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Preparation and characterization of PEGyated Concanavalin A for affinity chromatography with improved stability

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ABSTRACT

In order to improve its stability, immobilized Concanavalin A (Con A) on Toyopearl adsorbents was conjugated with monomethoxy poly(ethylene glycol) succinimidyl propionate (mPEG-SPA) with different molecular weight. A colorimetric method using ninhydrin is proposed to determine the degree of PEGylation; this method has proved to be easy applicable and reproducible. The PEGylation reaction was studied in detail to elucidate how parameters such as molar ratio of mPEG-SPA to Con A and molecular weight of mPEG-SPA affect the degree of PEGylation. The adsorption isotherms of glucose oxidase (GOD) onto native and PEGylated Con A adsorbents showed that the modification did not alter substantially the specificity of the carbohydrate binding ability of Con A. However, the binding capacity for GOD was slightly reduced probably due to the steric hindrance caused by mPEG chains. Adsorption kinetic studies revealed a lower adsorption rate after PEGylation which was attributed to the steric effect. The dynamic adsorption capacity for modified Con A depended very much on the degree of PEGylation and the molecular weight of mPEG derivatives. The adsorption capacity could be highly preserved for Toyopearl Con A modified by mPEG2k (90% of the original adsorption capacity) even with a degree of PEGylation up to 20% (the ratio of primary amino groups of PEGylated immobilized Con A to that of native immobilized Con A). Studies show that the binding capacity of PEGylated Con A was highly preserved under mild process conditions. PEGylated Con A also exhibited obviously higher stability against more stressful conditions such as the exposure to organic solvents and high temperatures. Conjugation of Con A with mPEG2k provided better adsorption performance thus has greater potential for application in affinity separation processes compared with mPEG5k. The fact that PEGylation stabilizes the properties of Con A may greatly expand the range of applications of unstable proteins to bioprocessing (e.g. biocatalysis and downstream separation) as well as other protein applications (e.g. medication, industrial use, etc.).

1. Introduction

Affinity chromatography has proved to be of great significance for the separation and purification of biological macromolecules in biochemical technology [1,2]. Affinity chromatography using lectins as ligands has been extensively employed for the purification of glycoconjugates due to their carbohydrate binding specificity [3–6]. However, the industrial application of lectin ligands is sometimes hampered by their toxicity, as with Con A [7], and the instability of their protein-based structure under more stressful operating conditions, which may involve the presence of organic solvents or elevated temperatures. The employment of more stable ligands is expected to facilitate their extensive industrial application (e.g. in affinity chromatography).

The generation of robust proteins can be achieved by either genetic or biochemical approaches. Genetic approaches have yielded significant results in obtaining much more stable proteins against harsh operational conditions [8,9]. However, the main drawback of the genetic approach is that the knowledge of the properties gained by site-directed mutagenesis or evolution processes cannot be used as a general method to be applied to other proteins [10]. On the other hand, the chemical modification seems to be a more universal approach to improve intrinsic properties of proteins, and deeper knowledge of gene or protein structure is not required. Several chemical methods have been employed

Abbreviations: Ads, adsorbents; C, chloroform; Con A, Concanavalin A; DP, degree of PEGylation; GOD, glucose oxidase; HRP, Horseradish peroxidase; M, methanol; mPEG-SPA, monomethoxy poly (ethylene glycol) succinimidyl propionate; TC, native Toyopearl Concanavalin A adsorbents; PVPAA, poly (vinylpyrrolidone-*co*-acrylic acid).

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to obtain more stable proteins, including immobilization, cross-linking, attachment to polysaccharides, and chemical modification with amphiphilic polymers [11,12]. One of the most successful approaches is to modify proteins with poly(ethylene glycol) (PEG), a process commonly known as PEGylation. PEG is a synthetic, non-toxic, non-immunogenic, amphiphilic and most importantly, highly biocompatible polymer. Conjugation of proteins with PEG, linear or branched, via a covalent linkage can eliminate some drawbacks of native proteins and improve their physicochemical, biomedical and pharmacological properties. PEGylation often results in a protein with improved solubility and temperature stability, enhanced stability against enzymatic degradation, increased serum half-life and anti-tumour potency, decreased renal clearance and immunogenicity, while normally maintaining a high percentage of its biological activity [13–16]. It has to be pointed out that although the application of PEGylation has been extensively studied, the mechanism of these effects caused by PEG chains is still not fully understood and well accepted.

This work will take the jack bean lectin, Concanavalin A (Con A), as an example to study the PEGylation of a proteinaceous affinity ligand and the influence of modification on its adsorption performance in the affinity separation process. PEGylation of Con A has been studied by several authors for various purposes. Ueno et al. investigated the *in vivo* induction of anti-tumour cytotoxicity in mice by the treatment with Con A modified with PEG. They found that PEGylated Con A exhibited reduced immunogenicity and prolonged clearance time in mice blood [15]. Kim and Park used Con A for delivery of modulated insulin in sol–gel phase-reverse hydrogel systems, and found PEGylated Con A had improved aqueous solubility, enhanced long-term stability, and higher glucose sensitivity compared to native Con A [17]. Liu et al. studied a Con A based glucose-responsive insulin delivery system, which could be applied for long-term diabetes treatment. They found the conjugation of Con A with PEG grafted by hydrophilic poly (vinylpyrrolidone-*co*-acrylic acid) (PVPAA) exhibited substantially improved solubility at pH 7.4 while preserving its sugar binding characteristics [18]. On the basis of the above observations, the present work describes the preparation and characterization of PEGylated Con A adsorbents, focusing on their applications for affinity chromatography. A method to determine the PEGylation degree by ninhydrin is proposed in this study and the parameters influencing the degree of PEGylation were studied in detail. The binding specificity and binding rate of PEGylated Con A to glucose oxidase (GOD) were investigated by adsorption isotherm and adsorption kinetic experiments. GOD is a glycoprotein with a high-mannose type carbohydrate content (10–16%, w/w), which binds well into the Con A binding pocket [19]. The binding capacities of GOD under both normal and harsh conditions were determined and compared by affinity chromatography for both PEGylated and native Con A adsorbents.

2. Materials and methods

2.1. Materials

Toyopearl AF-Tresyl-650M (hereafter called Toyopearl for short) was obtained from Tosoh Bioscience (Stuttgart, Germany). Con A (type V), Horseradish peroxidase (HRP) (EC 1.11.1.7), methyl-α-D-mannopyranoside, ninhydrin, Bradford reagent and all the organic solvents, including tetrahydrofuran, chloroform and methanol, were purchased from Sigma (Munich, Germany). Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4) was delivered from Serva (Heidelberg, Germany). Monomethoxy poly(ethylene glycol) succinimidyl propionate (mPEG-SPA) with different molecular weight was from Nektar (Huntsville, AL, USA). All the chemicals were of analytical reagent grade unless otherwise stated.

2.2. Immobilization of Concanavalin A

Con A was immobilized onto Toyopearl supports according to the procedure stated in our previous work [6]. The protein concentration was determined by the Bradford method [20].

2.3. Conjugation of immobilized Concanavalin A with mPEG derivatives (mPEG-SPA)

Immobilized Con A was coupled with PEG using mPEG-SPA, which is active towards primary amines. mPEG-SPA was dissolved in 0.1 M phosphate buffer, pH 8.0, with added methyl-α-D-mannopyranoside for protection of the binding sites of Con A. To vary the molar ratio of Con A to mPEG-SPA in the reaction solution, the amount of mPEG-SPA was changed while Con A was kept constant. The molecular weight of the Con A molecule is 104,000 g/mol, while those of mPEG-SPAs were 2000 g/mol, 5000 g/mol and 20,000 g/mol (mPEG2k-SPA, mPEG5k-SPA, and mPEG20k-SPA), respectively. In typical PEGylation experiments, the reaction mixture was gently shaken at room temperature for 2 h, and then 0.1 M acetate buffer, pH 4.0, was introduced to the system to terminate the reaction. The modified Con A adsorbents were filtrated and washed extensively with 0.1 M acetate buffer, containing 0.1 M NaCl, 1 mM Ca^{2+}, Mn^{2+}, and Mg^{2+}, pH 6.0 (hereafter called buffer A), and then stored in the same buffer.

2.4. Determination of the degree of PEGylation of immobilized Concanavalin A

mPEG-SPA was conjugated with Con A through the unreacted primary amino groups during its immobilization. The indirect determination of the degree of PEGylation is performed by comparison of the amount of primary amines of immobilized Con A before and after modification with mPEG-SPA. Ninhydrin is one of the commonly used reagents to determine the concentration of amino acids or proteins, since it reacts with the primary amines developing a typical purple color. A ninhydrin method was developed to determine the degree of PEGylation in this work. A typical procedure was carried out as follows.

The PEGylated and unPEGylated Con A adsorbents were vacuum evacuated after being thoroughly washed with distilled water. Affinity adsorbents with an amount of immobilized Con A between 0.5 mg and 2.5 mg were placed in a 15 mL test tube and then gently mixed with 2 mL purified water and 1 mL ninhydrin reagent. The mixture was then heated in a boiling water bath for 10 min. After cooling down to the room temperature, 5 mL 95% ethanol water solution was added to the mixture and mixed well with the adsorbents. The mixture was then centrifuged and the absorbance of the supernatant at a wavelength $\lambda = 570$ nm was measured (UV spectrophotometer, Carl Zeiss, Jena, Germany). The same mixture without affinity adsorbents was taken as blank. The degree of PEGylation was calculated from the slopes of the plots of PEGylated and unPEGylated Con A adsorbents.

2.5. Adsorption isotherms of glucose oxidase onto Concanavalin A adsorbents

The adsorption isotherms of GOD to Con A affinity adsorbents were performed by batch experiments. 5 mL GOD solutions in buffer A with increasing concentrations were mixed with 0.1 g (wet weight, about 0.15 mL) PEGylated, and unPEGylated Con A Toyopearl affinity adsorbents, respectively, in 15 mL plastic tubes. The suspensions were allowed to equilibrate for 15 h at 25 °C in a rotary water bath with a speed of 150 rpm. After the adsorbents were settled by centrifugation, the supernatant obtained from each tube was utilized to determine the protein concentration by measuring

the absorbance at λ = 280 nm. The equilibrium adsorption capacity was calculated by the mass balance as shown in Eq. (1).

$$q* = \frac{(c_0 - c^*)V_{GOD}}{V_{Ads}} \quad (1)$$

where q^* is the equilibrium adsorption capacity of the affinity adsorbent for GOD (mg/mL); c_0 and c^* are the initial and equilibrium concentration of GOD in the aqueous phase of the mixture, respectively (mg/mL); V_{GOD} is the total volume of the GOD aqueous solution (mL); and V_{Ads} is the volume of the Con A adsorbents (mL).

The adsorption isotherms of GOD onto different Con A adsorbents were fitted with the Langmuir equation presented in Eq. (2).

$$q^* = \frac{q_m c^*}{K_d + c^*} \quad (2)$$

where q_m is the maximum adsorption capacity (mg/mL) and K_d the dissociation constant (M), which are the characterizing model parameters.

2.6. Adsorption kinetics of glucose oxidase onto Concanavalin A adsorbents

30 mL of about 0.4 mg/mL GOD was mixed with approximate 0.75 mL Con A adsorbents. The mixture was gently shaken at 120 rpm on an orbital shaker. The supernatant was withdrawn at time intervals to determine the GOD concentration in a UV spectrophotometer at λ = 280 nm.

2.7. Affinity chromatography of glucose oxidase onto Concanavalin A adsorbents

Dynamic adsorption of GOD was performed on a low-pressure liquid chromatography system from Bio-Rad (Munich, Germany). The system contains a gradient pump, a UV detector (280 nm), and a recorder. Affinity separation was carried out at room temperature and at a flow rate of 0.6 mL/min. About 1 mL Con A adsorbents was packed into a glass column (8 mm × 100 mm) and well equilibrated with buffer A. 250 μL of 4 mg/mL GOD was applied to the Con A adsorbents from the top of the column. The residence time of GOD solution contacting the Con A adsorbents was 10 min. The column was then thoroughly washed with buffer A till no protein appeared in the eluate. The specific desorption was achieved with the same buffer containing 0.1 M methyl-α-D-mannopyranoside. The desorbed fraction was collected to determine the protein concentration with the Bradford method [20].

2.8. Stability against organic solvents

All the fresh prepared native and PEGylated Con A adsorbents were incubated in various organic solvents for 30 min, then packed into a 8 mm (inner diameter) column. The column was washed and equilibrated with buffer A for another 30 min. 1 mg GOD or HRP was applied to the column and the affinity separation was conducted at a flow rate of 0.6 mL/min. The following operations were the same as described in Section 2.7. All the experiments were repeated three times and the mean adsorption capacity was taken as the final result.

2.9. Stability against temperature

About 0.15 mL native and PEGylated Con A adsorbents was placed in a tube and mixed with 1 mL buffer A. The adsorbents were incubated at 55 °C for different periods of time. After cooling to room temperature, 4 mL of 0.3 mg/mL GOD was introduced into the tube and the mixture was equilibrated with gentle shaking for 15 h. The supernatant was withdrawn to determine the protein concentration and calculate the adsorption capacity.

3. Results and discussion

3.1. Design of the PEGylation route and definition of the degree of PEGylation

The structure and properties of a protein are the most important factors in the selection of the proper conjugation method. Since Con A exhibits many lysine groups on its surface and most of these lysine groups are not involved in its active sites, mPEG-SPA, a primary amine specific reagent, was chosen to modify Con A for the preparation of adsorbents for affinity chromatography. The PEGylation reaction can be finished within a short time, e.g. 15–60 min for mPEG-SPA with a native protein. However, the subsequent isolation and purification of PEGylated proteins is time-consuming by procedures such as dialysis, lyophilisation, and most frequently employed chromatography methods [21–23]. Because of the extremely high costs involved in the production of the native proteins it is important that the subsequent PEGylation process should be as efficient as possible and activity preserving for the protein as well. To simplify the process, Con A was first immobilized onto Toyopearl supports, and then the immobilized Con A was conjugated with mPEG-SPA. By this procedure unreacted mPEG-SPA and some sideproducts could be easily removed by filtration, which greatly facilitated the recovery of PEGylated Con A. This PEGylation route also saved process cost and time by reducing the number of unit operations.

The extent of the degree of PEGylation is highly related with the properties of the modified protein. To investigate how PEGylation influences the adsorption performance of modified Con A, it is necessary to know the degree of PEGylation. Since PEGylation is based on immobilized Con A adsorbents, it is needed to compare the difference of the adsorption performance before and after the modification of the immobilized Con A. Correspondingly, the degree of PEGylation in this work is defined as the ratio of primary amino groups of PEGylated immobilized Con A to that of native immobilized Con A.

3.2. Development of a new method for the analysis of the degree of PEGylation

Whatever the purpose for protein modification with mPEG, there is always a need for simple and rapid methods to determine the extent of modification. Methods such as size exclusion chromatography (SEC) [24], HPLC [25], SDS-PAGE [26], MALDI-TOF mass spectrometry [27], fluorometric assay [28], microfluidics assay [29] and TNBS assay [30] have been reported to determine the degree of PEGylation for free proteins. For immobilized PEG–protein conjugates, mostly physical methods such as contact angle measurement and transmission electron microscopy have been reported as summarized by Hooftman et al. [31]. However, these procedures are either complex, time consuming or not easy available. A simple and fast method is thus highly desired for the determination of the degree of PEGylation of immobilized PEG–protein conjugates. Since mPEG-SPA reacts with the primary amines of immobilized Con A by the formation of a stable amide linkage (see supplementary scheme), the degree of PEGylation could be calculated by comparing the number of primary amines before and after modification with PEG. Ninhydrin is a commonly used reagent for the determination of the protein concentrations since it can react with the primary amines in both free and immobilized states [32]. Here, this method was modified to determine the degree of PEGylation according to the procedure mentioned in Section 2.

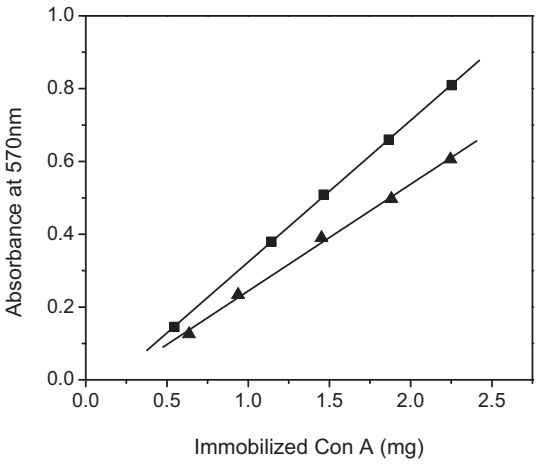

Fig. 1. Linear regression analysis of ninhydrin standard curve for native and PEGylated Con A immobilized onto Toyopearl supports. The regression equations are $y = 0.389x - 0.066$ for native immobilized Con A and $y = 0.294x - 0.050$ for PEGylated immobilized Con A, with the ordinate: absorbance at 570 nm and with the abscissa: the amount of immobilized Con A. The correlation coefficients are 0.9999, and 0.9986, respectively. (■) Native immobilized Con A; (▲) PEGylated immobilized Con A.

A good example for the determination of the degree of PEGylation is shown in Fig. 1. As can be seen, the relationship between the absorbance and the amount of native or PEGylated immobilized Con A is linear and both of the correlation coefficients of the plots are close to 1, which shows a very good reproducibility. The estimated degree of PEGylation of immobilized Con A in the example is 24%, calculated from the ratio of the slopes of the plots according to Eq. (3).

$$DP\ (\%) = \left(1 - \frac{b_1}{b_2}\right) \times 100 \quad (3)$$

where DP is the degree of PEGylation, b_1 is the slope of the plot of PEGylated Con A and b_2 is the slope of the plot of native Con A. The experiments show that the ninhydrin method is fast and easy reproducible even though it requires a relatively large amount of sample (milligram quantities).

It should be pointed out that the degree of PEGylation is just a mean value due to the heterogeneity in lysine substitution. Even for the same degree of PEGylation, the PEGylation may happen on the different lysine positions of large molecules such as Con A as well as on the heterogeneous adsorbents. Thus the extent of the PEGylation may be different for each Con A molecule. To minimize the experimental error caused by the heterogeneity of the Con A immobilization, all the comparisons were conducted between the same batch of immobilized Con A adsorbents.

3.3. PEGylation reaction studies

mPEG-SPA is one of the most popular derivatives for coupling PEG to proteins due to its fast reactivity, low toxicity and higher stability. Actually, during the PEGylation reactions the modification of proteins is competitive with the hydrolysis of mPEG-SPA. Studies indicate that the half-life of hydrolysis for mPEG-SPA is about 16 min (data from supplier) and the aminolysis (modification of protein) is always faster than the hydrolysis during the PEGylation reactions. In order to obtain reproducible modified products, quick and complete dissolution of mPEG-SPA and mixing of the reactants are necessary. Moreover, it is important to ensure that the active sites of proteins are not involved in the covalent linkage to mPEG derivatives [33]. Therefore, methyl-α-D-mannopyranoside was added to block the active sites of Con A during the PEGylation reactions. Primary studies of reaction time and reaction pH value show that the optimal condition for the conjugation of immobilized Con A with mPEG-SPA was performed in 0.1 M phosphate buffer, pH 8 for 2 h. In this section, the results of molar ratio of mPEG-SPA to Con A and the molecular weight of mPEG-SPA are presented to visualize their influences on the PEGylation. Unless otherwise stated, in each experiment, the same batch of immobilized Con A adsorbents were employed to make the results reliable and comparable.

3.3.1. Effect of the molar ratio of mPEG-SPA to immobilized Concanavalin A on the degree of PEGylation

The degree of PEGylation also depends very much on the molar ratio of mPEG-SPA to immobilized Con A. Because of the hydrolysis of mPEG-SPA, an excess amount of mPEG-SPA was introduced during the modification of immobilized Con A. As demonstrated in Fig. 2(a), the degree of PEGylation increased sharply with increasing molar ratio of mPEG2k-SPA to immobilized Con A from 10:1 to 30:1 and then tapered off until reaching a constant level at a ratio of 50:1.

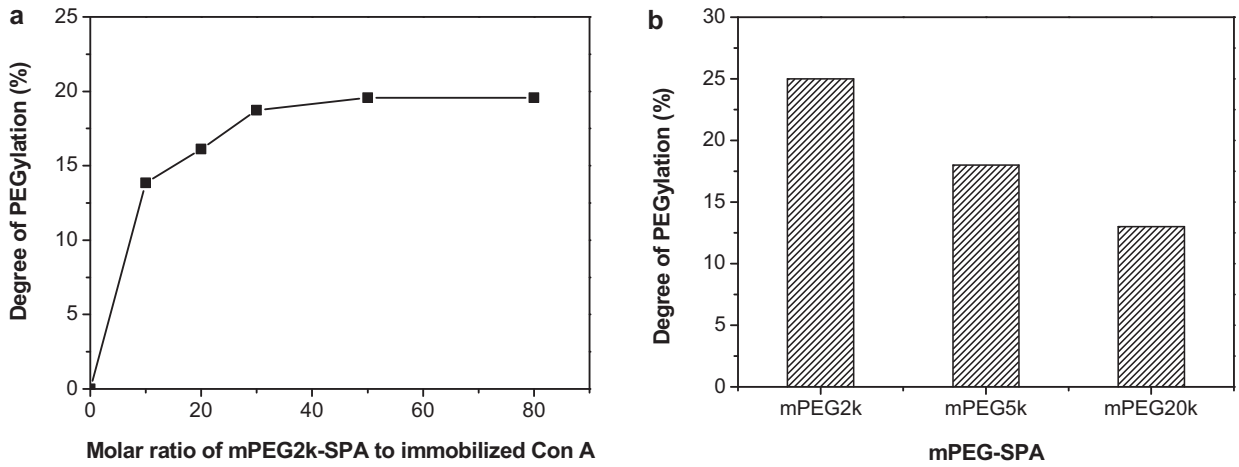

Fig. 2. PEGylation reaction studies. (a) Effect of the molar ratio of mPEG-SPA to immobilized Con A on the degree of PEGylation. Experimental conditions: variable amount of 1 mg/mL mPEG2k-SPA was dissolved in 0.1 M phosphate buffer, pH 8.0, and mixed with 0.15 mL immobilized Con A with a ligand density of 12 mg/mL for 2 h at room temperature. (b) Effect of the molecular weight of mPEG-SPA on the degree of PEGylation. mPEG-SPA was dissolved in 0.1 M phosphate buffer, pH 8.0, and mixed with immobilized Con A for 2 h at room temperature. The molar ratio of mPEG derivatives to immobilized Con A was 20:1.

3.3.2. Effect of the molecular weight of mPEG-SPA on the degree of PEGylation

To investigate the effect of the molecular weight of mPEG-SPA on the degree of PEGylation, mPEG2k-SPA, mPEG5k-SPA, and mPEG20k-SPA were selected to modify immobilized Con A with the molar ratio of mPEG-SPA to immobilized Con A as 20:1. As shown in Fig. 2(b), one may conclude that the degree of PEGylation of immobilized Con A is significantly influenced by the molecular weight of the mPEG chains. After 2 h reaction, the degree of PEGylation was about 25% for mPEG2k-SPA-Con A, while only 18%, and 13% for mPEG5k-SPA-Con A, and mPEG20k-SPA-Con A, respectively were reached. The effect of the length of mPEG chains on the degree of PEGylation was clearly caused by the molecular weight-dependent steric effect of mPEG derivatives. The accessibility of high molecular weight mPEG derivatives to the conjugation site of immobilized Con A could be more limited than that of low molecular weight mPEG derivatives. Thus under the same reaction conditions mPEG2k-SPA resulted in a higher degree of PEGylation to immobilized Con A than the other two higher molecular weight mPEG derivatives. Diwan and Park also observed the same phenomena in the modification of Interferon-α (IFN) with $mPEG_{2000}$-SPA and $mPEG_{5000}$-SSA [27]. In this case, the immobilization of Con A partially reduced the accessibility of the lysine-conjugation sites on the Con A surface caused by the big backbone volume of Toyopearl supports. mPEG-SPA had to overcome the diffusive resistance before the conjugation with Con A, which is highly related with the molecular size of mPEG-SPA. Therefore, it is not surprising that the degree of PEGylation of immobilized Con A is significantly affected by the molecular weight of mPEG-SPA.

3.4. Adsorption isotherms of glucose oxidase

Glucose oxidase was employed as target compound to study the adsorption isotherms and adsorption kinetics of native and PEGylated Toyopearl Con A. The results for the batch adsorption of GOD by native and PEGylated Toyopearl Con A adsorbents are shown in Fig. 3. The experimental data are modelled to the Langmuir isotherm. The graph proves that the data obtained follow the Langmuir isotherm. The corresponding fitted parameters are listed in Table 1. The dissociation constant K_d increased from 1.3×10^{-6} M (TC) to 2.4×10^{-6} M (TC-mPEG2k) and 3.6×10^{-6} M (TC-mPEG5k) when 25% and 16% of the free primary amine groups of immobilized Con A were modified by mPEG2k-SPA and mPEG5k-SPA, respectively. This indicates that the affinity interaction between GOD and PEGylated Con A was slightly lower than that between GOD and native Con A. However, this difference can be considered of little significance since it is similar to that observed by different preparations of native lectin [34]. Similarly, the studies of Liu et al. reflected that the coupling of Con A and mPEG-PVPAA did not impair the specificity of Con A [18]. Kim and Park found that PEGylation increased the binding affinity of glucose to Con A and preserved the binding affinity of allyl glucose to Con A when up to 5 mPEG molecules were coupled on Con A [17]. They also pointed out that only one lysine was involved in the binding sites of Con A according to its overall structure. Because the other lysine residues are far away from the saccharide binding sites, the PEGylated Con A is expected to preserve its binding activity after modification. Besides the location of the binding sites of lectins, the binding specificity can also be influenced by some other factors, such as PEGylation reaction conditions, PEGylation degree and the size of substrates employed [35]. Therefore, it will not be surprising if a slightly reduced binding affinity was found.

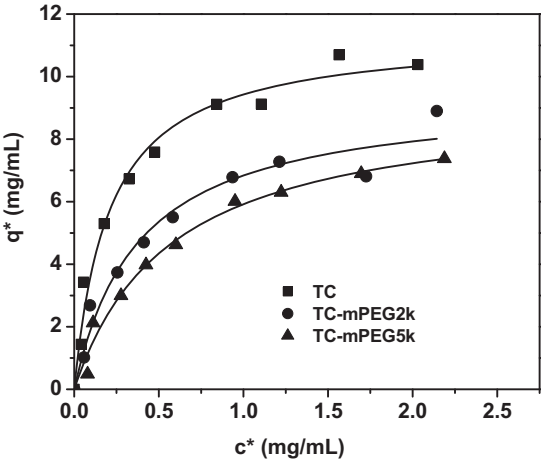

Fig. 3. Adsorption isotherms of GOD to native (TC) and PEGylated Toyopearl Con A affinity adsorbents. Experimental conditions: 0.1 M acetate buffer, pH 6.0, equilibrated for 15 h at $T = 25\,^{\circ}C$. Solid line: calculated from the Langmuir equation.

As can be found in Table 1, the maximum binding capacities of TC, TC-mPEG2k, and TC-mPEG5k were 11.4 mg/mL, 9.4 mg/mL, and 9.3 mg/mL, respectively. This indicates that the maximum binding capacity was somewhat reduced after PEGylation due to the steric hindrance caused by the introduced mPEG chains. Considering the large size of GOD (MW 160 kDa), the highly preserved binding capacity to PEGylated Con A (higher than 80% for both PEGylated Con A adsorbents) indicates that the degree of PEGylation is satisfactory for further technical application, such as affinity separations. Even though the degree of PEGylation of TC-mPEG5k (16%) was much lower than that of TC-mPEG2k (25%), the maximum adsorption capacity of TC-mPEG5k was similar to that of TC-mPEG2k. This is probably because that mPEG5k holds a larger hydrodynamic volume in contrast to mPEG2k and thus produces larger steric hindrance to the active binding sites.

3.5. Adsorption kinetics of glucose oxidase

The adsorption kinetics of GOD to native and PEGylated Toyopearl Con A affinity adsorbents were also investigated and the results are presented in Fig. 4. The data are modelled with an exponential decay of the form shown in Eq. (4) [36]:

$$\frac{c}{c_0} = a + be^{-t/\tau} \tag{4}$$

where c is the concentration of GOD in aqueous phase of the mixture at the evaluated time point (mg/mL); t is the adsorption time (min); a and b are constants and τ is the time constant.

Table 1
Parameters calculated from the Langmuir equation for different affinity adsorbents.

Matrix	Degree of PEGylation (%)	q_m (mg/mL)	K_d (mg/mL)	K_d (10^{-6} mol/L)	R^2
TC[a] (unPEGylated)	–	11.4 ± 0.5	0.21 ± 0.03	1.3 ± 0.2	0.984
TC-mPEG2k	25	9.4 ± 0.7	0.38 ± 0.09	2.4 ± 0.5	0.967
TC-mPEG5k	16	9.3 ± 0.5	0.57 ± 0.08	3.6 ± 0.5	0.986

[a] Hereafter Toyopearl Con A adsorbents can be abbreviated as TC.

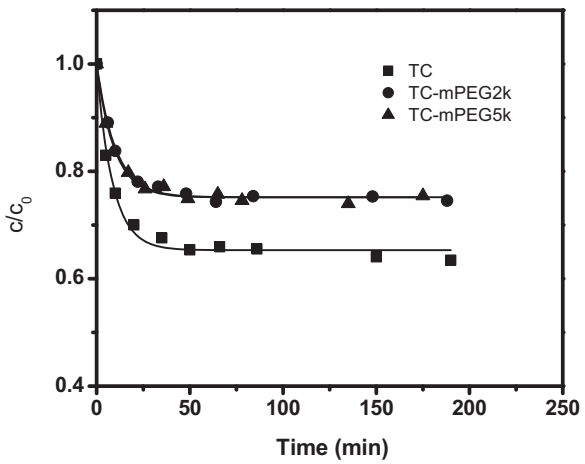

Fig. 4. Adsorption kinetics of GOD to native (TC) and PEGylated Toyopearl Con A affinity adsorbents. Experimental conditions: 30 mL of 0.386 mg/mL GOD in 0.1 M acetate buffer, pH 6.0, was mixed with about 0.75 mL Con A adsorbents with a ligand density of 13.4 mg/mL. $T = 25\,^{\circ}C$. Solid line: calculated from the first-order exponential decay form Eq. (4).

At the starting point of the adsorption experiment, namely $t = 0$, $c = c_0$, Eq. (4) can be written in the form of Eq. (5):

$$\frac{c_0}{c_0} = a + b = 1 \tag{5}$$

When the adsorption reaches the equilibrium, namely $c = c^*$, the following relation Eq. (6) establishes:

$$\frac{c*}{c_0} = a \tag{6}$$

Combining the Eqs. (1), (5) and (6), the equilibrium adsorption capacity q^* can be calculated from Eq. (7):

$$q^* = (c_0 - c^*)\frac{V_{GOD}}{V_{Ads}} = c_0 b \frac{V_{GOD}}{V_{Ads}} \tag{7}$$

The fitting data and calculated results are listed in Table 2. Fig. 4 shows that the adsorption of GOD onto both native and PEGylated Con A adsorbents was very fast; the equilibrium was reached after 50 min for all the three investigated Con A adsorbents. TC-mPEG2k and TC-mPEG5k exhibit very similar adsorption behaviour; their adsorption kinetic curves were partially overlapped. The modelling of the adsorption kinetic curves of different Con A adsorbents presents more information about the influence of PEGylation on the adsorption rate of GOD. As can be seen from the fitting data in Table 2, the time constant τ of adsorption kinetic curves increased after PEGylation. This indicates that PEGylation increased the diffusive resistance of the large GOD molecule onto the active binding sites of immobilized Con A. Thus it needs longer time for PEGylated Con A to reach the adsorption equilibrium. This result is in a good agreement with the fact that PEGylation is often utilized to prolong the circulation half-time of therapeutic proteins [16,17]. The longer equilibrium time for TC-mPEG2k when compared with that for TC-mPEG5k might be caused by its higher degree of PEGylation. The binding capacity of GOD calculated from Eq. (7) shows that PEGylation also reduced the binding capacity because of the steric hindrance of mPEG chains as indicated by the adsorption isotherm reactions. The parameter b reflects the binding rate. The experimental data reveal that initial adsorption velocity of both PEGylated Con A with the enzyme at the beginning is roughly 26% lower than with the unPEGylated immobilized lectin, which is due to the steric hindrance exhibited by the PEGylation. This reduction is relatively high, but will finally not constrain the employment of this method in technical processes.

Table 3
Purification of GOD by affinity chromatography onto different Con A adsorbents.

Con A adsorbents[a]	Molar ratio of mPEG to immobilized Con A	Degree of PEGylation (%)	Dynamic adsorption capacity of GOD (μg/mL)
TC	–	–	423 ± 12
TC-mPEG2k	3:1	15	416 ± 18
TC-mPEG2k	8:1	20	379 ± 2
TC-mPEG5k	3:1	13	319 ± 13
TC-mPEG5k	8:1	17	244 ± 15

[a] All the Con A adsorbents used here provide a ligand density of 6.5 mg/mL.

3.6. Affinity chromatography of glucose oxidase onto Concanavalin A adsorbents

It is necessary to study the adsorption performance of PEGylated Con A adsorbents by dynamic affinity chromatography processes since they are much nearer to practical separation applications as additionally hydrodynamic influences are encountered. About 1 mL of adsorbents were packed in a column to determine the dynamic adsorption capacity of GOD. In order to investigate the influence of the degree of PEGylation and the molecular weight of mPEG on the dynamic adsorption capacity, mPEG-SPA with molecular weight 2000, and 5000 was conjugated with immobilized Con A with varying molar ratio. 250 μL of 4 mg/mL GOD was applied to the column and each experiment was repeated 3 times. The results are summarized in Table 3. A similar tendency of dynamic adsorption capacity was obtained in accordance with the results observed by static adsorption experiments. Dynamic processes result mostly in a worse performance than static ones; the degree strongly depends on the operation conditions [4,37]. Here a pulse method was employed to apply GOD onto the analytical-scale affinity column. Since the aim of the dynamic adsorption experiment was to compare the dynamic adsorption behaviour before and after PEGylation, only 1 mg GOD (far less than the equilibrium adsorption capacity of immobilized Con A) was applied to the column. From the static adsorption kinetics experiment, one can see that the adsorption capacity at 10 min reached about 67% of the equilibrium adsorption capacity when GOD concentration was 0.386 mg/mL. The adsorption capacity should be higher than 67% of the equilibrium adsorption capacity when the applied GOD concentration was increased to 4 mg/mL in a dynamic adsorption experiment. Therefore, 10 min residence time should be satisfactory for the dynamic adsorption experiment. Table 3 shows that the dynamic adsorption capacity of GOD was also reduced after PEGylation. For TC-mPEG2k, 98%, and 89% of the adsorption capacity was preserved with a degree of PEGylation of 15%, and 20%, respectively. In case of TC-mPEG5k, only 75%, and 57% of the adsorption capacity was maintained with a degree of PEGylation of 13%, and 17%, respectively. The dynamic adsorption capacity studies show that

Table 2
Parameters estimated from the adsorption kinetics of GOD onto different affinity adsorbents.

Matrix	Degree of PEGylation (%)	b	q^* (mg/mL)	τ	R^2
TC	–	0.34 ± 0.01	5.3 ± 0.2	8.58 ± 0.79	0.990
TC-mPEG2k	25	0.25 ± 0.01	3.8 ± 0.2	10.13 ± 0.62	0.995
TC-mPEG5k	16	0.25 ± 0.01	3.8 ± 0.2	9.61 ± 0.91	0.992

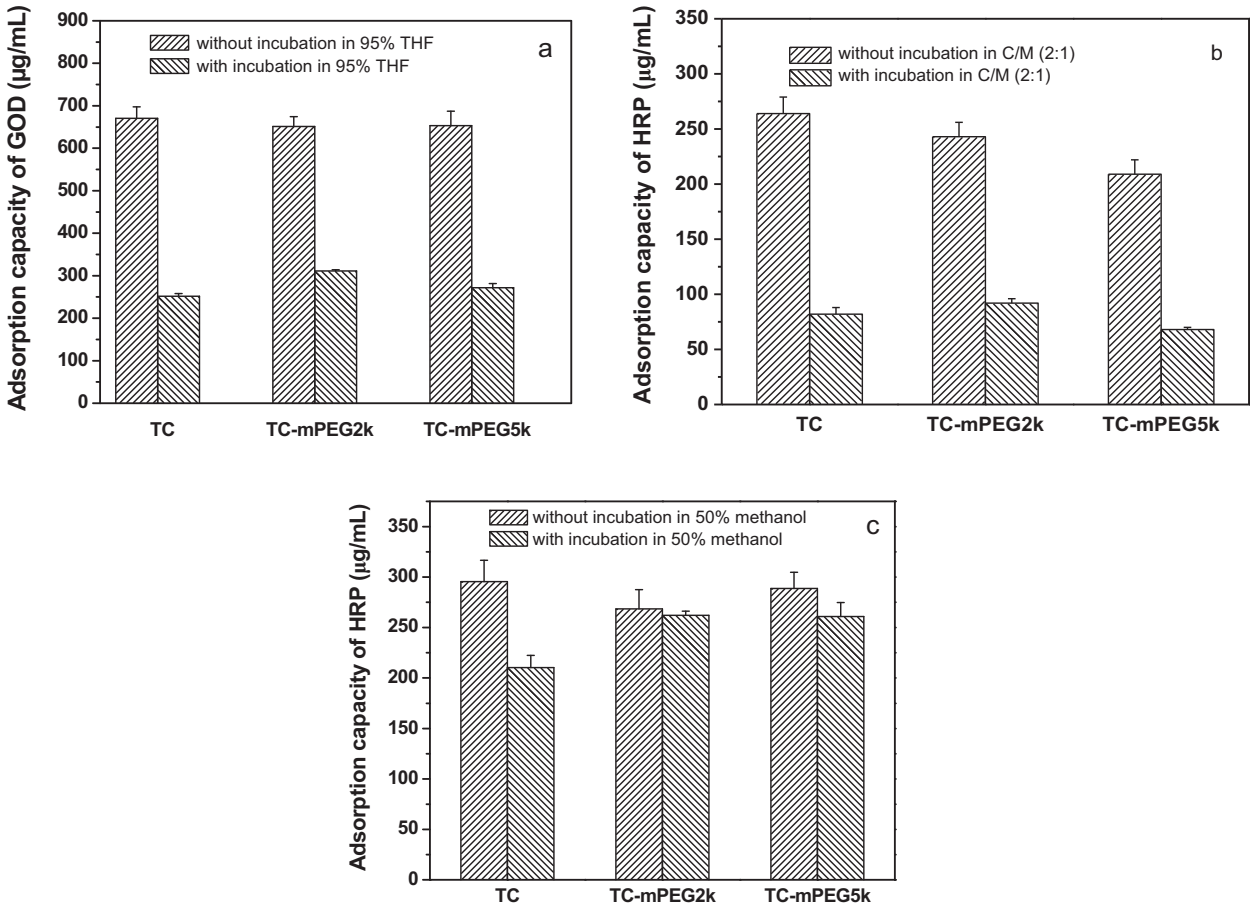

Fig. 5. (a) Adsorption capacities of GOD onto Toyopearl Con A before and after incubation in 95% THF acetate buffer solution for 30 min. The ligand density of all Con A adsorbents was 6.2 mg/mL. The molar ratio of mPEG derivatives to Con A was 1.25:1. (b) Adsorption capacities of HRP onto Toyopearl Con A adsorbents before and after incubation in C/M (2:1) for 30 min. The ligand density of all Con A adsorbents was 9.9 mg/mL. The molar ratio of mPEG derivatives to Con A was 2:1. (c) Adsorption capacities of HRP onto Toyopearl Con A adsorbents before and after incubation in 50% methanol for 30 min. The ligand density of all Con A adsorbents was 7.5 mg/mL. The molar ratio of mPEG derivatives to Con A was 1.25:1.

TC-mPEG2k exhibits much better adsorption performance than TC-mPEG5k. Especially when considering its high degree of PEGylation and the large molecular size of GOD, the dynamic adsorption capacity of TC-mPEG2k was quite satisfactory. In contrary, TC-mPEG5k showed a dramatic reduction of its dynamic adsorption capacity. Possible explanations might be that the contact time was not sufficient for TC-mPEG5k to bind GOD due to the slower diffusion rate, or because of the larger mPEG molecules immobilized onto the ligand, the substrate is unable to penetrate into the binding pocket to reach the binding sites. Therefore, TC-mPEG2k with an appropriate degree of PEGylation could be suggested as a satisfactory adsorbent for affinity separation, which also meets the required high internal transport rates.

3.7. Stability against organic solvents

On the basis of the observation that PEGylation could highly preserve the binding specificity and binding capacity of modified Con A, further investigations of the stability of PEGylated Con A against organic solvents and high temperature were performed. Lectin based affinity chromatography is particularly useful in aqueous solutions for the separation of glycoconjugates. Whereas the separation of micelle forming aggregates such as glycolipids by this aqueous method is difficult due to the formation of mixed glycolipid micelles. This problem might be resolved by using affinity separation in organic solvent–water mixture, which can conserve the carbohydrate-binding specificity of the lectin while eliminating glycolipid micelle formation [38,39]. However, this observation is only possible for fairly stable lectins. In case of other more unstable lectins, such as Con A, an organic solvent–water mixture can dramatically decrease their binding activity due to the instability of the lectin. It was reported that PEGylation could increase the stability of modified proteins in organic solvents [27,40,41]. On the basis of these observations, PEGylated Con A was expected to exhibit the ability to improve its stability in organic solvent–water mixture for the separation of intact glycolipids. 95% tetrahydrofuran (THF), chloroform/methanol (2:1, v/v), and 50% methanol were chosen as test media because these organic solvents are frequently employed in the extraction of glycolipids from natural sources. To make the detection easier, GOD or HRP (as an alternative glycosylated enzyme for the investigation) was applied as target compound to study the stability of PEGylated Con A in various organic media.

3.7.1. Stability against 95% THF buffer solution

It was reported 95% THF in water could disrupt the formation of mixed glycolipid micelles and thus induce single pure glycolipid molecule [39]. In order to employ this capability and to ensure at the same time the activity of Con A, the protein was PEGylated and added to the 95% THF solution, which was prepared in buffer A for the incubation of Con A adsorbents. The same batch of adsorbents but without incubation was applied as a control to measure the adsorption capacity of GOD. Fig. 5(a) shows the GOD adsorption capacity before and after 95% THF incubation. The adsorption capacity of GOD was merely slightly reduced after

PEGylation with good agreement with the observation described before. After incubation in 95% THF, the adsorption capacity of GOD was reduced from 670 μg/mL, 651 μg/mL, and 653 μg/mL to 252 μg/mL, 311 μg/mL, and 272 μg/mL for TC, TC-mPEG2k, and TC-mPEG5k, respectively. All the adsorption capacities were greatly reduced after incubation. However, the results show that the residual adsorption capacity was higher for both PEGylated Con A than that for unPEGylated Con A. For instance, 48% of the original adsorption capacity of GOD was kept for TC-mPEG2k, but only 37% for TC. This indicates that PEGylation could apparently improve the stability of Con A with the exposure to 95% THF.

3.7.2. Stability against chloroform/methanol (2:1)

Chloroform/methanol (C/M) (2:1, v/v) mixture is one of the most effective organic solvents for the extraction of glycolipids from natural sources, which was first developed by Folch [42]. The stability of PEGylated Con A against C/M (2:1) was investigated. The results are given in Fig. 5(b). First, the adsorption capacities of HRP onto Con A adsorbents were examined in buffer A. It was found that the PEGylation reduced the adsorption capacity of HRP a bit more than that of GOD. This might be caused by the higher degree of PEGylation (the molar ratio of mPEG derivatives to immobilized Con A is 2:1 for Fig. 5(b), while 1.25:1 for Fig. 5(a)), which can induce a larger shielding effect for the adsorption of HRP to modified Con A. Fig. 5(b) also shows that with the incubation in C/M (2:1) for 30 min, all Con A adsorbents proved significantly reduced adsorption capacities of HRP. However, PEGylated Con A, especially TC-mPEG2k, still possessed a higher residual adsorption capacity, which is 31% for TC and 38% for TC-mPEG2k when compared with their respective adsorption capacity without incubation in C/M.

3.7.3. Stability against methanol buffer solution

The stability of PEGylated Con A against methanol was also investigated as this extractant is often applied in biotechnological downstream processing. Fig. 5(c) presents the high stability of PEGylated Con A against 50% methanol in buffer A. As can be seen, the adsorption capacity of HRP onto PEGylated Con A was much higher than that onto unPEGylated Con A after incubation in 50% methanol. TC-mPEG2k and TC-mPEG5k maintained 98%, and 90%, respectively of their original adsorption capacities. However, only 71% of the original capacity was observed for TC. The investigated PEGylated Con A also showed much higher stability against 80% methanol compared with unPEGylated Con A. For example, with the incubation in 80% methanol for 30 min, the dynamic adsorption capacity of HRP was 99% preserved for TC-mPEG2k and 74% for TC (data not shown).

In conclusion, the above results reveal that PEGylated Con A exhibited obviously better stability against the organic solvents investigated (95% THF, C/M 2:1 and different methanol concentrations) than native immobilized Con A while the adsorption capacity was mostly maintained during the PEGylation process. The improvement of the stability of PEGylated Con A is more impressive after exposure to methanol buffer solutions than to 95% THF and to C/M 2:1. This should be highly related to the physicochemical properties of the organic solvents employed, which might result in different influence to the investigated system (such as the swelling degree of Toyopearl adsorbents as well as the degree of denature of the immobilized Con A). The three-dimensional structure of PEGylated HRP dissolved in benzene and toluene was similar to that in aqueous solutions [43] should be a quite good explanation for the high stability and activity of PEGylated enzyme in neat organic solvents. The enhanced stability in other organic solvents is also published for PEGylated proteins. For instance, Diwan and Park found that PEGylated Lysozyme and recombinant Interferon-α showed better stability against the exposure to dichloromethane during encapsulation [27]. PEGylated cellulase

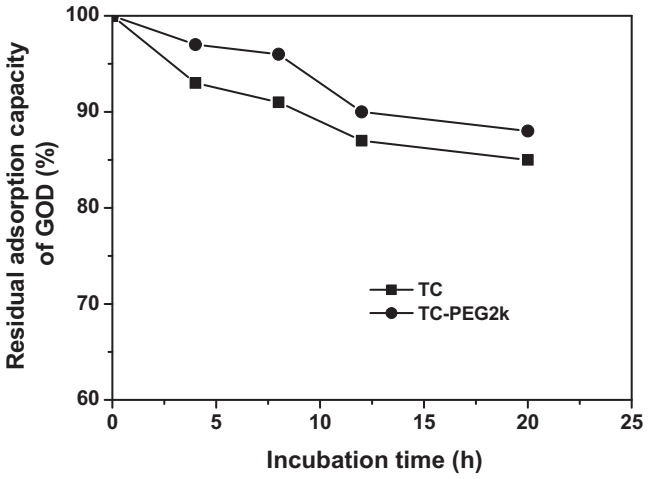

Fig. 6. Residual adsorption capacity of GOD onto Toyopearl Con A adsorbents after incubation at 55 °C for different periods of time. The ligand density of TC and TC-mPEG2k was 12.5 mg/mL, the degree of PEGylation of TC-mPEG2k was 20%.

exhibited greater stability in aqueous acetone and ethylalcohol than native cellulase [40]. Although this behaviour is frequently observed in protein (especially enzyme) PEGylation, the mechanism has not been clearly elucidated. Park and Kajiuchi [40] suggest a buffering action of mPEG chains modified onto enzyme surfaces. In our study, the stabilizing effect is likely due to the mPEG chains, which generate a hydrophilic environment for modified Con A, and thus create a buffering action against denaturation of the lectin protein in the background of the organic solvent. According to Combotz and Pettit [44], PEG is soluble in water and three water molecules are associated with one ethylene oxide unit of PEG to form hydrogen bonds. These water molecules are expected to form a protective hydration shell around PEG, which provide a hydrophilic microenvironment and thus also for the modified Con A in the water-organic solvents mixture. In addition, the introduction of mPEG chains could induce more hydrogen bonds or changes of the hydrophobic properties within modified Con A molecules and thus produce a more stable structure against the exposure to organic solvents.

3.8. Stability against temperature

Enhanced temperature is also often employed for affinity separation process such as in order to elute the tightly bond target molecules. Therefore, the stability of PEGylated Con A against temperature was also investigated in this work. Fig. 6 presents the residual adsorption capacity of GOD onto TC and TC-mPEG2k after incubation at 55 °C for different periods of time. PEGylated Con A always displayed slightly higher residual adsorption capacities (3–5%) than unPEGylated Con A, which indicates a higher stability of PEGylated Con A for at least up to an incubation time of 20 h and at high temperature. Previous works showed that PEGylation is an appropriate way to increase enzyme thermal stability [45]. The full explanation for improved thermostability by PEGylation remains uncertain. Some researchers proposed that PEG modification of proteins improved their thermostability due to the decreased structure mobility causing a decrease of the unfolding rate [10,46]. In fact, hydrophobic and electrostatic properties play an important role in determining the thermostability of proteins. Therefore, the change of hydrophobic and electrostatic properties of an enzyme surface has been applied to explain the thermal stabilization effect caused by PEG modification [47,48]. In addition, Longo and Combes proposed that the decreased thermal denaturation rate of α-chymotrypsin might result from the

increase of hydrophilicity of the enzyme's surface caused by PEG chains [49].

4. Conclusions

PEGylation of immobilized Con A and its adsorption behaviour in the affinity adsorption of GOD were investigated in this study. Immobilized Con A onto Toyopearl adsorbents was modified with mPEG-SPA with molecular weights of 2000, 5000, and 20,000 g/mol. A new method, ninhydrin method, was first developed to determine the degree of PEGylation of immobilized Con A, which has proved to be easy applicable and reproducible. The PEGylation reaction was studied in detail to elucidate how the parameters such as molar ratio of mPEG-SPA to Con A and molecular weight of mPEG-SPA affect the degree of PEGylation. The adsorption isotherms of GOD onto native and PEGylated Con A adsorbents showed that the modification did not alter substantially the specificity of the carbohydrate binding ability of Con A. However, the binding capacity for GOD was slightly reduced probably due to the steric hindrance caused by mPEG chains. The adsorption kinetic studies revealed the lower adsorption rate after PEGylation which was still attributed to the steric effect. The dynamic adsorption capacity for modified Con A depended very much on the degree of PEGylation and the molecular weight of mPEG derivatives. The adsorption capacity could be highly preserved for TC-mPEG2k (90% of the original adsorption capacity) even with a degree of PEGylation up to 20%. Conjugation of Con A with mPEG2k has shown better adsorption performance thus has greater potential for biotechnological application compared with mPEG5k. The adsorption properties of PEGylated Con A against some harsh operational conditions encountered in protein processing were also investigated. Studies prove that PEGylated Con A exhibited obviously higher stability against the exposure to organic solvents and high temperature. The reason for improved stability was investigated. The fact that PEGylation stabilizes the properties of Con A may greatly expand the range of applications of unstable proteins to bioprocessing (e.g. biocatalysis and downstream separation) as well as other protein applications (e.g. medication, industrial use, etc.). The drawbacks combined with the PEGylation procedure are often negligible and at relatively unimportant times (e.g. initial adsorption rate) for process design.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.04.018.

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