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Preparation and characterization of PEGylated 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. Colorimetric method using ninhydrin to determine the PEGylated degree was first proposed in this study, which has proved to be easy applicable and reproducible. The PEGylation reaction was studied in detail to elucidate how the parameters such as reaction time, pH value, molar ratio of mPEG-SPA to Con A, and molecular weight of mPEG-SPA affect the PEGylated degree. 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. 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 PEGylated degree 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 the PEGylated degree up to 20%. Studies show that PEGylated Con A not only highly preserved their binding capacities under mild process conditions, but also exhibited obviously higher stability against rigorous 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 the application in affinity separation process 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.).

Keywords: Protein stability; PEGylation; Concanavalin A; Glucose oxidase; Affinity adsorption

1. Introduction

Affinity chromatography has proved to be of great significance for the separation and purification of biological macromolecules in the 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 such as Con A [7] and the instability of their protein-based structure under some rigorous operation conditions, which may involve the presence of organic solvents or the evaluated temperatures. The employment of more stable ligands is expected to facilitate their extensive industrial application by 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 process cannot be used as a general method to be applied to other proteins [10]. On the other hand, chemical modification seems to be a more universal approach to improve intrinsic properties of proteins for which especially deeper knowledge of gene or protein structure is not required. Several chemical methods have been employed 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 protein 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. Proteins conjugation with PEG, linear or branched, via a covalent linkage can eliminate some drawbacks of native proteins and improve their physicochemical, biomedical and pharmacological

87 properties. PEGylation often induces a protein with improved solubility and
88 temperature stability, enhanced stability against enzymatic degradation, increased
89 serum half-life and anti-tumour potency, decreased renal clearance and
90 immunogenicity, while normally maintaining a high percentage of its biological
91 activity [13-16]. It has to be pointed out that although the application of PEGylation
92 has been extensively studied, the mechanism of these effects caused by PEG chains is
93 still not fully understood and well accepted.
94 This work will take the jack bean lectin, Concanavalin A (Con A), as an example to
95 study the PEGylation of proteinaceous affinity ligand and the influence of
96 modification on its adsorption performance in affinity separation process. PEGylation
97 of Con A has been studied by several authors for various purposes. Ueno et al.
98 investigated the *in vivo* induction of anti-tumor cytotoxicity in mice by the treatment
99 with Con A modified with PEG. They found that PEGylated Con A exhibited reduced
100 immunogenicity and prolonged clearance time in mice blood [15]. Kim and Park used
101 Con A for delivery of modulated insulin in sol-gel phase-reverse hydrogels system,
102 and found PEGylated Con A held improved aqueous solubility, enhanced long-term
103 stability, and higher glucose sensitivity compared to native Con A [17]. Liu et al.
104 studied a Con A based glucose-responsive insulin delivery system, which could be
105 used for long-term diabetes treatment. They found the conjugation of Con A with
106 PEG grafted by hydrophilic poly (vinylpyrrolidone-*co*-acrylic acid) (PVPAA)
107 exhibited substantially improved solubility at pH 7.4 while preserved its sugar binding
108 characteristics [18]. On the basis of 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 PEGylated degree by ninhydrin was first proposed in this study and the parameters influencing the PEGylated degree 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 kinetics 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 capacity of GOD under both normal conditions 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 (POD) (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, 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 derivative (mPEG-SPA)

Immobilized Con A was conjugated 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 additive of methyl-α-D-mannopyranoside presence 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-SPA are 2,000 g/mol, 5,000 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 PEGylated degree 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 PEGylated degree is actually the comparison of the amount of the 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. The typical ninhydrin reaction with amino acid is shown in Fig. 1.

Fig. 1

The ninhydrin method was developed to determine the PEGylated degree in this work. A typical procedure was carried out as follows:

The PEGylated and unPEGylated Con A adsorbents were sucked 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 λ= 570 nm was measured (UV spectrophotometer, Carl Zeiss, Jena, Germany). The same mixture without affinity adsorbents was taken as blank. The PEGylated degree 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 the speed of 150 rpm. After the adsorbents were settled by centrifugation, the supernatant obtained from each tube was used 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).

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

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).

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

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

40 ml of 0.4 mg/ml GOD was mixed with approximate 1.2 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

Typical 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 in 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 POD 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 PEGylated degree

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. 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 are 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 operation units. The scheme of PEGylation reaction is shown in Fig. 2.

Fig. 2

The extent of the PEGylated degree 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 extent of 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 PEGylated degree 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 PEGylated degree

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 PEGylated degree 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 [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 PEGylated degree 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 the indication in Fig. 2), the PEGylated degree 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 PEGylated degree according to the procedure mentioned in the experimental section.

A good example for the determination of the PEGylated degree is shown in Fig. 3. 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

Fig. 3

plots are close to 1, which shows a very good reproducibility. The estimated PEGylated degree of immobilized Con A in the example is 24%, calculated from the ratio of the slopes of the plots according to Eq. (3).

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

where *PD* is the PEGylated degree, 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 producible even though it requires a relatively large amount of samples (magnitude of milligram).

It should be pointed out that the PEGylated degree is just a mean value due to the heterogeneity in lysine substitution. Even for the same PEGylated degree, 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. In this section, the results of the reaction time, reaction pH value, 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 immobilized Con A adsorbents were used to make the results reliable and comparable.

3.3.1 Effect of reaction time on the PEGylated degree

Since longer reaction time could influence the binding activity of Con A, it is necessary to study the PEGylation kinetics. About 0.6 ml of 9.4 mg/ml Toyopearl Con A adsorbents was measured for five times and then placed into different tubes. 1.4 ml of 1 mg/ml mPEG2k-SPA was quickly dissolved in 0.1 M phosphate buffer, pH 8.0, and mixed with Toyopearl Con A with gentle shaking at room temperature. The reaction for each tube was stopped after different period of time by the introduction of 0.1 M acetate buffer, pH 4.0. The PEGylation degree against reaction time was determined and the result is shown in Fig. 4(a). As can be seen, the PEGylated degree linearly increased in the first 30 min. Then the reaction rate was decreased till it

Fig. 4(a)

312 reached to a constant level (with the PEGylated degree of 18%) after 2 h. Therefore, 2
313 h was selected as the optimal reaction time for the modification of immobilized Con
314 A since a further increase in the reaction time did not cause a significant increase of
315 the PEGylated degree.

316 **3.3.2 Effect of pH value on the PEGylated degree**

317 0.1 M phosphate buffer with pH 6.0, 7.0, and 8.0 were selected to study the effect of
318 the pH value on the PEGylated degree. As shown in Fig. 4(b), the pH value of the
319 reaction medium greatly influenced the PEGylated degree as expected. Keeping the
320 other conditions constant, the higher PEGylated degree was obtained at higher pH
321 values. For example, the PEGylated degree of immobilized Con A was 20% at pH
322 8.0, while only 12% conversion degree was found at pH 6.0. The reason is that the
323 conjugation of mPEG derivative to Con A requires a nucleophilic attack of
324 unprotonated amine groups to succinimidyl groups in mPEG-SPA [34]. The similar
325 phenomenon has also been observed by Kim during the PEGylation of recombinant
326 human epidermal growth factor (rhEGF) [35]. Con A still shows high activity at pH
327 9.5 [36]. Thus in this work pH 8.0 was chosen for the PEGylation reactions if not
328 stated otherwise.

Fig. 4(b)

329 **3.3.3 Effect of the molar ratio of mPEG-SPA to immobilized Concanavalin A on**
330 **the PEGylated degree**

The PEGylated degree 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 the immobilized Con A. As demonstrated in Fig. 4(c), the PEGylated degree was increased sharply with increasing the molar ratio of mPEG2k-SPA to immobilized Con A from 10:1 to 30:1 and then tapered off till reached a constant lever at the ratio of 50:1. Further increasing the molar ratio of two reactants did not make any sense for increasing the PEGylated degree.

Fig. 4(c)

3.3.4 Effect of the molecular weight of mPEG-SPA on the PEGylated degree

To investigate the effect of the molecular weight of mPEG-SPA on the PEGylated degree, 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. 4(d), one may conclude that the PEGylated degree of immobilized Con A is significantly influenced by the molecular weight of the mPEG chains. After 2 h reaction, the PEGylated degree was about 25% for mPEG2k-SPA-Con A, whilst only 18%, and 13% for mPEG5k-SPA-Con A, and mPEG20k-SPA-Con A, respectively. The effect of the length of mPEG chains on the PEGylated degree 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

Fig. 4(d)

mPEG2k-SPA resulted in a higher PEGylated degree 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 conquer 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 PEGylated degree 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. 5. The experimental data are fitted to the Langmuir isotherm. The graph proves that the data obtained, obey well to 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

Fig. 5

Table 1

373 slightly lower than that between GOD and native Con A. However, this difference
374 can be considered of little significance since it is similar to that observed by different
375 preparations of native lectin [37]. Similarly, the studies of Liu et al. reflected that the
376 coupling of Con A and mPEG-PVPAA did not impair the specificity of Con A [18].
377 Kim and Park found that PEGylation increased the binding affinity of glucose to Con
378 A and preserved the binding affinity of allyl glucose to Con A when up to 5 mPEG
379 molecules were coupled on Con A [17]. They also pointed out that only one lysine
380 was involved in the binding sites of Con A according to its primary structure.
381 Because the other lysine residues are far away from the saccharide binding sites, the
382 PEGylated Con A is expected to preserve its binding activity after modification.
383 Besides the location of binding sites of lectins, the binding specificity can also be
384 influenced by some other factors, such as PEGylation reaction conditions,
385 PEGylation degrees and the size of substrates employed [38]. Therefore, it will not
386 be surprising if a slightly reduced binding affinity was found.

387 As can be found in Table 1, the maximum binding capacities of TC, TC-mPEG2k,
388 and TC-mPEG5k were 11.4 mg/ml, 9.43 mg/ml and 9.28 mg/ml, respectively. This
389 indicates that the maximum binding capacity was somewhat reduced after
390 PEGylation due to the steric hindrance caused by the introduced mPEG chains.
391 Considering the large size of GOD (MW 160 kDa), the highly preserved binding
392 capacity to PEGylated Con A (higher than 80% for both PEGylated Con A
393 adsorbents) indicates that the PEGylated degrees are satisfactory for the further
394 affinity separations. Even though the PEGylated degree of TC-mPEG5k (16%) was

much lower than that of TC-mPEG2k (25%), the maximum adsorption capacity of TC-mPEG5k was lower than 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. 6. The data are modelled with an exponential decay of the form shown in Eq. (4) [39]:

Fig. 6

$$c/c_0 = a + be^{-t/\tau} \quad (4)$$

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. The parameter b is the slope of the curve at various times and expresses the velocities of adsorption versus desorption rates. At the beginning it only gives an account of the adsorption rate, as the desorption velocity is zero, there. 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):

$$c_0/c_0 = a + b = 1 \quad (5)$$

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

$$c^*/c_0 = a \quad (6)$$

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

$$q^* = (c_0 - c^*)V_{GOD} / V_{ads} = c_0 b V_{GOD} / V_{ads} \quad (7)$$

The fitting data and calculated results are listed in Table 2. Fig. 6 shows that the adsorption of GOD onto both native and PEGylated Con A adsorbents were very fast, the equilibrium were reached after 50 min for all the three investigated Con A adsorbents. TC-mPEG2k and TC-mPEG5k exhibit very similar adsorption behavior; 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 PEGylated degree. 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 ConA with the enzyme at

Table 2

437 the is roughly 26% lower than with the unPEGylated immobilized lectin, which is
438 due to the steric hindrance exhibited by the PEGylation. This reduction is relatively
439 high, but will finally not constrain the employment of this method in technical
440 processes.

441 **3.6 Affinity chromatography of glucose oxidase onto Concanavalin A adsorbents**

442 It is necessary to study the adsorption performance of PEGylated Con A adsorbents
443 by dynamic affinity chromatography processes since they are much nearer to practical
444 separation applications as additionally hydrodynamic influences are encountered.
445 About 1 ml adsorbents was packed in a column to determine the dynamic adsorption
446 capacity of GOD. In order to investigate the influence of the PEGylated degree and
447 the molecular weight of mPEG on the dynamic adsorption capacity, mPEG-SPA with
448 molecular weight 2000, and 5000 was conjugated with immobilized Con A with
449 varying molar ratio. 1 mg GOD was applied to the column and each experiment was
450 repeated for 3 times. The results are summarized in Table 3. A similar tendency of
451 dynamic adsorption capacity was obtained in accordance with the results observed by
452 static adsorption experiments. Dynamic processes result mostly in a worse
453 performance than a static one; the degree strongly depends on the operation
454 conditions [4,40]. The dynamic adsorption capacity of GOD was also reduced after
455 PEGylation. For TC-mPEG2k, 98%, and 89% of the adsorption capacity was
456 preserved with a PEGylated degree of 15%, and 20%, respectively. In case of
457 TC-mPEG5k, only 75%, and 57% of the adsorption capacity was maintained with a

Table 3

458 PEGylated degree of 13%, and 17%, respectively. The dynamic adsorption capacity
459 studies show that TC-mPEG2k exhibits much better adsorption performance than
460 TC-mPEG5k. Especially when considering its high PEGylated degree and the large
461 molecular size of GOD, the dynamic adsorption capacity of TC-mPEG2k was quite
462 satisfactory. In contrary, TC-mPEG5k showed a dramatic reduction of its dynamic
463 adsorption capacity. Possible explanations might be that the contact time was not
464 sufficient for TC-mPEG5k to bind GOD due to the slower diffusion rate; or according
465 to the larger mPEG molecules immobilized onto the ligand, the substrate is unable to
466 penetrate into the binding pocket to reach the binding sites. Therefore, TC-mPEG2k
467 with an appropriate PEGylated degree could be suggested as a satisfactory adsorbent
468 for affinity separation, which also meets the required high internal transport rates.

469 **3.7 Stability against organic solvents**

470 On the basis of the observation that PEGylation could highly preserve the binding
471 specificity and binding capacity of modified Con A, further investigations of the
472 stability of PEGylated Con A against organic solvents and high temperature were
473 performed. Lectin based affinity chromatography is particularly useful in aqueous
474 solutions for the separation of glycoconjugates. Whereas the separation of micelle
475 forming aggregates such as glycolipids by this aqueous method is difficult due to the
476 formation of mixed glycolipid micelles. This problem might be resolved by using
477 affinity separation in organic solvent-water mixture, which can conserve the
478 carbohydrate-binding specificity of the lectin while eliminating glycolipid micelle

formation [41,42]. However, this observation is only possible for fairly stable lectins. In case of other more unstable lectins, such as Con A, organic solvent-water mixture can dramatically decrease their binding activity due to the instabilization of lectin. It was reported that PEGylation could increase the stability of modified proteins in organic solvents [27,43,44]. 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 POD (for investigating another glycosylated enzyme) 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 induce intact glycolipids and disrupt the micelle structure [42]. 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 control to measure the adsorption capacity of GOD. Fig. 7(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.

Fig. 7(a)

After incubation in 95% THF, the adsorption capacity of GOD was reduced from 0.67 mg/ml, 0.65 mg/ml, and 0.65 mg/ml to 0.25 mg/ml, 0.31 mg/ml, and 0.27 mg/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 [45]. The stability of PEGylated Con A against C/M (2:1) was investigated. The results are given in Fig. 7(b). First, the adsorption capacities of POD onto Con A adsorbents were examined in buffer A. It was found that the PEGylation reduced the adsorption capacity of POD a bit more than that of GOD. This might be caused by the higher PEGylated degree (the molar ratio of mPEG derivatives to immobilized Con A is 2:1 for Fig. 7 (b), while 1.25:1 for Fig. 7(a)), which can induce a larger shielding effect for the adsorption of POD to modified Con A. Fig. 7(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 POD. However, PEGylated Con A, especially TC-mPEG2k, still possessed a higher residual adsorption capacity, which is

Fig. 7(b)

521 31% for TC and 38% for TC-mPEG2k when compared with their respective
522 adsorption capacity without incubation in C/M.

523 **3.7.3 Stability against methanol buffer solution**

524 The stability of PEGylated Con A against methanol was also investigated as this
525 extractant is often applied in biotechnological downstream processing. Fig. 7(c)
526 presents the high stability of PEGylated Con A against 50% methanol in buffer A. As
527 can be seen, the adsorption capacity of POD onto PEGylated Con A was much higher
528 than that onto unPEGylated Con A after incubation in 50% methanol. TC-mPEG2k
529 and TC-mPEG5k maintained 98%, and 90%, respectively of their original adsorption
530 capacities. However, only 71% was reserved for TC. The investigated PEGylated Con
531 A also showed much higher stability against 80% methanol compared with
532 unPEGylated Con A. For example, with the incubation in 80% methanol for 30 min,
533 the dynamic adsorption capacity of POD was 99% preserved for TC-mPEG2k and
534 74% for TC.

Fig. 7(c)

535 In conclusion, the above results reveal that PEGylated Con A exhibited obviously
536 better stability against the organic solvents investigated (95% THF, C/M 2:1 and
537 different methanol concentrations) than native Con A while the adsorption capacity
538 was mostly maintained during the PEGylation process. The enhanced stability in other
539 organic solvents is already published for PEGylated proteins. For instance, Diwan and
540 Park found that PEGylated Lysozyme and recombinant Interferon-α showed better
541 stability against the exposure to dichloromethane during encapsulation [27].

PEGylated cellulase exhibited greater stability in aqueous acetone and ethylalcohol than native cellulase [43]. Although this behaviour is frequently observed in protein (especially enzyme) PEGylation, the mechanism has not been clearly elucidated. Park and Kajiuchi [43] 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 [46], 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. 8 presents the residual adsorption capacity of GOD onto TC and TC-mPEG2k after

Fig. 8

562 incubation at 55 °C for different periods of time. The data of inactivation kinetics are
563 also fitted with the exponential decay similar to Eq. (4):

564 $$\frac{Q'}{Q_0} = a' + b' e^{-t'/\tau} \qquad (8)$$

565 Where Q' is the adsorption capacity of GOD onto Con A adsorbents after incubation
566 at 55 °C at the evaluated time point (mg/ml); Q_0 is the adsorption capacity of GOD
567 onto Con A adsorbents without incubation at high temperature (mg/ml); *t'* is the
568 incubation time (h); *a'* and *b'* are constants and τ is still the time constant.

569 The modelled data are listed in Table 4. The time constant τ of inactivation kinetics
570 increased after PEGylation, indicating the inactivation reaction rate of PEGylated Con
571 A was slower than that of native Con A. The data also prove ccc Fig. 8 shows that
572 PEGylated Con A always displayed slightly higher residual adsorption capacities (3 to
573 5%) than unPEGylated Con A, which indicates a higher stability of PEGylated Con A
574 for at least up to an incubation time of 20 h and at high temperature. Previous works
575 showed that PEGylation is an appropriate way to increase enzyme thermal stability
576 [47]. The full explanation for improved thermostability by PEGylation is also
577 versatile and remains uncertainty. Some researchers proposed that PEG modification
578 of proteins improved their thermostability due to the decreased structure mobility
579 causing a decrease of the unfolding rate [48,49]. In fact, hydrophobic and electrostatic
580 properties play an important role in determining the thermostability of proteins.
581 Therefore, the change of hydrophobic and electrostatic properties of an enzyme

Table 4

surface has been applied to explain the thermal stabilization effect caused by PEG modification [50,51]. 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 [52].

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 were modified with mPEG-SPA with molecular weights of 2,000, 5,000, and 20,000 g/mol. A new method, ninhydrin method, was first developed to determine the PEGylated degree 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 reaction time, pH value, molar ratio of mPEG-SPA to Con A, and molecular weight of mPEG-SPA affect the PEGylated degree. 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 PEGylated degree 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 PEGylated degree up to 20%. Conjugation of Con A with mPEG2k has shown better adsorption performance thus has greater potential for the biotechnological application compared with mPEG5k. The adsorption properties of PEGylated Con A against some harsh operational conditions encountered in protein processing and application were also investigated. Studies prove that PEGylated Con A exhibited obviouslyhigher stability against the exposure to organic solvents and high temperature. The reason for improved stability was shortly discussed. 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 minor times (e.g. initial adsorption rate) to be considered for process design. But at no point they prohibit the application of PEGylation for technical applications.

625 **References**

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627 [1] S.R. Narayanan, J. Chromatogr. A 658 (1994) 237.

628 [2] J. Turkova, Bioaffinity Chromatography, Elsevier, Amsterdam, 1993.

629 [3] H. Helmholz, S. Cartellieri, L. He, P. Thiesen, B. Niemeyer, J. Chromatogr. A

630 1006 (2003) 127.

631 [4] H. Rosenfeld, J. Aniulyte, H. Helmholz, J. Liesiene, P.H. Thiesen, B. Niemeyer,

632 A. Prange, J. Chromatogr. A 1092 (2005) 76.

633 [5] J. Aniulyte, J. Liesiene, B. Niemeyer, J. Chromatogr. B 831 (2006) 24.

634 [6] Z. Wen, B. Niemeyer, J. Chromatogr. B 857 (2007) 149.

635 [7] R. Ballerstadt, C. Evans, R. McNichols, A. Gowda, Biosens. Bioelectron. 22
636 (2006) 275.

637 [8] J.R. Cherry, M.H. Lamsa, P. Schneider, J. Vind, A. Sevendsen, A. Jones, A.H.

638 Pedersen, Nature Biotechnol. 17 (1999) 379.

639 [9] S. Gülich, M. Linhult, P.-Å. Nygren, M. Uhlén, S. Hober, J. Biotechnol. 80 (2000)

640 169.

641 [10] H. García-Arellano, B. Valderrama, G. Saab-Rincón, R. Vazquez-Duhalt,

642 Bioconjugate Chem. 13 (2002) 1336.

643 [11] E.Y. Shami, A. Rothstein, M. Ramjeesingh, Trends Biotechnol. 7 (1989) 186.

644 [12] C. Ó'Fágáin, Enzyme Microb. Technol. 33 (2003) 137.

645 [13] D.H. Na, Y.S. Youn, E.J. Park, J.M. Lee, O.R. Cho, K.R. Lee, S.D. Lee, S.D.

646 Yoo, P.P. Deluca, K.C. Lee, J. Pharm. Sci. 93 (2004) 256.

647 [14] Y. Tsutsumi, T. Kihira, S. Tsunoda, T. Kanamori, S. Nakagawa, T. Mayumi, Br.

648 J. Cancer 71 (1995) 963.

649 [15] T. Ueno, K. Ohtawa, Y. Kimoto, K. Sakurai, Y. Kodera, M. Hiroto, A.

650 Matsushima, H. Nishimura, Y. Inada, Cancer Detect. Prev. 24 (2000) 100.

651 [16] R.-G. Hu, Q.-W. Zhai, W.-J. He, L. Mei, W.-Y. Liu, Int. J. Biochem. Cell Biol.

652 34 (2002) 396.

653 [17] J.J. Kim, K. Park, Pharm. Res. 18 (2001) 794.

654 [18] F Liu, S.C. Song, D. Mix, M. Baudyš, S.W. Kim, Bioconjugate Chem. 8 (1997)
655 664.
656 [19] S. Hayashi, S. Nakamura, Biochim. Biophys. Acta. 657 (1981) 40.
657 [20] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
658 [21] C.J. Fee, J.M. Van Alstine, Chem. Eng. Sci. 61 (2006) 924.
659 [22] M. Cisneros-Ruiz, K. Mayolo-Deloisa, T.M. Przybycien, M. Rito-Palomares,
660 Sep. Purif. Technol. 65 (2009) 105.
661 [23] A. Moosmann, J. Christel, H. Boettinger, E. Mueller, J. Chromatogr. A 1217
662 (2010) 209.
663 [24] C.J. Fee, J.M. Van Alstine, Bioconjugate Chem. 15 (2004) 1304.
664 [25] P. Yu, G. Qin, D. Qin, Z. Zhang, Anal. Biochem. 396 (2010) 325-327.
665 [26] H. Sato, Adv. Drug Deliv. Rev. 54 (2002) 487.
666 [27] M. Diwan, T.G. Park, Int. J. Pharm. 252 (2003) 111.
667 [28] S.J. Stocks, A.J.M. Jones, C.W. Ramey, D.E. Brooks, Anal. Biochem. 154
668 (1986) 232.
669 [29] T. Yu, J.A. Traina, E. Pungor Jr., M. McCaman, Anal. Biochem. 359 (2006) 54.
670 [30] A.S.F.A. Habeeb, Anal. Biochem. 14 (1966) 328.
671 [31] G. Hooftman, S. Herman, E. Schacht, J. Bioact. Compat. Polym. 11 (1996) 135.
672 [32] G.T. Hermanson, A.K. Mallia, P.K. Smith, Immobilized Affinity Ligand
673 Techniques, Academic Press, California, USA, 1992, pp.282.
674 [33] O. Schiavon, P. Caliceti, P. Ferruti, F.M. Veronese, Il Farmaco. 55 (2000) 264.
675 [34] O.B. Kinstler, D.N. Brems, S.L. Lauren, A.G. Paige, J.B. Hamburger, M.J.
676 Treuheit, Pharm. Res. 13 (1996) 996.
677 [35] T.H. Kim, H. Lee, T.G. Park, Biomaterials. 23 (2001) 2311.
678 [36] V.G. Shore, B. Shore, Biochemistry. 12 (1973) 502.
679 [37] D. Solís, M. Vallejo, T. Díaz-Mauriño, Anal. Biochem. 209 (1993) 117.
680 [38] H.-C. Chiu, S. Zalipsky, P. Kopečková, J. Kopećek, Bioconjugate Chem. 4
681 (1993) 290.
682 [39] R.H. Clemmitt, H.A. Chase, Biotechnol. Bioeng. 82 (2003) 506.

683 [40] H.A. Chase, J. Chromatogr. 297 (1984) 179.
684 [41] B.V. Torres, D.F. Smith, Anal. Biochem. 170 (1988) 209.
685 [42] D.F. Smith, B.V. Torres, Methods Enzymol. 179 (1989) 30.
686 [43] J.W. Park, T. Kajiuchi, Biotechnol. Bioengin. 45 (1995) 366.
687 [44] J.E. Beecher, A.T. Andrews, E.N. Vulfson, Enzyme Microb. Technol. 12 (1990)
688 955.
689 [45] J. Folch, M. Lees, G.H. Sloance-Stanley, J. Biol. Chem. 226 (1957) 497.
690 [46] W.R. Combotz, D.K. Pettit, in: K. Park, R.J. Mrsny (Eds.), Controlled Drug
691 Delivery Designing Technologies for the Future, Oxford University Press, UK, 2000,
692 pp.110.
693 [47] I.J. Castellanos, W. Al-Azzam, K. Griebenow, J. Pharm. Sci. 94 (2005) 327.
694 [48] H. García-Arellano, B. Valderrama, G. Saab-Rincón, R. Vazquez-Duhalt,
695 Bioconjugate Chem. 13 (2002) 1336.
696 [49] Zh. He, Z. Zhang, M. He, Process Biochem. 35 (2000) 1235
697 [50] Zh. He, Z. Zhang, J. Protein Chem. 18 (1999) 557.
698 [51] D. Garcia, F. Ortega, J.-F. Marty, Biotechnol. Appl. Biochem. 27 (1998) 49.
699 [52] M.A. Longo, D. Combes, J. Chem. Technol. Biotechnol. 74 (1999) 25.

700 **Figure captions**

701 Fig. 1. Scheme of ninhydrin reaction with amino acid

702 Fig. 2. Scheme of PEGylation of immobilized Con A

703 Fig. 3. Linear regression analysis of ninhydrin standard curve for native and
704 PEGylated Con A immobilized onto Toyopearl supports. The regression equations are
705 y=0.389x-0.066 for native immobilized Con A and y=0.294x-0.050 for PEGylated
706 immobilized Con A, where y=absorbance at 570 nm and x=the amount of
707 immobilized Con A. The correlation coefficients are 0.9999, and 0.9986, respectively.
708 ■: native immobilized Con A. ▲: PEGylated immobilized Con A.

709 Fig. 4. PEGylation reaction studies.

710 (a). PEGylation kinetics of immobilized Con A onto Toyopearl supports. The ligand
711 density of Toyopearl Con A was 9.4 mg/ml. The concentration of mPEG2k-SPA was
712 1 mg/ml, the molar ratio of mPEG2k-SPA to immobilized Con A was 13:1 and the
713 reaction was performed in 0.1 M phosphate buffer, pH 8.0, at room temperature.
714 (b). Effect of pH value on the PEGylated degree of immobilized Con A onto
715 Toyopearl supports. The ligand density of Toyopearl Con A was 12 mg/ml and the
716 concentration of mPEG2k-SPA was 1 mg/ml, the molar ratio of mPEG2k-SPA to
717 immobilized Con A was 40:1 and the reaction was performed for 2 h at room
718 temperature.
719 (c). Effect of the molar ratio of mPEG-SPA to immobilized Con A on PEGylated
720 degree. Experimental conditions: variable amount of 1 mg/ml mPEG2k-SPA was
721 dissolved in 0.1 M phosphate buffer, pH 8.0, and mixed with 0.15 ml immobilized
722 Con A with ligand density of 12 mg/ml for 2 h at room temperature.
723 (d). Effect of the molecular weight of mPEG-SPA on PEGylated degree. mPEG-SPA
724 was dissolved in 0.1 M phosphate buffer, pH 8.0, and mixed with immobilized Con A
725 for 2 h at room temperature. The molar ratio of mPEG derivatives to immobilized Con
726 A was 20:1.
727 Fig.5. Adsorption isotherms of GOD to native ('TC') and PEGylated Toyopearl Con
728 A affinity adsorbents. Experimental conditions: 0.1 M acetate buffer, pH 6.0,
729 equilibrated for 15 h at T= 25 °C. Solid line: calculated from the Langmuir equation.

730 Fig.6. Adsorption kinetics of GOD to native ('TC') and PEGylated Toyopearl Con A
731 affinity adsorbents. Experimental conditions: 30 ml of 0.386 mg/ml GOD in 0.1 M
732 acetate buffer, pH 6.0, was mixed with about 0.75 ml Con A adsorbents with ligand
733 density of 13.4 mg/ml. T=25 °C. Solid line: calculated from the first-order
734 exponential decay form.
735 Fig.7(a). Adsorption capacities of GOD onto Toyopearl Con A before and after
736 incubation in 95% THF acetate buffer solution for 30 min. The ligand density of all
737 Con A adsorbents was 6.2 mg/ml. The molar ratio of mPEG derivatives to Con A was
738 1.25: 1.
739 Fig. 7(b). Adsorption capacities of POD onto Toyopearl Con A adsorbents before and
740 after incubation in C/M (2:1) for 30 min. The ligand density of all Con A adsorbents
741 was 9.9 mg/ml. The molar ratio of mPEG derivatives to Con A was 2:1.
742 Fig. 7 (c). Adsorption capacities of POD onto Toyopearl Con A adsorbents before and
743 after incubation in 50% methanol for 30 min. The ligand density of all Con A
744 adsorbents was 7.5 mg/ml. The molar ratio of mPEG derivatives to Con A was 1.25:1.
745 Fig. 8. Residual adsorption capacity of GOD onto Toyopearl Con A adsorbents after
746 incubation at 55 °C for different periods of time. The ligand density of TC and
747 TC-mPEG2k was 12.5 mg/ml, the PEGylated degree of TC-mPEG2k was 20%.