 1983, 1984). In the current study, we are trying to apply this strategy to immobilize a common α-glucan to amine-activated gel beads (EAH Sepharose 4B). The glucan we used in this study, named as YCP (YCP is the acronym of Yancheng polysaccharide) was purified from the mycelium of *Phoma herbarum* YS4108 that inhabits the sediment in the Yellow Sea area around Yancheng, China. It has a backbone of

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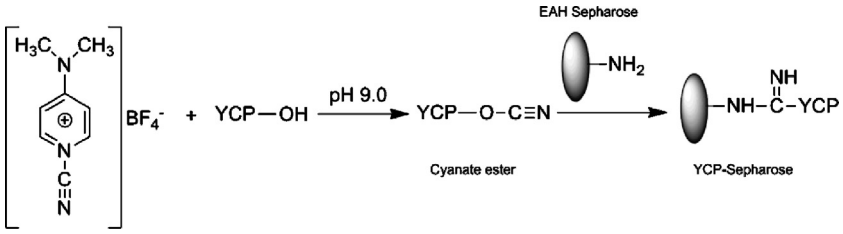

Fig. 1. The reaction pathway for the activation of YCP by CDAP and the binding of EAH Sepharose 4B.

α-1,4-D-glucan with a lower proportion of α-1,6-linked glucopyranosyl and glucuronic acid residues as non-reducing terminals (Chen, Guo, Gao, & Qian, 2005; Chen et al., 2009; Yang et al., 2005).

The Reaction pathway for the activation of YCP by CDAP and the binding of EAH Sepharose 4B is shown in Fig. 1. CDAP is slowly pipeted into the solution of YCP, the YCP hydroxyl groups are converted to cyanate ester. With the presence of primary amines in EAH Sepharose 4B, the amine and cyanate ester form an isourea bond. Thus, the YCP-Sepharose is synthesized and YCP is exposed at the surface of the Sepharose. Since we previously found that YCP possesses a great antitumor potential via enhancement of host immune through Toll-like receptor (TLR) 2 and TLR4 signaling (Zhang et al., 2013), the YCP-immobilized absorbent was further used as a tool in the study of YCP:TLR2 and YCP:TLR4 interaction. Collectively, our findings provide an alternative approach to immobilize common carbohydrate-based molecules for the study of carbohydrate–protein interaction.

2. Methods and materials

2.1. Materials

YCP was isolated and characterized in our lab previously (Yang et al., 2005). BioTrace NT nitrocellulose membrane was purchased from PALL (Ann Arbor, MI, USA). Enhanced Chemiluminescence (ECL) Detection Kit (Beyotime Biotech, China). 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). EAH Sepharose™ 4B was purchased from GE Healthcare (Uppsala, Sweden). The micro bicinchoninic acid (BCA) protein assay kit was from Beyotime Biotech (Shanghai, China). ProteoExtract Transmembrane Protein Extraction Kit was purchased from Novagen (Darmstadt, Germany). Anti-mouse TLR2 (CD282) and anti-mouse TLR4 were purchased from eBioscience (San Diego, CA, USA). Reagents used for cell culture were purchased from Gibco (Grand Island, NY, USA). All other reagents used in this study were of the highest available quality.

2.2. Experimental animals

Male BALB/C mice of clean grade (6–8 weeks old) weighing 18–22 g, were purchased from the Comparative Medicine Centre of Yangzhou University and acclimatized for 1 week prior to use. Throughout the study, the animals were fed by rodent laboratory chow pellets and tap water and were kept in five in each plastic cage in a well-ventilated room, maintained at 21 ± 2 °C with humidity of 50 ± 10% and a 12/12 h light/dark cycle. All the procedures conducted by the Comparative Medicine Centre of Yangzhou University were carried out in strict accordance with the PR China legislation on the use and care of laboratory animals and were approved by the university committee for animal experiments.

2.3. Preparation of the YCP-Sepharose affinity absorbent

2.3.1. Pre-treatment of EAH Sepharose 4B

EAH Sepharose 4B was washed with distilled water and adjusted to pH 4.5 with HCl on a sintered glass filter, followed by 0.5 M NaCl (80 mL, pH 7.0) and the residual water was removed by gentle suction. The drained gel (1 g) was suspended to 1 g/mL of distilled water and the pH of the suspension was confirmed to be 9.0 by 0.25 M NaOH at room temperature. The gel was divided into two equal portions: one to be used for the synthesis of YCP-Sepharose and the remaining was stand-by as blank group.

2.3.2. Synthesis of YCP-Sepharose

YCP (10 mg) was dissolved in distilled water (1 mL). CDAP (designed weight) was dissolved in acetonitrile (50 μL). CDAP was slowly pipeted into the solution of polysaccharide and 1 min later 0.25 M NaOH was added to adjust the pH to 9.0. At designed time, the solution of activated YCP was added to the gel (designed volume) after pre-treatment. The reaction mixture was slowly shaken in the incubator shaker in the 4 mL EP Tube for designed time at 25 °C. The mixture was centrifuged while the supernatant was collected. After washing by distilled water, the polysaccharide concentration was determinated through phenol-sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The ligand density of YCP-Sepharose (mg/g) was calculated by the mass balance according to the following equation.

$$q = \frac{m_y - (c_s V_s + c_w V_w)}{m_e}$$

where q is the ligand density of YCP-Sepharose (mg/g); c_s and c_w are the concentrations of YCP in the supernatant and washing distilled water of the mixture, respectively (mg/mL); v_s and v_w are the volumes of YCP in the supernatant and washing distilled water of the mixture, respectively (mL); m_y and m_s are the weights of YCP and EAH Sepharose 4B.

2.3.3. Treatment of Sepharose

Sepharose as blank group was slowly shaken in the incubator shaker in the 4 mL EP Tube for same time as YCP-Sepharose at 25 °C. In order to verify the difference between YCP-Sepharose and Sepharose, their superficial morphologies were studied by scanning electron microscopy (SEM).

2.4. Optimization of the synthesis of YCP-Sepharose affinity absorbent

An orthogonal L_9 $(3)^4$ test design was used to investigate the optimal coupling conditions in the synthesis of polysaccharide-Sepharose. The pH of synthesis experiment was fixed at 9.0 (Lees, Nelson, & Mond, 1996; Shafer et al., 2000), As seen from Table 1, the synthesis experiments were carried out with 4 factors and 3 levels, as molar ratio of CDAP to dextran in YCP (1:2, 1:4, 1:8), activation time of YCP by CDAP (2.5, 5, 10 min), volume of gel (0.1, 0.2, 0.4 mL) and incubation time of activated YCP and gel (24, 48, 72 h).

Table 1
Factors and levels for orthogonal test.

Variable	Level		
	1	2	3
A, molar ratio of CDAP to dextran in YCP (n)	1:8	1:4	1:2
B, activation time of YCP by CDAP (min)	2.5	5	10
C, volume of gel (ml)	0.1	0.2	0.4
D, incubation time of activated YCP and gel (h)	24	48	72

All processes of synthesis were maintained at 25 °C. The range of each factor level was based on the results of preliminary single factor experiments. The optimal solution obtained from the above 9 tests was operated following the method in Section 2.3.

2.5. Extraction of B cells membrane proteins

Murine spleens were harvested from BALB/C inbred mice and minced in RPMI-1640 medium at room temperature. Dispersed splenocytes were passed through a 22 μm sieve. Recovered splenic cells were centrifuged at 1000 rpm for 5 min. After centrifugation, supernatant was removed and pellet was resuspended in lysis buffer (0.15 M NH_4Cl, pH 7.4) for 10 min to remove sheep erythrocytes. After centrifugation, pellet was resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated (56 °C for 30 min) FCS. Spleen cells were incubated for 6 h in flask at 37 °C with 5% CO_2 humidified atmosphere to remove adherent cells such as macrophages. The suspended cell populations were collected and used as the lymphocyte populations.

T cells and B cells were prepared by using the nylon-wool method (Litvin & Rosenstreich, 1984). Briefly, T cells were separated over nylon wool column and B cells were eluted from the nylon wool column with RPMI-1640 medium supplemented with 10% heat-inactivated (56 °C for 30 min) FCS. The purity of B lymphocytes was higher than 90% as described (Pellegrino, Ferrone, & Theofilopoulos, 1976).

Separated B cells were centrifuged at 1000 rpm for 5 min. The pellet was resuspended and washed by PBS (0.01 M, pH7.4). B cells membrane proteins were extracted by ProteoExtract Transmembrane Protein Extraction Kit. The concentration of B cells membrane proteins was determined by a bicinchoninic acid assay using a by BCA protein assay kit.

2.6. Affinity adsorption assay

0.5 mL of the Sepharose group and 0.5 mL of the YCP-Sepharose group were both prepared for the affinity adsorption of B cells membrane proteins to identify the B cell receptors of YCP. Both of the gels were washed 5 times with 1 mL Tris–NaCl (0.1 M Tris, 0.15 M NaCl, pH 7.4, 0.1% Triton X-100) till equilibrated and ready for use. Added 100 μL B cells membrane proteins solution to each gel, and the mixture was shaken softly in the incubator shaker for 1 h at 25 °C. After centrifugation, two groups of gels were washed with 1 mL Tris–NaCl (0.1 M Tris, 0.15 M NaCl, pH 7.4, 0.1% Triton X-100) for 5 times and the supernatant was collected after washing every time. The collection of supernatant was combined together, respectively named Sepharose washing sample (SWS) and YCP-Sepharose washing sample (YWS). Then both of the gels were washed with 1 mL Tris-NaCl (0.1 M Tris, 1.5 M NaCl, pH 7.4, 0.1% Triton X-100) for 5 times and the supernatant was collected after washing every time. The collection of supernatant was combined together, respectively named Sepharose elution sample (SES) and YCP-Sepharose elution sample (YES). After dialysis and freeze drying, the volume of each sample was concentrated from 5 mL to 100 μL. The protein concentration was determined by a bicinchoninic acid assay using a by BCA protein assay kit.

To remove the excess of uncoupled ligand that remains after coupling, the adsorbent was washed alternatively with high pH (0.1 M Tris–HCl, 0.5 M NaCl, pH 8.5) buffer solution and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5) buffer solution three times. This procedure ensured that no free ligand remains ionically bound to the immobilized YCP.

2.7. Western blot analysis

In order to identify the B cell receptors of YCP, SDS-PAGE and western bolt were used. Samples for electrophoresis (equal amount) were placed in sample buffer and heated for 5 min at 100 °C. Proteins were separated by SDS-PAGE in 12% polyacrylamide gels and electrically transferred onto BioTrace NT nitrocellulose membrane. The membrane was incubated in TBST buffer (0.05 M Tris, pH 7.6, 0.15 M NaCl, 0.05% Tween-20) containing 3% bovine serum albumin for 2 h at 37 °C. Then the membrane was incubated with the indicated primary antibody (anti-TLR2, anti-TLR4) overnight at 4 °C, followed by the corresponding secondary antibody for 1 h at 37 °C. The membrane was exposed by using an enhanced chemiluminescence (ECL) detection kit after washing with TBST.

3. Results and discussion

3.1. Effects of various parameters on the ligand density of YCP-Sepharose

3.1.1. Effect of molar ratio of CDAP to dextran in YCP

The ligand density of YCP-Sepharose affected by parameters is seen in Fig. 2. Fig. 2A shows the effects of molar ratio of CDAP to dextran in YCP (1:32, 1:16, 1:8, 1:4, 1:2) on the ligand density while the other factors (activation time of YCP by CDAP, volume of gel, incubation time of activated YCP and gel) are fixed at 2.5 min, 0.4 mL, 48 h. The ligand density of YCP-Sepharose increases with the increasing CDAP and reaches the critical value (9.229 ± 0.402 mg/g) when molar ratio of CDAP to dextran in YCP ranging from 1:8 to 1:2.

3.1.2. Effect of activation time of YCP by CDAP

Fig. 2B indicates the effects of activation time of YCP by CDAP (2.5 min, 5 min, 10 min, 20 min, 40 min) on the ligand density while the other factors (molar ratio of CDAP to dextran in YCP, volume of gel, incubation time of activated YCP and gel) are fixed at 1:4, 0.4 mL, 48 h. The ligand density of YCP-Sepharose varies with the increasing activation time and reaches the critical value (9.336 ± 0.096 mg/g) when activation time of YCP by CDAP ranging from 2.5 to 10 min.

3.1.3. Effect of incubation time of activated YCP and gel

Fig. 2C illustrates the effects of incubation time of activated YCP and gel (12 h, 24 h, 48 h, 72 h, 96 h) on the ligand density while the other factors (molar ratio of CDAP to dextran in YCP, activation time of YCP by CDAP, volume of gel) are fixed at 1:4, 2.5 min, 0.4 mL. The ligand density of YCP-Sepharose changes with the increasing incubation time and reaches the threshold value (9.213 ± 0.250 mg/g) when incubation time of activated YCP and gel ranging from 24 to 72 h.

3.2. Optimization of the synthesis of YCP-Sepharose

Since various parameters potentially affect the synthesis of YCP-Sepharose affinity absorbent, the optimization of the experimental factors is necessary and vital. In fact, molar ratio of CDAP to dextran in YCP, activation time of YCP by CDAP, incubation time of activated YCP and gel, volume of gel are generally considered to be the most

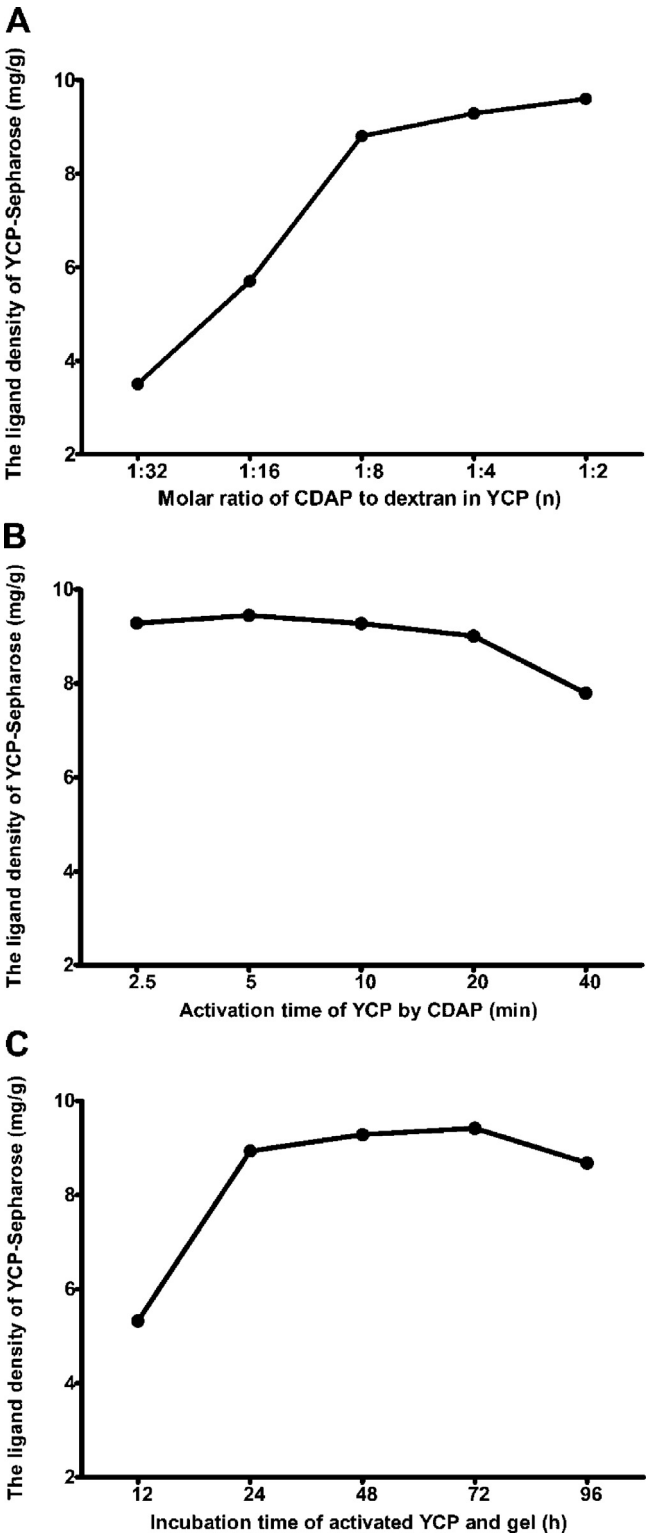

Fig. 2. The ligand density of YCP-Sepharose affects by various parameters. A: The effects of molar ratio of CDAP to dextran in YCP (1:32, 1:16, 1:8, 1:4, 1:2) on the ligand density while the other factors (activation time of YCP by CDAP, volume of gel, incubation time of activated YCP and gel) are fixed at 2.5 min, 0.4 mL, 48 h. B: The effects of activation time of YCP by CDAP (2.5 min, 5 min, 10 min, 20 min, 40 min) on the ligand density while the other factors (molar ratio of CDAP to dextran in YCP, volume of gel, incubation time of activated YCP and gel) are fixed at 1:4, 0.4 mL, 48 h. C: The effects of incubation time of activated YCP and gel (12 h, 24 h, 48 h, 72 h, 96 h) on the ligand density while the other factors (molar ratio of CDAP to dextran in YCP, activation time of YCP by CDAP, volume of gel) are fixed at 1:4, 2.5 min, 0.4 mL.

important factors. An orthogonal $L_9(3)^4$ test design is used to investigate the optimal synthesis of YCP-Sepharose. Orthogonal Design is generated in SPSS 19.0 and the factors and levels are added as described in Table 1. The data of orthogonal test is analyzed by univariate statistics as General Linear Model in Table 2. K_1, K_2 and K_3 are defined as the sum of the evaluation indexes of all levels (1, 2, 3) in each factor (A, B, C, D), and R^2 is defined as the range between the maximum and minimum value of K and used for evaluating the importance of the factors. The larger R^2 means a greater importance of the factor (Chuanwen, Feng, Yuguo, & Shuyun, 2010). The factors influence the ligand density are listed in a decreasing order: C > A > D > B according to the R value. Due to the experimental error cannot be determined by the range analysis, an ANOVA analysis is necessary to obtain the magnitudes of the factor affecting the index. The F value of each factor indicates the ratio of the sum of the square of each factor's mean deviations to that of the experimental error range analysis. Therefore, the F value is used to indicate the magnitudes of the factor effects and the data are analyzed by a F-test (Buljak & Maier, 2011; RenJie, 2008; Wu & Leung, 2011). F_α is constant and defined as the critical value of F-value for different inspection level. The factor for the results is prominent when test F is larger than F_α (0.5%). The percentage contribution can indicate the relative power of a factor to reflect each factor's influence (Ghambarian, Yamini, Saleh, Shariati, & Yazdanfar, 2009). From the results of the ANOVA in Table 3, those parameters that the molar ratio of CDAP to dextran in YCP, the volume of gel and the incubation time of activated YCP and gel, have been regarded to be the significant factors. Therefore the above three designing parameters are noticeably variable factors as the resources which increasing the ligand density of YCP-Sepharose. The last column indicates the percentage of contribution (P) of each factor on the total variation and the degree of influence. Based on the above discussion, the optimal ligand density is obtained while volume of gel, molar ratio of CDAP to dextran in YCP, incubation time of activated YCP and gel, activation time of YCP by CDAP are C1 A3 D3 B2 (0.1 mL, 1:2, 72 h and 5 min).

The volume of gel is found to be the most important determinant of the ligand density. There are reasonable explications. First of all, in this ligand coupling reaction under certain conditions, with the increasing of the volume of resin, the number of activated YCP is constant and the concentration is degraded. In other words, the number of activated YCP per volume is decreased which causes the decay of effective collision probability and the reduction of coupling probability (Bruno, Kasapis, & Heng, 2012; Claverie et al., 2009; Coltrin & Malik, 1982; Plass & Cooks, 2003). Secondly, due to the steric effect, the raise of amino exposed to YCP is weaker than the addition of volume of resin. The probability of occupation of activated of YCP to amino is cut down (Curtis et al., 2011). And then, the space which is taken by coupling YCP would trend the spatial structure to be more polygons which abate the collision probability between particles (Li, Chen, Wang, Guo, & Li, 2013). However, ligand density is lessened with the increasing volume of sepharose; the amount of coupling YCP is inevitably enlarged. In order to couple YCP at utmost, we synthesized the affinity absorbent several times by adopting the optimal scheme which could synthesize 0.1 mL one time.

3.3. Characterization of YCP-Sepharose affinity absorbent

3.3.1. Synthesis of YCP-Sepharose affinity absorbent

YCP-Sepharose affinity absorbent was synthesized with the optimal solution obtained from Section 3.2 following the method in Section 2.3. According to Table 4 which displays the weights of YCP before and after synthesis, the ligand density of YCP-Sepharose is calculated as 34.74 ± 1.19 mg/g. The ligand density of

Table 2
Analysis of L_9 $(3)^4$ test results.

No.	A, molar ratio of CDAP to dextran in YCP (n)	B, activation time of YCP by CDAP (min)	C, volume of gel (ml)	D, incubation time of activated YCP and gel (h)	The ligand density of YCP-Sepharose (mg/g).
1	1	1	1	1	20.60
2	1	2	2	2	11.80
3	1	3	3	3	7.20
4	2	1	2	3	19.46
5	2	2	3	1	7.91
6	2	3	1	2	24.31
7	3	1	3	2	9.60
8	3	2	1	3	30.38
9	3	3	2	1	15.71
K_1	39.60	49.66	75.29	44.22	–
K_2	51.68	50.09	46.97	45.71	–
K_3	55.69	47.22	24.71	57.04	–
R^2	16.09	2.87	50.58	12.82	–

Refers to the result of extreme analysis

Table 3
Results of the analysis of variance.

Designing parameter	Sum of squares	Degrees of freedom	Test F	$F\alpha = 0.5\%$	SD	P (%)
A, molar ratio of CDAP to dextran in YCP (n)	46.77	2	29.28	19.00	*	9.18
B, activation time of YCP by CDAP (min)	1.60	2	1.00	19.00		0.31
C, volume of gel (ml)	428.43	2	268.27	19.00	*	84.08
D, incubation time of activated YCP and gel (h)	32.77	2	20.52	19.00	*	6.43
Error		0				
Total	509.57	8				100

P: percentage of contribution; *: test $F \leq F\alpha - 0.5\%$.

YCP-Sepharose is moderate (Hjertén, Rosengren, & Pahlman, 1974). When the ligand density is high enough, although the apparent binding capacity of the affinity adsorbent would maintain at a constant, the adsorption ability is enhanced. When the ligand density is high beyond the threshold, there will not only emerge multipoint association between YCP and resin that may lead to alterations in the structure of YCP, but also produce irreversible adsorption due to the combination is too firm (Laas, 1975; Maisano, Belew, & Porath, 1985; Tanford, 1972). The optimal solution was used in the synthesis of YCP-Sepharose affinity absorbent while the ligand density was moderate in order to maintain biological properties of YCP on the beads.

3.3.2. Scanning electron microscope observation

The scanning electron microscope micrographs emerging the surface structure of EAH Sepharose 4B and YCP-Sepharose affinity absorbent are presented in Fig. 3A and B, respectively. EAH Sepharose 4B displays a smooth and glossy surface structure. There is amount of granules on the surface of YCP-Sepharose. These bulbiform small bulges make it appear to be coarser. The detailed morphological findings by SEM indicate the difference between microstructures of YCP-Sepharose and Sepharose which means YCP has been immobilized on sepharose beads successfully.

3.3.3. Western blot

B cells were separated on the basis of the difference in their affinity to nylon fibers and the population was sufficient purity

Table 4
The weights of YCP before and after synthesis ($n = 5$).

EAH Sephorase 4B (g)	YCP (mg)	
	Before the synthesis	After the synthesis
0.1	10.97	7.62
		7.63
		7.36
		7.42
		7.48

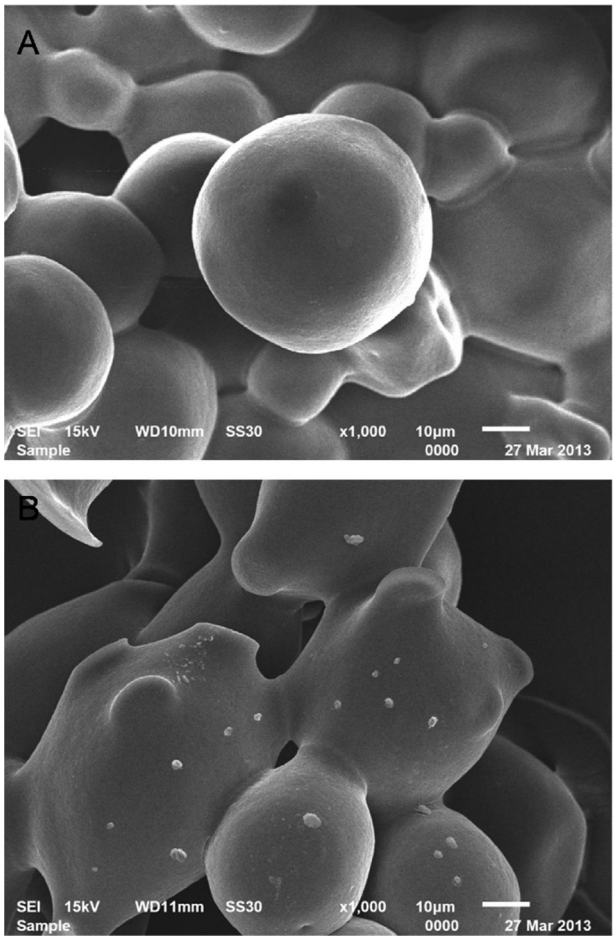

Fig. 3. Scanning electron micrographs showing surface morphology. A: EAH Sepharose 4B. B: YCP-Sepharose affinity absorbent.

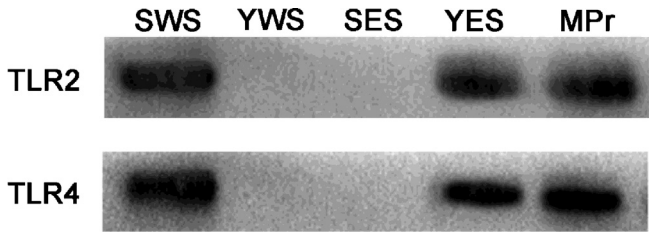

Fig. 4. Each sample of affinity adsorption subjected to Western blot analysis probing with anti-TLR2 and anti-TLR4. SWS: Sepharose washing sample; YWS: YCP-Sepharose washing sample; SES: Sepharose elution sample; YES: YCP-Sepharose elution sample; MPr: B cell membrane protein.

(approximately 90%, data not shown). After extracting of B cells membrane proteins and binding the proteins with the affinity absorbent, we collected Sepharose washing sample (SWS), YCP-Sepharose washing sample (YWS), Sepharose elution sample (SES) and YCP-Sepharose elution sample (YES). The volume of each of the samples which was harvested from affinity adsorption was concentrated from 5 mL to 100 μL through dialysis and freeze drying. And concentrations of B cells membrane proteins, SWS, YWS, SES, YES were 2.201, 1.793, 1.584, 0.242, 0.492 mg/mL determined by a bicinchoninic acid assay using a by BCA Protein Assay Kit.

Our previous study indicated that the activation of B cells induced by YCP was dependent on TLR2 and TLR4, as both specific binding and immunological activities of YCP in B cells were significantly decreased by blocking antibody to TLR2 and TLR4 (Zhang et al., 2013). In order to verify this point by further experiment, we designed a western blot to indentify the protein gained from affinity absorbent. As shown in Fig. 4, there is a statistically significant difference among the effects of SWS and YES on protein levels of TLR2 and TLR4, respectively. While the signals of SES on protein levels of TLR2 and TLR4 are not detected. The results indicate that SWS and YES contained TLR2 and TLR4, in the other word, TLR2 and TLR4 are the receptors of YCP on B cells. Meanwhile, our YCP-Sepharose affinity absorbent has effectively specific affinity for the receptors of YCP due to the difference between SWS and YWS on protein levels of TLR2 and TLR4. The results of western blot indicate that YCP can interact with TLR2 and TLR4 specifically at comparable level on B cells and YCP still maintains biological properties after being immobilized.

The optimized technology of immobilizing polysaccharide could not only identify the receptors of immobilized polysaccharide, but also seek for the antibody and specifically combined ligands (Lin et al., 2011; Monzo, Bonn, & Guttman, 2007; Roy, Sardar, & Gupta, 2000; Tozzi, Anfossi, & Giraudi, 2003; Zhao et al., 2008). Great convenience will be brought in the research of polysaccharides on the aspect of immunity and anti-tumor therapy if the scheme widely exploited and utilized in future.

Acknowledgements

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