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Konjac glucomannan microspheres for low-cost desalting of protein solution

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ABSTRACT

In this study, low-cost konjac glucomannan (KGM) microspheres used for desalting were developed by an inverse dispersion method. High concentration of KGM was pretreated to reduce viscosity by acid hydrolysis method under the condition of high temperature and pressure. The selectivity of the obtained cross-linked KGM gels with different degree of crosslinking was studied by constructing calibration curves (K_{av}) of standard molecular weight substances. The stability of the gels was investigated, which showed that the KGM microspheres are tolerant to a wide range of pH and stable in all commonly used aqueous buffers, and insensitive to autoclaving as well. Furthermore, protein-desalting performances of GM-1250, a cross-linked KGM microsphere, were evaluated with two proteins, bovine serum albumin and filamentous hemagglutinin, which turned out that GM-1250 is comparable to the widely used commercial product – Sephadex G25 Fine. From economic considerations, KGM gel is a prospective alternative for dextran gels in protein desalting process.

1. Introduction

Konjac glucomannan (KGM) is the main component of konjac which has long been used in China, Japan, and South East Asia as a food source and a traditional medicine (Chua, Baldwin, Hocking, & Chan, 2010). Recently KGM has been marketed for its potential use in the treatment of obesity (Kraemer et al., 2007), obesity-related dyslipidemia (Vasques et al., 2008), and diabetes (Vuksan et al., 2000, 2001) since it is a good dietary fiber. KGM has also shown promise in controlled drug delivery systems (Chen, Liu, & Zhuo, 2005; Du et al., 2004; Yu & Mao, 2008), plasma substitutes (Li et al., 2010), and in the potential of antimicrobial materials (Xu et al., 2008). These studies have proved pivotal to the increasing demand for KGM, hence prompting studies on industrial applications should be conducted in order to increase its market potential.

As one kind of polysaccharides, KGM has good hydrophilicity and biocompatibility. It is composed of a linear chain of β-1,4-linked D-glucose and D-mannose residues in a molar ratio of 1:1.6, lightly branched through β-1,6-glucosyl units (Maeda, Shimahara, & Sugiyama, 1980; Shimahara, Suzuki, Sugiyama, & Nisizawa, 1975). This structure is very similar to dextran which has been widely used as separation media (Stone & Carta, 2007; Tao & Carta, 2008; Thommes, 1999) especially for desalting and small molecules, but the price of KGM is much lower than that of dextran (about 10%). According to its structure and economic considerations, KGM microspheres may also be a good choice for bio-separation.

A patent on KGM chromatography media appeared in 1987 in Japan. The starting material was an ester KGM dissolved in a solvent, which was then suspended in an aqueous solution. By evaporation of the solvent from the droplets, solidified particles were formed (Yoshiaki, 1987). In 1993, another patent application was filed describing a kind of cross-linked KGM particles which could be used for biomacromolecule separation (Xiao & Wang, 1993). However, few reports are available on solving the problem of low solubility and on the preparation of microspheres for desalting.

Protein desalting is the process of removing dissolved salts and minerals from aqueous solution. The main technologies used in desalting are dialysis, diafiltration, and gel filtration. Gel filtration provides certain advantages over dialysis (Shimoni, Reuveni, & Cais, 1993). In fact, dialysis is generally a slow technique that requires large volumes of buffer and carries the risk that material and target protein activity will be lost during the process (Berry & Kauvar, 1993; Vanderkaay & Vanhaastert, 1995). As for gel

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filtration, sample volumes up to 30% of the total volume of the desalting column can still be conducted. The high speed and capacity of the separation allows even relatively large sample volumes to be carried out rapidly and efficiently in the group separation process (Porath, 1981). In order to achieve a good effect of separation, several characteristics of gel filtration medium are very important, such as pore size and matrix rigidity. The matrix should also have an appropriate molecular exclusion limit with a dense structure, from which the large molecular weight solute can be excluded. The matrix should have an appropriate molecular exclusion limit with a dense structure, from which the large molecular weight solute can be excluded. Sephadex-G25, a dextran-based commercial medium has been widely used for desalting protein and DNA preparations (Blondeau, 1985). However, dextran, which is a bacterial-derived polysaccharide generally produced by enzymes from certain stains of *Leuconostoc* or *Streptococcus* (Levesque & Shoichet, 2006), has relatively high-cost. The price of dextran powder is about $80 per kilogram while the price of crosslinked dextran hydrogel beads jumped more than 25 times, selling at a whopping $2100 a kilo. On the other hand, the fine flour of konjac is very low cost (Beneke, Viljoen, & Hamman, 2009; Zhou et al., 2012), with a price of $12.2 a kilo. Thus, it is potential for KGM microspheres becoming a low-cost and effective materials for industrial application.

In the present work, we have attempted to fabricate a low-cost KGM medium for desalting and small molecules separation. But owing to the extreme high molecular weight and viscosity of KGM, it is difficult to prepare KGM solutions with high concentration. A gel is formed in the presence of $\geq$1% KGM concentration (w/w), which has little fluidity (Alonso-Sande, Teijeiro-Osorio, Remunan-Lopez, & Alonso, 2009; Kobayashi, Tsujihata, Hibi, & Tsukamoto, 2002), making a challenge for KGM microsphere preparation. Although various chemical modifications such as carboxylmethylation can be processed to increase its solubility (Kobayashi, Tsujihata, Hibi, & Tsukamoto, 2002), it is not practical to obtain KGM solution with concentration high enough to prepare the microspheres. In this study, we circumvented this disadvantage by cutting down the chain length to decrease its molecular weight. Acid hydrolysis under the condition of high temperature and pressure was used to cleave the original long chain. Moreover, we applied online viscosity testing to determine the degree of hydrolysis and control the molecular weight. The KGM microspheres were synthesized by inverse suspension method, and the structure was characterized by SEM image analyzer. We evaluated the molecular weight fractionation range, the stability, and desalting effect of KGM particles. The performance of KGM microspheres was also compared with that of the commercial product Sephadex G25.

2. Materials and methods

2.1. Materials

The fine flour of konjac (the content of KGM was approximately 65%) was purchased from Hu-Bei Konson Konjac Gum Co. Ltd. (China). Span80 used as emulsifier in the oil phase was purchased from Farco Chemical Supplies (China). Liquid paraffin and petroleum ether were chosen as oil phase and were supplied by Sinopharm and Tianjin Jin Dong Tian Zheng Precision Chemical Reagent Factory (China), respectively. Epichlorohydrin used as the cross-linking agent was purchased from Xilong Co. Ltd. (China). Sephadex G25 and gel filtration LMW calibration kit were products from GE Healthcare. Other reagents were of analytical purity and were purchased from Beijing Chemical Reagent Company (China).

2.2. Degradation of KGM

The KGM was swelled in 0.5 M hydrochloric acid solution (4/3, w/w), and then the jelly was heated at 115 °C for 55 min, followed by adding 45% sodium hydroxide containing a small percentage of sodium borohydride with vigorous stirring. Finally, the obtained solution was filtered and the filtrate was collected. The concentration of KGM in the filtrate was about 12%, and the viscosity was 362 mPa s at 35 °C.

2.3. Determination of molecular weight

20 g deionized water was added into 3.0 g degraded KGM solution prepared in 2.2. The pH was adjusted to 7.0 with HCl, and the concentration of KGM in the solution was adjusted to 1% (w/w). The samples were determined by multi-angle laser light scattering, MALLS (Wyatt Technology Corporation, WTC) combined with gel permeation chromatography (GPC). The operation was under the following conditions: Agilent 1100 high performance liquid chromatography instrument, TSK-GEL G5000 HHR column (7.8 mm × 300 mm), inject volume 100 μL, mobile phase 50 mM sodium phosphate and 100 mM sodium sulfate, pH 7.0. The velocity of flow was 0.5 mL/min, and operation time was 40 min. Samples were filtered by 0.2 μm membrane before injecting into the HPLC column. Astra software was utilized for data acquisition and analysis.

2.4. Preparation of KGM gel

A 2 L three-necked reaction flask equipped with a semicircular paddle stirrer was filled with 1.2 L oil phase comprised of 1 L liquid paraffin, 0.2 L petroleum ether, and 3.5% (w/w) of Span80. The reaction flask was placed in a water-bath at 60 °C and the oil was stirred for 30 min until well-mixed. 12% KGM solution (400.0 g) was added into the flask and the mixture was stirred for 1 h. Epichlorohydrin, the cross-linking agent, was added dropwise into the reaction flask in about 30 min. The reaction was then lasted for 8 h. Finally, the formed microspheres were washed with 95% ethanol, ligroin, and deionized water successively. KGM microspheres with 50% cross-linker (200.0 g) were named as GM-1250, while microspheres with 25% cross-linker (100.0 g) were named as GM-1225.

2.5. Characterization of the KGM gel

2.5.1. SEM observation of KGM microspheres

The shape and surface feature of KGM microspheres after drying were observed by a JEM-6700F scanning electron microscopy (JEOL, Japan). The sample was placed on a metal stub and coated with platinum under vacuum by an ion sputter (JFC-1600, JEOL).

2.5.2. Particle size distribution

The particle size distribution of KGM microspheres was measured by a laser diffractometry using Ls230 Coulter (Coulter Co., USA).

2.6. Chromatographic procedure

2.6.1. Column packing procedure for KGM gel

The KGM gel and Sephadex G25 used in the chromatographic experiments were packed, respectively, according to the following procedure. The column (XK16/30 column, GE Healthcare) was mounted vertically, and then 20 mL well-mixed gel slurry (80% (v/v) KGM gel in deionized water) was poured into the column. When the gel had settled down (after 2 h), the column was washed with deionized water at a flow rate of 1.0 mL/min for 1 h. And then

the flow rate was set sequentially at 2.0, 3.0, and 4.0 mL/min for 30 min. The gel bed was then compressed about 2 mm by pushing down the top adaptor with a bed height between 27 and 28 cm.

The number of theoretical plates (N) was determined by injection of 100 μL acetone (20%) and elution at a flow rate of 0.5 mL/min. All columns used in the study have more than 1000 plates per meter.

2.6.2. Gel filtration chromatography using KGM gels

Gel filtration chromatography was used to determine the fractionation range. For these determinations, the mobile phase was a 20 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. These experiments were done with an ÄKTA Purifier (GE Healthcare) system using a UV-900 detector and 100 μL injections of individual sample at 0.5 mL/min (35 cm/h). The K_{av} values were calculated as Eq. (1):

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad (1)$$

where V_e is the peak retention volume, V_o is the extraparticle volume, and V_t is the included or total mobile phase volume. Blue dextran 2000 (2.0×10^3 kDa) was used to determine V_o.

2.7. Desalting of proteins

For desalting purposes, the gel was packed in TRICORN column (1.0 × 10 cm, GE Healthcare) with a bed height between 9 and 10 cm. The mobile phase was a 20 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. All of the experiments were carried out at room temperature.

2.8. Stability of KGM gel under different conditions

The stability of the gels (GM-1250; GM-1225) in various aqueous solutions was measured. The KGM gels were washed with deionized water on a glass-filter funnel, sucked dry with a water

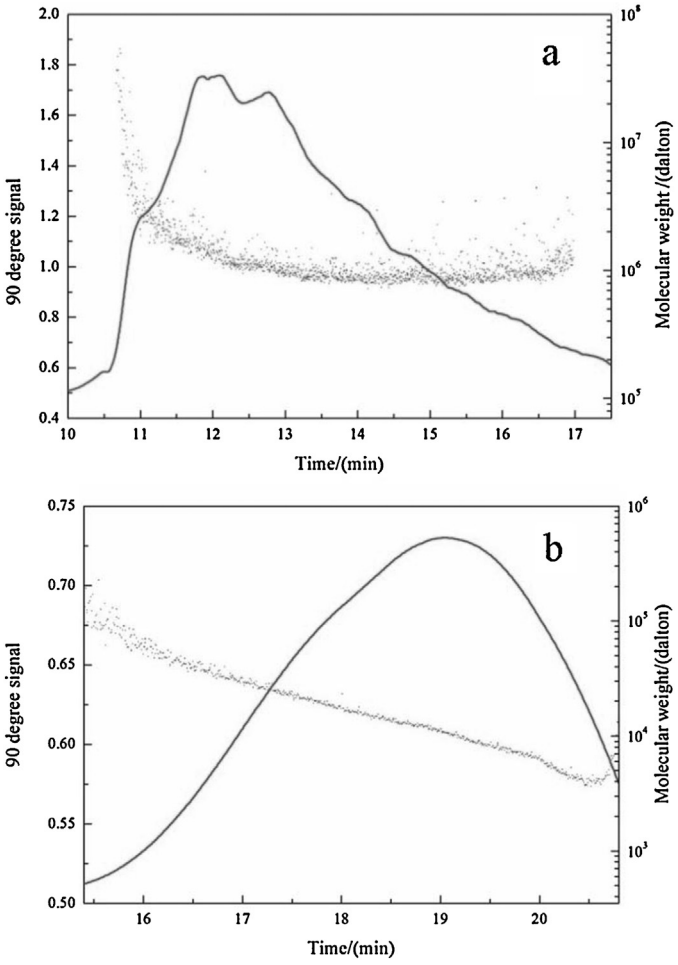

Fig. 1. Determination of KGM molecular weight by GPC–MALLS. (a) KGM and (b) degradation products of the KGM.

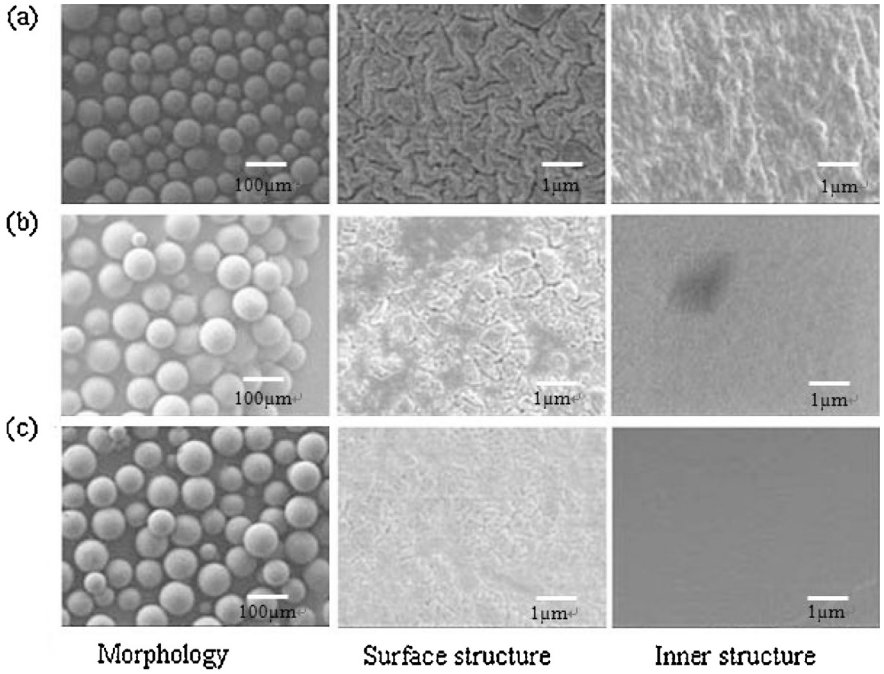

Fig. 2. SEM images of the surface and inner structure of Sephadex G25 and KGM microspheres. (a) Sephadex G25 Fine. (b) KGM microspheres with 50% cross-linker (GM-1250). (c) KGM microspheres with 25% cross-linker (GM-1225).

aspirator, and kept at 40 °C for 7 days in different solutions (or autoclaved in deionized water). The storage solution was as follows: 0.1 M HCl, 1 M NaOH, 6.0 M guanidine hydrochloride, 8.0 M urea and 0.5 M HCl. The gels were kept in the solution at 40 °C for one week or one week at room temperature. The autoclaving was carried out at 121 °C for 20 min. After storage or autoclaving, the gels were washed with deionized water and packed in TRICORN column for desalting tests. Desalting conditions: 1.5 mL BSA in 0.02 M sodium phosphate, 0.5 M NaCl, eluted at a flow rate of 2 mL/min (152 cm/h).

3. Result and discussion

3.1. Material properties

Konjac glucomannan consists of 1,4-linked β-D-mannose and D-glucose units in the ratio 1.6:1 with a low degree of acetyl groups (approximately 1 acetyl groups per 19 residues) at the C-6 position, which is very similar to that of dextran. MALLS combined with GPC were used to determine molecular weight of KGM (Fig. 1), which indicated that the molecular weight distribution of KGM became broader after the degradation. The molecular weight of KGM decreased significantly, with the polydispersity index $M_w/M_n = 1.386$, M_n (number average molecular weight), M_w (weight average molecular weight) 8.029 kDa and 11.13 kDa, respectively. Before degradation the molecular weight of KGM was 1.234×10^3 kDa, and it became a gel easily when the concentration of KGM solution was 1%. After the degradation we can adjust the KGM concentration from 5% to 20% while it was also flowing. The viscosity is a macroscopic characterization of the molecular weight of KGM which can be controlled in the range of 200.0 mPa s to 300.0 mPa s.

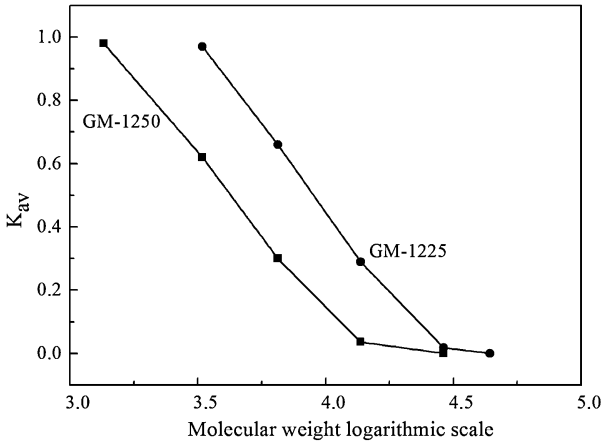

Fig. 3. K_{av} of standard substances on KGM gels versus molecular weight logarithmic scale.

3.2. Observation of the microsphere morphology

The KGM microspheres were prepared by an inverse dispersion method with a narrow size distribution after sieving. As shown in Fig. 2, the morphology of all microspheres was spherical. However, the structures of KGM microspheres and Sephadex G25 are slightly different. The surface structure of Sephadex G25 is drape, and cracks are observed in the fracture surface. While the surface structure of KGM microspheres has some irregular pores, and the fracture surface presents reticulation structures, in which there are innumerably regular interspaces. KGM is a linear molecule; nevertheless, in the process of forming particles, the KGM chains are intertwined and crosslinked by ECH. Consequently,

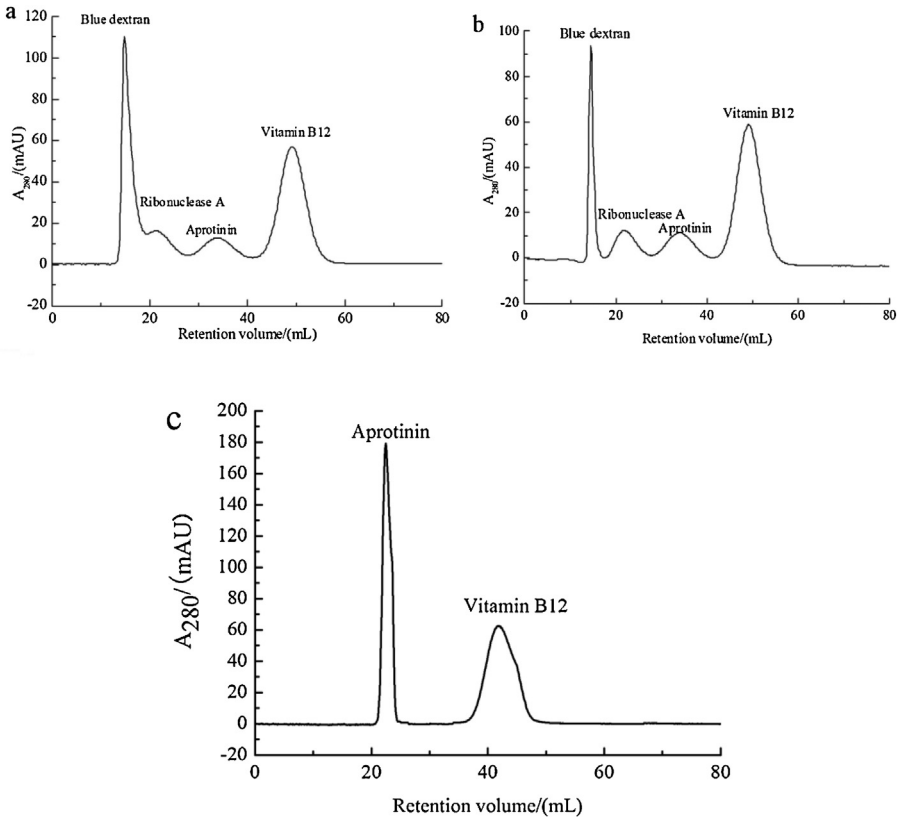

Fig. 4. Chromatography profiles of mixed standard substances separated by KGM media. (a) GM-1250; (b) GM-1225; and (c) Sephadex G25. Column: XK16 × 40 (I.D. 16 mm × 40 mm).

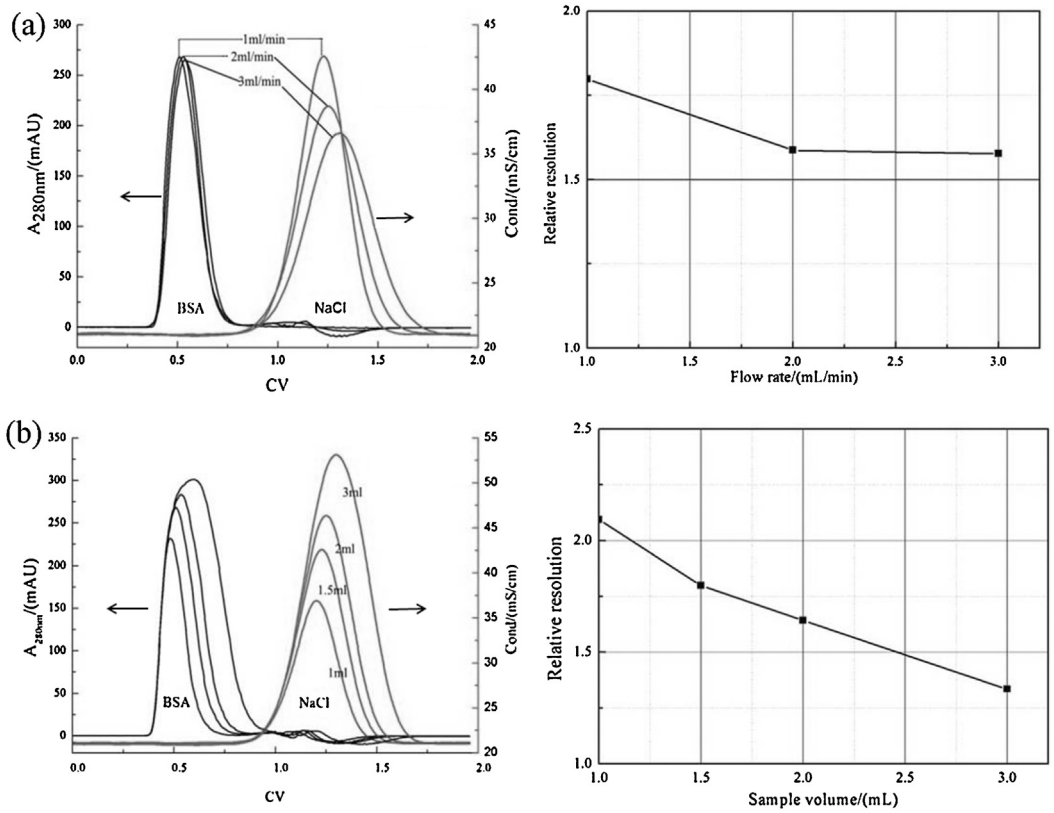

Fig. 5. Influence of flow rate and sample volume on desalting using GM-1250 gel. Medium: GM-1250. Column: TRICORN 1/10, packed bed 1.0 cm × 10 cm. Sample: BSA, 2 mg/mL in 0.5 M NaCl, 0.02 M sodium phosphate, pH 7.0. Buffer: 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.0. (a) Sample volume: 1.5 mL. Flow rate: 1, 2, 3 mL/min. (b) Sample volume: 1, 1.5, 2, 3 mL. Flow rate: 1 mL/min.

the interstice between chains formed very small pores. The KGM concentration and crosslinking degree also affected the mechanical strength and fractionation range of KGM particles. Therefore, we can adjust them by changing KGM content or crosslinking degree.

The size and distribution of the microspheres are shown in Table 1. The mean particle diameters of two KGM microspheres are 152.7 and 151.5 μm, respectively, and the relative widths of the distributions (span value) are 0.470 and 0.553, respectively. Compared with Sephadex G25 fine, the sizes of two KGM microspheres are much bigger while the span values are far more narrow. To perform a separation, efficient column packing is essential. Gel filtration media with high bed uniformity (smaller and more uniform particles) will produce decreased peak widths and better resolution. Meanwhile, the uniformity of the packed bed and the particles influences the uniformity of the flow profile and hence affects the column efficiency which is defined in terms of theoretical plates per meter (N). The theoretical plate height (H) of 0.026–0.032 cm, as measured with acetone as solute, has been routinely achieved in packed columns of all the 3 kinds of media.

Table 1
Size and distribution of KGM microspheres compared with Sephadex G25 microbeads.

	$d_{(0.1)}$ (μm)	$d_{(0.5)}$ (μm)	$d_{(0.9)}$ (μm)	Span
Sephadex G25 Fine	48.9	77.9	113.3	0.826
GM-1250	118.9	152.7	190.6	0.470
GM-1225	112.8	151.5	196.7	0.553

3.3. Fractionation range of KGM gel

Gel filtration chromatography profiles for a range of standard substances were studied. The detail of the standard substances was as followed: Blue dextran 2000 (2.0×10^3 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa), GLP-1 (3.27 kDa), and vitamin B_{12} (1.35 kDa). The selectivities of GM-1250 and GM-1225 measured by K_{av} for proteins on these two KGM gels are shown in Fig. 3. GM-1250 gel was found to be suitable for fractionation of

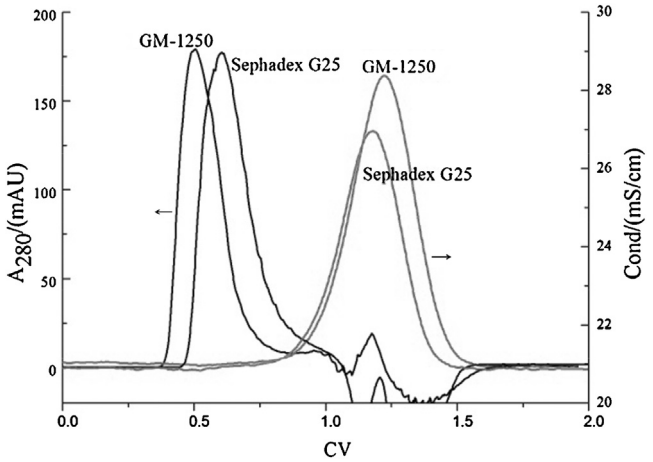

Fig. 6. The desalting effect of filamentous hemagglutinin on Sephadex G25 and GM-1250 gel.

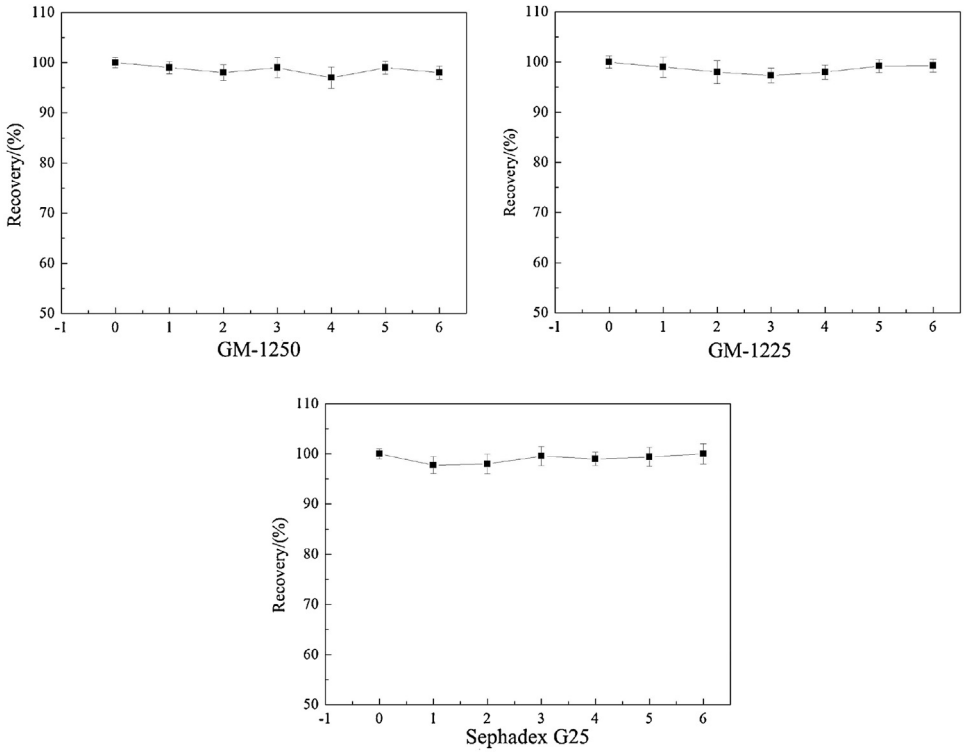

Fig. 7. Stability of KGM gels measured as change in the recovery of proteins stored in various solutions or after autoclaving. Storage solutions: 0, control; 1, 0.1 M HCl; 2, 1 M NaOH; 3, 6.0 M guanidine hydrochloride; 4, 8.0 M urea 5, 0.5 M HCl; 6, autoclaving. Storage test (1–5): the gel was kept in the solutions at 40 °C for one week (experiments 1–4) and one week at room temperature (experiment 5). Autoclaving: experiment 6, 121 °C for 20 min. Buffers were same as Fig. 5.

globular proteins in the molecular weight range 1–10 kDa. While the fractionation range of GM-1225 was found to be 4–30 kDa for globular proteins. The detailed separation results are illustrated in Fig. 4. Since Sephadex G25 has a molecular weight exclusion limit of 5 kDa for globular protein, it can be seen only Vitamin B_{12} is not excluded from the gel matrix. The KGM contents of GM-1225 and GM-1250 are both 12%, and their crosslinking degrees are 25% and 50%, respectively. Variations in the degree of crosslinking could create the different KGM gels and influence their degree of swelling and selectivity for specific molecular sizes.

3.4. Desalting experiments of proteins

The exclusion limit of GM-1250 for globular proteins was 10 kDa. Therefore, any protein having a molecular weight of 10 kDa or above can be desalted easily. The desalting behavior was investigated for GM-1250, and the influence of flow-rate and sample volume on desalting of bovine serum albumin (BSA) was studied. Results are shown in Fig. 5. Similar to other polysaccharides, during the cross-linking of the gels, a small amount of carboxyl groups were introduced (Gupta, Sahni, Rathaur, Narang, & Mathur, 1979; Hellberg, Ivarsson, & Johansson, 1996). To suppress ionic interactions between these groups and positively charged solutes, a low salt concentration (0.15 M NaCl) was added during desalting and in the final sample buffer. The recovery of proteins in all the tests was above 98%, but resolution will be reduced with higher flow rate or larger sample volumes. Fig. 5a shows that the resolution was approximately the same at two different flow rates, 152 and 229 cm/h when the sample volume was 19% of the total column volume (1.5 mL). With the increase of sample volume, the resolution was significantly deteriorated. Of the parameters studied, sample volume has the greatest effect on resolution (Fig. 5b).

Fig. 6 shows the comparison of desalting effect of filamentous hemagglutinin (FHA) on GM-1250 gel and Sephadex G25. FHA is a 220 kDa large surface-associated adherence protein of *Bordetella pertussis*, which is a component of some new acellular pertussis vaccines. The recoveries for FHA on both columns were over 95%, and their desalting results were comparative.

3.5. Chemical and physical stability

The stability of the KGM gels stored in various aqueous solutions at 40 °C for 1 week was studies by measuring the recovery of BSA before and after storage (Fig. 7). In each case, no change in recovery was observed, indicating that KGM gels are stable in all commonly used aqueous buffers and denaturing agents (8 M urea or 6 M guanidine hydrochloride). Meanwhile, autoclaved gel also had similar recovery of BSA as untreated gel. The same experiment was carried out to test the stability of Sephadex G25, and the findings were not significantly different from the KGM microspheres.

These results showed that the cross-linked KGM gels are excellent in chemical stability and can be used over the whole pH range (1–14). They can be stored in 0.1 M HCl or 1 M NaOH at 40 °C for 1 week without any change in chromatographic properties. Moreover, the microspheres can be used in high concentrations of guanidine hydrochloride and urea. They could be reused for multiple cycles in the desalting experiment without noticeable change of chromatographic behavior.

4. Conclusions

In the study, we prepared novel Konjac glucomannan microspheres which have excellent chemical stability for low-cost desalting of protein solution. The fractionation range of microspheres can be controlled by KGM content or crosslinking degree of the particles, for example GM-1250 gel is suitable for fractionation of globular proteins with molecular weight between 1 and 10 kDa while the GM-1225 is suitable for 4–30 kDa globular proteins. The

KGM microspheres are stable in a wide range of pH, i.e. pH 1–14, and in all commonly used aqueous buffers, and insensitive to autoclaving as well. The superior performance and manufacture economy of KGM gel will make it a prospective alternative material for protein desalting in industry. However, as for the mechanical stability, KGM microspheres, like other polysaccharides, are satisfactory only under moderate pressures. Work on the development of other gels based on KGM is in progress.

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