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Preparation and Characterization of Macroporous Carrageenan Beads for Enhanced Gel Filtration

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خلاصة : تم تحضير خرز ك - كاراقينان كبيرة المسام بطريقة الإستحلاب ذي الخطوتين، بتداخل الحلقات كيميائياً والتمييز، ومن ثم الإعداد للترشيح الهلامي. لقد عرف حديثاً أن التصعد (اي حركة السائل الكلي) من خلال المسام الكبيرة (الماكروبورز) طريقة فعالة من أجل الإسراع بعملية الفصل الكروماتوغرافي بالتحويل الكتلي. هدفت هذه الدراسة لتحديد ما إذا كان هذا المفهوم صحيحاً بالنسبة لخرز الكاراقينان كبيرة المسام. تم تحليل بناء مسامات الخرز بالمجهر الألكتروني للمسح البيئي وتمييزها بقياس مساحة السطح الكلية وكثافة التعبئة. وجد أن الزيادة في كمية التولوين للمستحلب الأول تلعب دوراً هاماً في خلق المزيد من المسامات وتحديد بنائها. بالإضافة الى ذلك كانت المعالجة الحرارية للخرز فعالة جداً في زيادة كثافة تداخل الحلقات وتقليص حجم الخرز المتضخم في الملح ولكن المعالجة بالبخار قلصت مساحة السطح الكلية للخرز مما يوحي بتغيير في بناء المسامات. كان هلام الخرز ذو الحلقات المتداخلة ثابتاً في مدى الرقم الهايدرولوجي ١ - ١٣ وفي محلول كلوريد البوتاسيوم بتركيز ٠,٠٧ مليمول. كما زاد حجم الخرز بنقصان الشدة الأيونية. وعند الشدة الأيونية المنخفضة، وجد أن اثنين من البروتينات التي درست، سايتوكروم سي وليسوزايم يمتصان خرز الكراقينان. كما أن الشدة الأيونية أثرت على مدى تجزئة الخرز لأجل الترشيح الهلامي. وتقلص حجم التصفية لمختلف البروتينات غير الممتصة بنقصان الشدة الأيونية. كما أن كفاءة (أي انحلال واختزال ارتفاع اللوح) خرز الكاراقينان في الفصل الكروماتوغرافي كان أحسن منه في خرز السفاروز سي إل ٦ بي التجاري، خاصة عند معدلات الدفق العالية.

ABSTRACT: Macroporous *K*-carrageenan beads were prepared by a two-step emulsion method, chemically cross-linked, characterized, and then employed for gel filtration. It has recently been recognized that convection (i. e. bulk fluid movement) through large pores (i.e. macropores) is an effective way of enhancing the performance of chromatographic separation by improving mass transfer. The aim of our study was to determine if this concept was true for macroporous carrageenan beads. The pore structure of the beads was analyzed by environmental scanning electronic microscopy and characterized by measuring the total surface area and packing density. An increasing toluene content in the first emulsion was found to play an important role in creating more pores and in determining the pore structure. In addition, heat treatment of the beads was very effective for increasing the crosslinking density and reducing the bead volume swelling in saline. However, treatment with steam reduced the bead surface area suggesting a change in pore structure. The crosslinked hydrogel beads were stable in the pH range 1-13 and in KCl solutions at a concentration over 0.07 M. The bead size increased with a decrease in ionic strength. At low ionic strength, two of the proteins studied, cytochrome C and lysozyme, were found to adsorb to the carrageenan beads. The ionic strength also affected the fractionation range of the beads for gel filtration. The elution volume of various non-adsorbed proteins was reduced with a decrease in ionic strength. The efficiency (i. e. resolution and reduced plate height) of carrageenan beads in chromatographic separation was better than that of commercial Sepharose CL-6B beads, especially at high flow rates.

Convection through pores has recently been recognized as an effective means for enhancing the performance of porous beads in catalytic reactions (Rodrigues and Ferreira, 1988; Rodrigues et. al., 1982; Creswell, 1985; Cogan et. al., 1982), immobilized cell culture (Prince et. al., 1991; Stephanopoulos and Tsiveriotis, 1989) and chromatographic separation (Fulton, et. al., 1991; Afeyan et. al., 1991; Lloyd 1991; Li et. al., 1995). Due to the presence of convection, solutes carried by a fluid are directly transported into or out of a macroporous system (Figure 1). It improves mass transfer in porous beads and their efficiency in catalytic reactions and chromatographic separation. One advantage of macroporous beads is their remarkable mass transfer efficiency at high flow rates. The apparent effective diffusivity was dramatically increased when the Reynolds number was over 10 (Knight, 1989; Janson and Hedman, 1982). Afeyan et. al. (1990a) developed perfusion macroporous beads, based on poly(styrene-divinyl benzene), for high performance liquid chromatography (HPLC). The reduced plate height of these rigid perfusion beads showed little dependence and, in some cases, independence from the fluid flow in very high

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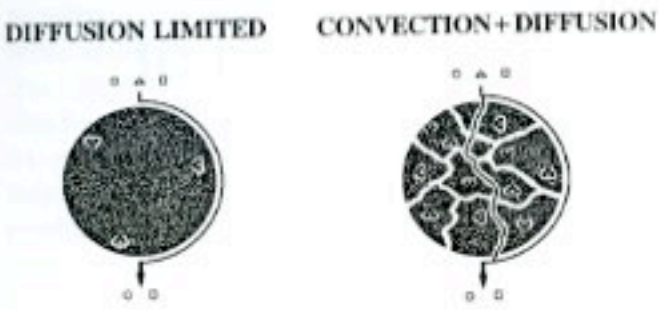

Figure 1. Comparison of mass transport in conventional beads and macroporous beads.

flow rate regions (i.e. Peclet number > 100) depending on the type of beads (Afeyan et. al., 1990b). The processing time of this perfusion chromatography was reduced by a factor of 10 (from 30 minutes to 3 minutes) and the productivity increased by a factor of 50 when compared to conventional HPLC.

Convection can only occur in a porous system if the pore size is large enough. The pore size should be greater than 4000 Å before intraparticle convection can occur (Lloyd 1991, Frey et. al., 1993, Lloyd & Warner, 1990). For the beads with 4000 Å pores, the apparent diffusivity was dependent on the flow rate (Frey et. al., 1993). In addition, the column pressure drop was also 40% lower when compared to beads with 1000 Å pores. Some newly developed macroporous bead systems (Fulton et. al, 1991; Afeyan et. al., 1991; Afeyan 1990b) adopted a heterogeneous pore structure containing both large and small pores. The large pores allow for convection, while small pores allow for molecular diffusion. For example, the beads developed by Afeyan et. al. (1991) had pores ranging from 6000 to 8000 Å and from 800 to 1500 Å.

Two methods, emulsion (Nilson et. al., 1986; Lim et. al., 1992) and aggregation (Frey et. al. 1993), have been used for preparation of macroporous beads. For production by emulsion methodology, a pore forming reagent was uniformly mixed with a polymer hydrogel. Large pores were created after the reagent was removed and the polymer gels solidified. The pore structure was greatly dependent on the content of the pore-forming reagent. For the aggregation method, sub-particles were aggregated to form larger particle. The large channels in these beads consisted of the space between the aggregated sub-particles.

Various gels, such as dextran, polyacrylamide, agarose, and cellulose, have been widely employed for chromatographic separation. Advantages of these gels include low non-specific adsorption, good chemical stability, easy chemical derivatization, and simple column preparation when compared to rigid packing materials such as silica and glass (Janson and Hedman, 1982). However, one major drawback is gel softness which prevents their use in high performance liquid chromatography with its associated high pressure drop which would physically deform soft gel beads. Efforts have been directed towards improving the rigidity of gel beads in order to reduce the particle size and boost their performance in chromatographic separation (Knight, 1989). The mechanical strength of gel matrices may be improved by increasing the degree of crosslinking and by using composite materials. For example, highly cross-linked Superose agarose beads, have a wet particle size of 20-40 μm compared to 45-165μm for Sepharose beads. On the other hand, the composite beads Superdex (agarose and dextran) and Sepharyl (allyl dextran and acrylamide) are 22-75 μm in diameter in the wet state. Other materials, such as alginate (Kierstan et. al., 1992), chitosan (Seo and Kinenura, 1989) and carrageenan (Chibata et. al., 1981) have also been investigated for chromatographic applications.

K-Carrageenan composed of unit structures of ß-D-galactose sulphate and 3, 6-anhydro-α-D-galactose has been applied as a packing material for chromatographic separation of proteins (Chibata et. al., 1981). This polysaccharide produced rigid gels with a mechanical strength ranging from 1 to 2.4 kg/cm^2 in the presence of cations, such as K^+,Rb^+,Cs^+,Ca^{++}, Al^{+++}, and NH_4^+, at low temperature (Guiseley, 1989; Chao et. al., 1986). One popular model to describe the gelation mechanism of carrageenan is the aggregation of the cation mediated double helixes of carrageenan to form microcrystalline junction zones (Yalpani, 1988; Therkelsen, 1993). This gel structure is similar to the network of agarose gels (Guiseley, 1989). However, compared to agarose gels, *K*-carrageenan gels contain extra sulphate groups. Even a small sulphate group content in the gels resulted in high protein adsorption (Porath, 1971). Sulphate groups must normally be removed before the polymer can be used for gel filtration.

Carrageenan beads have been prepared for gel filtration and affinity chromatography based on a modified procedure employed by Chibata et. al. (1981). This procedure was originally used for producing agarose beads and later successfully utilized for preparing agar beads for gel filtration (Porath, 1971). *K*-Carrageenan beads were first crosslinked with epichlorohydrin at high pH and then desulphated in base solution at 120°C. In spite of this work, however, there is still a lack of detailed information on the gel filtration properties of carrageenan beads, such as stability, protein adsorption and fractionation range.

In the present study a two-step emulsion method was investigated for preparing carrageenan and carrageenan-gelatin beads with toluene used as the pore forming reagent. Since the toluene content in the first

emulsion was responsible for creating large pores in the beads, part of the investigation focused on the effect of toluene on the pore structure. The pore structure of the beads was analyzed by environmental scanning electronic microscopy (ESEM) and characterized by measuring the total beads surface area and the packing density. In addition, heat treatment to control volume swelling of the beads by increasing the degree of carrageenan crosslinking is reported. Bead stability at varying pH and ionic strength was also examined. We further assessed the effect of eluent ionic strength on protein adsorption and fractionation by the beads.

Experimental

MATERIALS: *K*-Carrageenan, epichlorohydrin, Span 60, surfactant Tween 80 and molecular weight marker standard kit, MW-G-F-1000, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), gelatin (bloom 300 from swine skin) from Aldrich (Milwaukee, WI, U.S.A.), glutaraldehyde from BDH Inc. (Toronto, Canada), and Araldite 502, Epon 812, dodecenyl succinic anhydride (DDSA), and tri[dimentylaminomethy]phenol (DMP-30) from J. B. EM Services Inc. (Montreal, Canada). Toluene, ethanol, acetone and buffers from pH 1 to pH 10 were supplied by BDH Inc. (Toronto, Canada). All other chemicals were of analytical grade.

PREPARATION OF MACROPOROUS CARRAGEENAN BEADS BY EMULSION: The preparation of macroporous beads was based on a procedure developed by Nilsson et. al. (1986) (Figure 2). *K*-Carrageenan, 4 g, was dissolved in 100 ml water at 80°C. The solution was then transferred into 250 ml toluene with 4% (w/v) Span 60. The mixture was stirred at 1200 rpm with a 2" turbine propeller (Cole-Parmer Instrument Co., Anjou, Canada) for 5 min at 80°C to give an emulsion. The temperature of the emulsion was lowered to 25°C under continuous stirring to allow the gel beads to form. After the stirring was stopped, the beads were allowed to settle (15-20 min.) and the bulk toluene on top was removed. The recovered beads were washed with ethanol, followed by acetone, and finally sieved in acetone in a range 75-300 μm (sieve No. 200 and No. 50, Endecotts Ltd., London, England).

For the preparation of macroporous carrageenan-gelatin beads, gelatin, 5g, was dissolved in 50 ml water at 50°C. Then, 2 g carrageenan was added to the solution and dissolved by raising the temperature to 80°C. While continuously stirring the polymer solution at 2800 rpm (2" three-blade propeller, Cole-Parmer Instrument Co., Anjou, Canada), a specific volume of toluene ranging from 0 to 50 ml, with 5% (w/v) Tween 80, was added to make the first emulsion. The

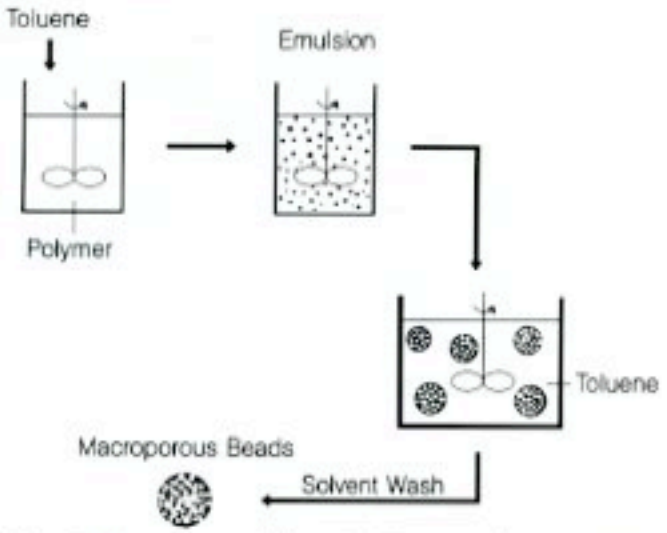

Figure 2. A two-step emulsion method for preparing macroporous beads.

polymer solution was the continuous phase in the first emulsion. The emulsion was kept at 80°C and transferred into a beaker containing 300 ml toluene. This mixture was stirred at 1200 rpm (2" turbine propeller) and formed the second emulsion. In the second emulsion, the extra toluene was the continuous phase while the first emulsion containing tiny toluene droplets inside a polymer solution became the separated phase. Under constant stirring, the temperature of the second emulsion was dropped below 20°C with a cold water bath, allowing the beads to form by gelation. The beads were placed in 400 ml ethanol to remove the internal toluene, and then washed thoroughly with acetone.

CROSSLINKING AND DESULPHURIZATION OF CARRAGEENAN BEADS: Crosslinking and desulphurizing of carrageenan beads was done according to the method of Chibata et. al. (1981). This technique was originally designed by Porath et. al., (1971) for agar and agarose beads. Swollen carrageenan beads, 200 ml, were thoroughly washed with 0.3 *M* KCl followed by 2 *M* KOH. The beads were then suspended in 500 ml of 2 *M* KOH with 2.5 g sodium borohydride ($NaBH_4$) and 25 ml epichlorohydrin at 60°C for 1 h with stirring. The reacted mixture was filtered and the crosslinked beads were washed with 0.3 *M* KCl and resuspended in 1 L of 2 *M* KOH with 5 g sodium borohydride. The mixture was autoclaved at 120°C for 20 minutes. The crosslinked and desulphated carrageenan beads were then thoroughly washed with 0.1 *M* KCl.

The carrageenan-gelatin beads, 100 ml gel beads, were washed thoroughly with 1% KCl solution. They were then fully swelled in 0.1 M phosphate buffer with 1% KCl at pH 7. Glutaraldehyde, 10 ml 40-50% (w/v), was added for 1h at 25°C to crosslink the beads. The beads were then washed with acetone and dried in

vacuum or by evaporation at 25 degrees centigrade overnight. Dry beads were further treated in an oven at 120°C for 2 h, or treated with steam at 120°C for 1 h to increase the degree of crosslinking.

MEASUREMENT OF SURFACE AREA: The surface area of dried carrageenan-gelatin beads was measured by a nitrogen isothermal adsorption method (BET; Flowsorb II 2300, Micromeritics Instrument Corp., Norcross, U.S.A.) at 30% nitrogen and 70% helium. Dried samples, 0.5-1 g, were placed in a sample tube and then immersed in liquid nitrogen and allowed to reach equilibrium. At this stage, the samples were saturated with adsorbed nitrogen. The liquid nitrogen was later removed and the sample was brought to room temperature. The nitrogen began to desorb from the sample as the temperature increased. The amount of nitrogen released during this drying is proportional to the surface area of the beads.

DETERMINATION OF SWELLING VOLUME: Dried beads, 2 ml, were swollen in 1% (w/v) KCl solution with stirring. The solution was then allowed to settle for 4 h at 25 C. Gels are usually fully swollen in solution within half an hour (Monke, 1990). The final volume of the swollen beads, V, was taken and compared to the initial bead volume at time zero, V_0 = 2 ml, to give the volume increase $(V-V_0)/V_0$.

ANALYSIS OF BEAD CROSS-SECTION: Dried beads for sectioning were embedded in epoxy resin (Hayat , 1970). Araldite 502, 83 ml, was mixed with 105 ml Epon 812 and 250 ml DDSA at 80°C, stirred for 3 h and then stored at -20°C ready for use. A small number of beads, about 100, were placed in a 1 ml microtube and then 0.6 ml of the embedding resin was added with 1 droplet DMP-30 per ml resin. The suspension was gently stirred until uniformly mixed with the resin. The tube was baked at 65 degrees C for 36 h for polymerization. The beads were sectioned with a razor blade under a microscope.

BEAD STABILITY: The stability of carrageenan beads in solution was investigated at varying pH and ionic strengths. Carrageenan beads, 10 ml each, were suspended in 50 ml buffer at a pH ranging from 1 to 10 with 0.1 *M* KCl for one week. In addition, 10 ml of beads were put into 50 ml of 2 *M* KOH solution at pH 13. The bead integrity (i.e. breakage) was then observed visually under a microscope. Effects of ionic strength on bead stability were assessed in a similar manner. The beads were first thoroughly washed with distilled water to remove all ions. The beads, 2-4 ml, were added to 10 ml KCl or NaCl solution at concentrations ranging from 0.00 *M* to 2 *M* for one day. The solution was refreshed every 4-5 h. The change in particle size was determined with an image analysis system as described below.

PARTICLE SIZE DETERMINATION: Several thousand carrageenan beads were placed under an optical microscope. The particle image from the microscope was taken by a video camera (Sony AVC-D5, Sony Co., Japan) and transferred to an on-line monitor and a computer. The image recorded in the computer was further analyzed by a software package (JAVA, Jandel Scientific, Corte Madera, U.S.A.). The mean particle size was determined from a population of 250-350.

PROTEIN ADSORPTION BY CARRAGEENAN BEADS: The adsorptive ability of carrageenan beads was determined from the adsorption isotherm of cytochrome C at room temperature. Dried carrageenan beads, 4-5 g, were swollen in 150 ml of 0.1 *M* phosphate buffer with varying KCl concentrations ranging from 0.1 *M* to 0.5 *M* at pH 7. A cytochrome C solution (10 mg/ml in 0.01 M phosphate buffer) containing the desired KCl concentration was added to the suspension, 1 or 2 ml each time. The suspension was stirred for 1 h and then allowed to settle for 10 min. A sample was taken from the supernatant to determine its protein concentration. The same procedure of adding protein solution and measuring its concentration was repeated about 10 times. After measurement, the beads were filtered and thoroughly washed with the 0.1 *M* phosphate buffer until no more protein was found in solution. The washed beads were then ready for more continuous studies. Protein concentration in solution was determined by measuring the absorbance at 280 nm with a UV/vis spectrophotometer (Philips PU8700Pye Unicam Ltd., Cambridge, England).

GEL FILTRATION COLUMN SET-UP: A slurry of carrageenan gel beads (bead diameter 75-180μm) in 0.1 M phosphate buffer with 2% KCl and 0.02% sodium azide at pH 7 was loaded onto a 1.6 cm (I.D.) glass column (LBK Instruments Inc., Maryland, U.S.A.). The beads were then compacted at a flow rate of 50-100 ml/h for 1 h. A phosphate buffer (0.01 *M* to 0.1 *M*) with KCl ranging from 0.1 *M* to 2 *M* and 0.02% sodium azide at pH 7 was washed through the column for 1 d at a flow rate 10 ml/h. Protein samples dissolved in the phosphate buffer, 0.4-2 ml, were loaded onto the column from the top. The eluent was collected with a fraction collector (LKB 2070 UltroRac II, Pharmacia Biotech Inc., Baie D'Urie, Canada) at 0.5-2 ml/sample and analyzed at 280 nm.

Results and Discussion

MICROBEAD PORE STRUCTURE: The pore structure of the beads was greatly affected by the toluene content in the first emulsion as shown by environmental scanning electronic microscopy (ESEM) (Figure 3). Solid carrageenan-gelatin beads without toluene added to the emulsion showed no internal pore structure; although the bead surface was quite rough (Figure 3A and 3B). At 20% (v/v) toluene content, many discrete isolated compartments ($<10\mu m$) were formed in the beads (Figure 3C and 3D). When the toluene content was over 30% (v/v), large numbers of pores with varying sizes were produced in the beads. At this toluene content, the pore structure of the beads became sponge-like. This could easily be seen on the surface and the cross-section (Figure 3E and 3H). More importantly, it was very interesting to find that the embedding polymer, an epoxy resin employed for the ESEM analysis, actually ended up inside the macroporous beads (Figure 3H and 4 arrow). The detailed fine pore structure seen on the bead surface (Figure 3G) could not be seen in the cross-section (Figure 3H). The liquid epoxy could have easily entered the beads when the beads were being embedded. This suggests that as a higher toluene content increasingly produces a large quantity of pores in the beads, the walls between these pores become thinner and weaker. The weakened walls could easily be ruptured during the ethanol washing process, and broken and enlarged during the further drying process. Many pores in the beads were well connected in this manner to form channels. Apparently, connected channels or paths allowed for the epoxy fluid to pass through. Continuous connected channels were also reported in drug solid-polymer mixed systems at solid loadings over 20-25 vol% (Bawa et. al., 1985). While the dissolved drug diffused out from these channels, water entering the polymer followed the same route but in the opposite direction.

The effect of toluene on the pore structure was also revealed by measuring the total surface area of the macroporous beads (Figure 5A). As the toluene content was increased from 0% to 50% (v/v), the surface area soared from 0.22 to 72 m^2/g, an increase by a factor of 300, for the original beads with no heat treatment. It reached a maximum when the toluene content was about 45-50% v/v. The maximum may have been due to partial bead collapse beyond 50% v/v toluene. The high surface area indicated highly porous beads, which is consistent with the ESEM observations. The measurement of the bead packing density confirmed the increasing porosity at the high toluene content. The packing density of the beads decreased from 0.6 g/cm^3 to 0.2 g/cm^3 with an increase in the toluene content from 0% to 50% (v/v)(Figure 5B).

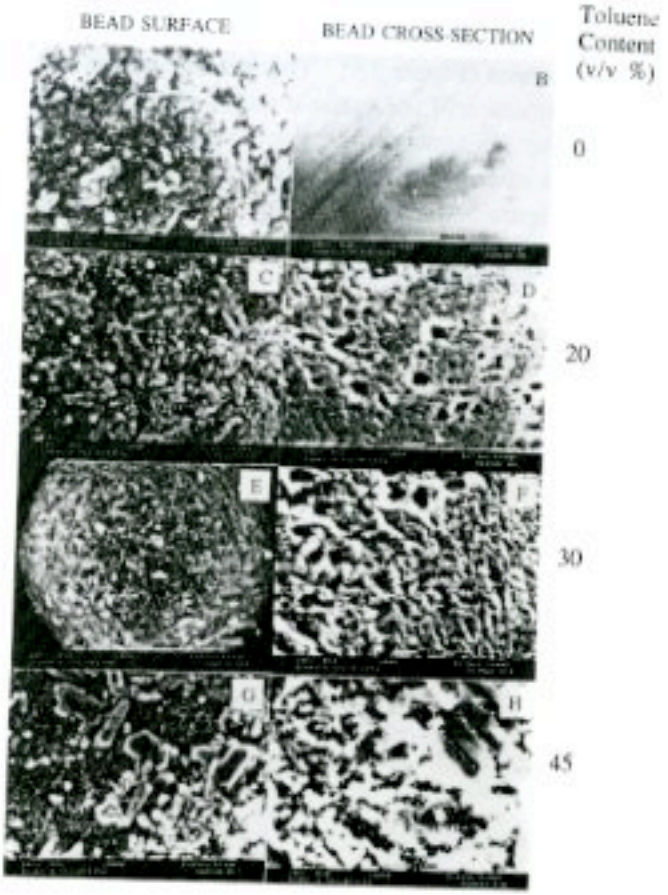

Figure 3. Scanning electron microscopy of macroporous carrageenan beads (100-300 μm). The toluene content was 0% (v/v) in A and B, 20% in C and D, 30% in E and F, and 45% in G and H.

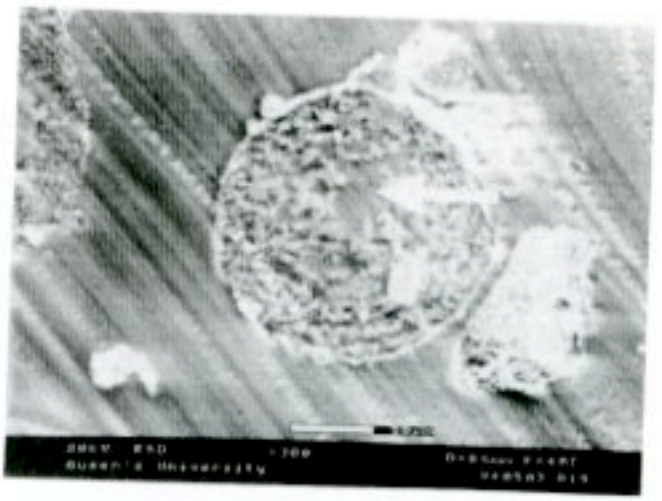

Figure 4. Embedding epon present within pore structure of carrageenan beads (arrow).

The surface area of the beads could be significantly changed by using different heat treatments. After the beads were treated in hot air at 120°C for two hours, to enhance glutaraldehyde crosslinking of the polysaccharide, there was almost no apparent change in the surface area compared with the original beads without treatment (Figure 5A). This result suggests that hot air treatment will not cause a major change in the pore structure. In contrast, there was a reduction in the surface area of beads treated with steam at 120°C for one hour (Figure 5A). The pores in the beads probably collapsed during the steam treatment, which resulted in a decrease in the surface area.

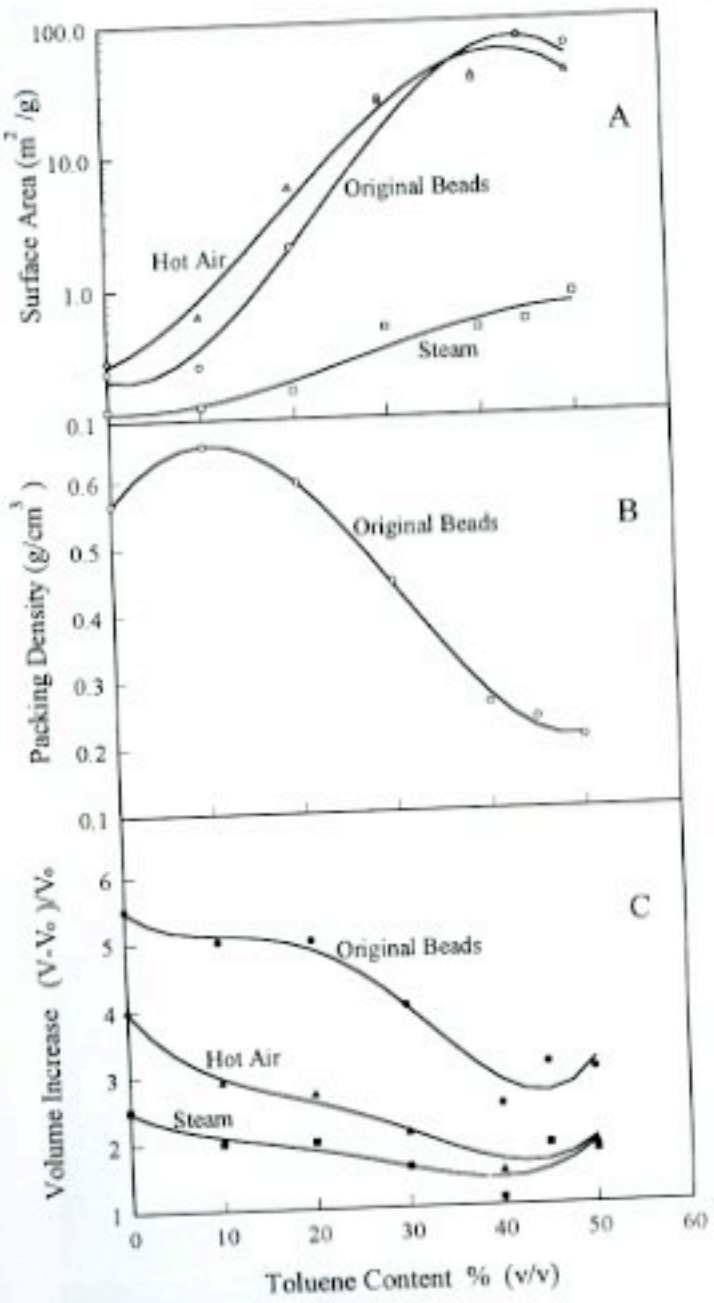

Figure 5. A: The total surface area, B: the packing density, C: the volume increase as a function of the toluene content in the emulsion.

The swelling of the beads in solution was significantly reduced by the heat treatment (Figure 5C). Dried carrageenan-gelatin beads without heat treatment swelled from 6 to 6.5 times their original dried volume when they were incubated in the saline. With a heat treatment of either hot air or steam, bead swelling was reduced to about 2 to 5 times its initial volume. Steam treatment was more effective in depressing bead swelling than hot air treatment. The reduction in volume swelling indicated tightly cross-linked gel matrices. Obviously, the heat treatment greatly increased the cross-linkage of gel matrices and reduced the bead swelling.

All of the experiments on pore structure had been done with carrageenan-gelatin beads. Gelatin had been added to the carrageenan solution to produce beads with increased strength. Unfortunately, gelatin contains proteins as well as unspecified substances, making carrageenan-gelatin beads unsuitable for chromatographic separation. This route for microbead preparation was therefore abandoned. All subsequent studies were performed with pure carrageenan beads.

BEAD STABILITY: Carrageenan beads in pH buffer with 0.1 *M* KCl at a pH range from 1 to 10 kept their integrity with no observed change in shape over the one week observation period. Similarly, the beads were also stable in 2 *M* KOH at pH 13. However, at low ionic strength (0.07 *M* KCl), the beads swelled (Figure 6). There was a 70% increase in bead size in distilled water. In the presence of a gelling ion, K^+, the bead size quickly decreased with an increase in KCl concentration. The bead size appeared to be constant at KCl concentrations over 0.07 *M*. In contrast, the bead size decreased very slowly with an increase in NaCl concentration. At 2 *M* NaCl, the size of the beads was still larger than that of the beads in 0.07 *M* KCl solution.

The high sensitivity of bead size to potassium ions indicates that carrageenan beads were not only chemically crosslinked by epichlorohydrin but also ionically crosslinked by the gelling ion potassium. In water without potassium ions, the hydrogel structure in the beads disappears. Many molecular chains are released from ordered arrangement and become free. These free chains repulse each other due to the same electrical charge. The bead size increased dramatically (i.e. the KCl curve in Figure 6). In distilled water, the carrageenan beads were kept stable by epichlorohydrin crosslinking. The hydrogel structure in the beads recovered in the presence of potassium ions in solution and the bead size decreased rapidly. When the concentration of KCl was over 0.07 *M*, most molecular chains in the beads returned to an ordered structure as the hydrogel configuration and therefore the bead size

stabilized. For non-gelling Na^+ ions, on the other hand, an increase in ion concentration reduced the repulsive force between molecular chains, and the bead size decreased. The shrinkage of the carrageenan beads in the presence of increasing concentration of Na^+ was much slower than that in the case of K^+ ions because no hydrogel formed. The optical examination of the microbeads under different conditions, though, can only be a preliminary measure of the long-term stability and mechanical strength of the beads.

PROTEIN ADSORPTION: Adsorption of cytochrome C on carrageenan beads was demonstrated by isotherms at various ionic strengths at 20°C (Figure 7). At low ionic strength, there was strong adsorption of cytochrome C to the beads. For example, at 0.1 *M* KCl, 0.45 mg of cytochrome C was absorbed to 1 ml swollen beads at 0.2 mg/ml bulk protein concentration. As the ionic strength increased from 0.1 *M* to 0.5 *M*, protein adsorption was dramatically reduced. When the KCl concentration was over 0.5 *M*, only a very small amount of cytochrome C, 0.033 mg, absorbed to the beads at the 0.2 mg/ml bulk protein concentration. In contrast, commercial beads, Sepharose CL-6B, showed almost no protein adsorption even at low ionic strength.

Gel filtration of cytochrome C and lysozyme also confirmed the results of the adsorption study. The elution volume of both proteins (i.e. Ve = 97 ml for cytochrome C and Ve = 187 ml for lysozyme at 0.3 *M* KCl eluent concentration) was much greater than the total volume of the column (i.e. 78 ml) at low ionic strength (Figure 8). With an increase in ionic strength, the elution volume dramatically decreased to about 80

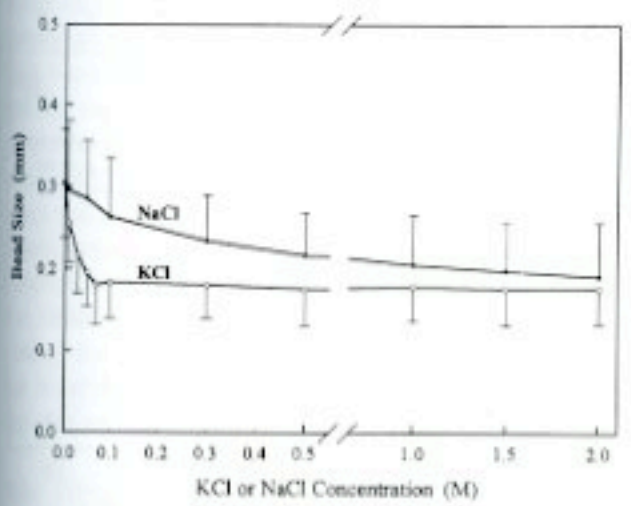

Figure 6. The size of carrageenan beads as a function of ionic strength. Bars represent the standard deviation. Only one side of the bars has been shown to avoid confusion. Each data point consists of at least 10 measurements of bead diameter. The data was generated from a single set of experiments.

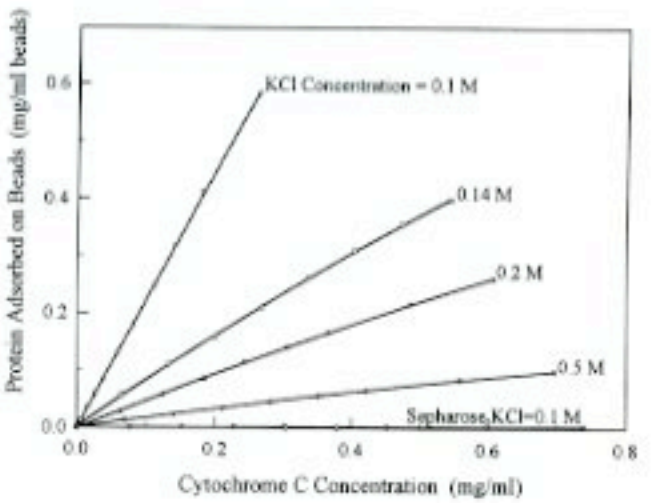

Figure 7. Cytochrome C adsorption isotherms with carrageenan beads at varying ionic strengths. Sepharose CL-6B beads were used as a reference.

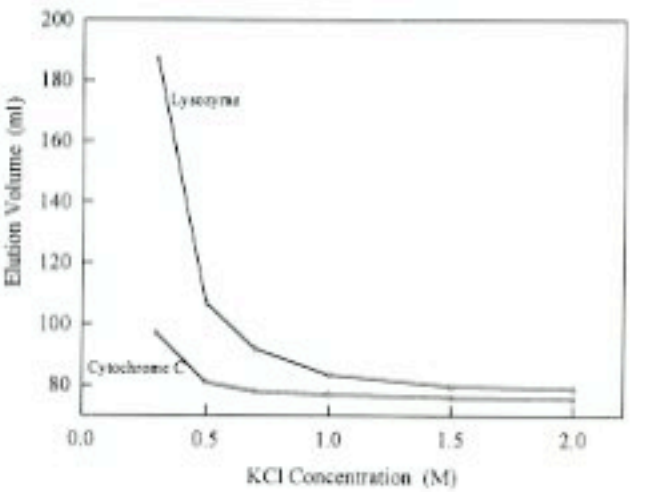

Figure 8. The elution volume of cytochrome C and lysozyme from a carrageenan column (38.8X1.6 cm I.D.) as a function of ionic strength.

ml. The elution volume became constant at KCl concentrations over 1.0 M. The decrease in the elution volume suggests less interaction between proteins and gel beads. The elution volume of cytochrome C at 2 *M* KCl strength was 75.5 ml, which fell reasonably within the normal gel filtration range (i.e. between the excluding volume of 27.1 ml and the total column volume of 78 ml). The interaction of the protein with the beads was greatly minimized at high ionic strength. This result was quite consistent with that observed in the adsorption experiments.

The strong ionic effect on protein adsorption on carrageenan beads indicated that the adsorption was an ionic interaction between proteins and gel beads. If both the geometry and the electrical distribution of

proteins, such as cytochrome C and lysozyme, matched those of gel beads, a strong adsorption was inevitable. Sulphate groups, $-OSO_3^-$, existing in the carrageenan gels were believed to be responsible for the high protein adsorption. Porath et. al. (1971) showed that the adsorption capacity of cytochrome C was proportional to the content of sulphate residuals on agar and agarose gel beads. Our results suggest that the desulphate reaction at 120°C for 20 minutes used in our investigation might not completely remove all the sulphate groups from the carrageenan beads. Further studies on effectively removing these sulphate groups from carrageenan gels are needed.

Although two proteins, cytochrome C and lysozyme, showed strong adsorption to carrageenan beads at low ionic strength; five other proteins investigated, thyroglobulin, apoferritin, amylase, albumin, and carbonic anhydrase, did not exhibit any apparent adsorption to the gels. The elution volume of all these proteins from a carrageenan column fell between the excluding volume and the total column volume. This is the normal separation range for gel filtration. In particular, almost all these proteins could be fully recovered from the carrageenan column when each protein was run through the column on its own (Table 1). The recovery rate for each of five proteins ranged from 99.2% to 100%. It is therefore reasonable to conclude that these protein standards passed through the carrageenan column without adsorbing to the beads.

CALIBRATION CURVES FOR CARRAGEENAN COLUMNS: Calibration curves for a carrageenan column were obtained with a molecular weight marker standard kit, MW-GF-1000, which included blue dextran (MW 2,000,000), thyroglobulin (MW 669,000), apoferritin (MW 443,000), amylase (MW 200,000), albumin (MW 66,000), and carbonic anhydrase (MW 29,000). Carrageenan gels exhibited a wide separation range for proteins with molecular weight from 30 *K* to over 700 *K*, which indicated a broad pore size distribution in the gel matrices (Figure 9). The dimensionless elution volume was 1.49 for thyroglobulin and 2.62 for carbonic anhydrase at 0.1 *M* KCl with 0.01 *M* phosphate buffer. These calibration curves were significantly affected by the ionic strength of eluent. Proteins at low ionic strength were eluted much faster than at high ionic strength (i.e. smaller elution volume). As the KCl concentration decreased from 0.3 *M* to 0.1 *M*, the dimensionless elution volume was reduced from 2.2 to 1.49 for thyroglobulin, from 2.89 to 2.20 for albumin, and from 3.07 to 2.62 for carbonic anhydrase. The calibration curve for carrageenan gels at 0.01 *M* phosphate buffer with 0.1 *M* KCl was quite close to that of Sepharose CL-6B gels (Figure 9). However, the slope of the carrageenan calibration curve in this case was not as steep as that of the Sepharose curve (i.e. the absolute value of slope 1.2 versus 2.1 respectively).

TABLE 1

The mean recovery rate(and SD)of various proteins in 0.01 M phosphate buffer with 0.1 M KCl at pH 7 from a Sepharose column (46.3X1.6 cm I.D.) or a carrageenan column (38.8X1.6 cm I.D.) based on two to six runs.

Proteins	Protein Recovery (% w/w)	
	Carrageenan Beads	Sepharose CL-6B
Thyroglobulin (MW 669,000)	99.8 ± 0.9	100 ± 0.7
Apoferritin (MW 443,000)	100 ± 0.2	99.3 ± 0.2
Amalyse (MW 200,000)	100 ± 0.3	100 ± 1.0
Albumin (MW 66,000)	99.7 ± 0.8	99.8 ± 0.4
Carbonic Anhydrase (MW 29,000)	100 ± 0.4	99.2 ± 0.5

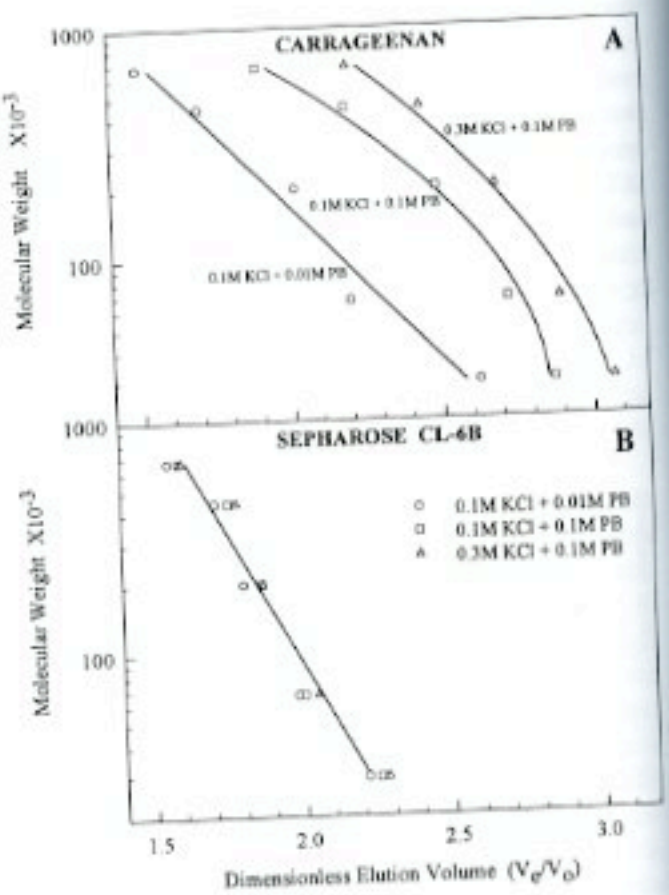

Figure 9. Ionic strength effects on molecular calibration curves for A: carrageenan column (51.3X1.6 cm I.D.) and B: Sepharose CL-6B column (54X1.6 cm I.D.). Blue Dextran (MW 2,000,000) was used as reference to obtain the excluding volume, V_o = 39.9 ml for the Sepharose column. PB stands for phosphate buffer.

This suggests that the pore size distribution of carrageenan beads at low ionic strength was narrower than that of Sepharose beads. The elution volume of the standard proteins in the carrageenan column increased with an increase in ionic strength. At high ionic strength, the elution volume of the smaller proteins, molecular weight below 100 *K*, became similar. The differences between the dimensionless elution volume of albumin and carbonic anhydrase, for example, was 0.42 at 0.1 *M* KCl and only 0.18 at 0.3 *M* KCl. This indicates that the pore size of the carrageenan gels increased at high ionic strength. The opposite shrinkage behavior has been found with polyvinyl alcohol hydrogel networks (Bachtsi and Kiparissides, 1995). The separation of smaller proteins in the carrageenan column was more difficult at high ionic strength. In contrast, the elution volume of the proteins in the Sepharose column changed only slightly at varying ionic strengths.

The increase in the elution time could result either from a decrease in protein size and/or an increase in pore size of the gels. A high concentration of ions in solution could diminish the repulsive force among various side groups on a macromolecule and therefore reduce its size. Figure 9 shows that eluent ionic strength did not have an effect on protein size as shown by the almost constant elution volumes of proteins in the Sepharose column at different KCl concentrations. These conditions, however, may not be optimal for the Sepharose (i.e., to demonstrate the possible effect of ionic strength). On the other hand, in the case of carrageenan beads, eluent ionic strength had a very large effect on protein elution volume. This could therefore be attributed to a change in gel pore size. The variation in pore size constitutes a major contribution affecting the elution volume. Carrageenan gels were formed by multiple ionic reactions between gelling cations and anionic polymer chains. We can speculate that at high ionic concentration, the polymer chains were more tightly bundled together. This left more space between the polymer bundles for the protein macromolecules to pass through (Figure 10). In contrast, a loose polymer structure probably resulted at the lower ionic concentration. This reduced the pore size of the gels significantly, making it more difficult for the diffusing proteins to enter, and in turn reducing the elution volume in gel infiltration.

GEL FILTRATION: The separation of three proteins, thyroglobulin (MW 690,000), albumin (MW 66,000) and carbonic anhydrase (MW 29,000), using a carrageenan column (51.7X1.6 cm I.D.) at varying eluent flow rates was assessed (Figure 11). This was compared with results from a Sepharose CL-6B column (54X1.6 cm I.D.). The three proteins were separated

TABLE 2

The separation resolution of thyroglobulin, albumin and carbonic anhydrase from a carrageenan column and a Sepharose column at varying flow rates. The eluent was 0.01 M phosphate buffer with 0.1 M KCl.

Flow Rate ml/h	Separation Resolution R*			
	Carrageenan Column (51.7X1.6 cm I.D.)		Sepharose Column (54x1.6 cm I.D.)	
	Thyroglobulin-Albumin	Albumin-Carbonic Anhydrase	Thyroglobulin-Albumin	Albumin-Carbonic Anhydrase
2.6	5.2	4.0	4.1	2.7
11	3.3	2.9	2.6	1.8
35	2.2	1.6	1.7	1.0

*$R = 2(V_{e2}-V_{e1})/(W_1+W_2)$, where V_{e1} and V_{e2} are elution volumes of different elutes, and W_1 and W_2 are the peak widths respectively.

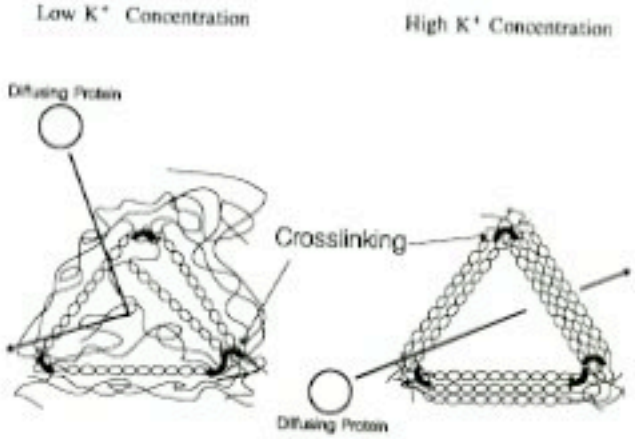

Figure 10. Suggested change in gel structure with K^+ concentration and its effect on protein diffusion.

more efficiently (i.e. better resolution) in the carrageenan column than in the Sepharose column, especially at high flow rates. At a flow rate of 35 ml/h, the resolution, R, between each pair of proteins was greater than 1.58, indicating very good separation. In contrast, for the Sepharose column, R = 1.04 for the separation between albumin and carbonic anhydrase. The elution volume of the proteins appeared to be farther apart from each other in the carrageenan column, compared to Sepharose, suggesting a comparatively narrow distributed pore size in carrageenan beads. For Sepharose beads, the pore size distribution was probably much wider than that of the carrageenan beads, which resulted in elution volumes being closer between different proteins, especially for albumin and carbonic anhydrase. For example, the difference between the dimensionless elution volumes of albumin and carbonic anhydrase was 0.42 in the carrageenan column, compared with only 0.23 in the Sepharose column. At high flow rate (>30 ml/h), both proteins could not be well separated from the Sepharose

column (i.e., resolution was 1.04), while the carrageenan column still gave a good resolution (i.e., the resolution was 1.58, Table 2).

The reduced plate height for a Sepharose CL-6B column (46.3X1.6 cm I.D.) and a carrageenan column (51.7X16 cm I.D.) was determined by separately running albumin and thyroglobulin through the columns at varying flow rates (Figure 12). Both coordinates in Figure 12 employed reduced parameters, the reduced plate height, $h = H/d_p$, and the reduced velocity , $\nu = d_p\mu/D$, where H is the plate height (determined from the column height, L, divided by the plate number, N) d_p the bead diameter, u the linear velocity of eluent (determined from L divided by the void elution time, t_o), and D the protein diffusivity in solution. The results showed that the reduced plate height for carrageenan beads (75-180 μm) was slightly better (i.e. smaller plate height) than that of Sepharose CL-6B (40-140 μm). The reduced plate height for the carrageenan beads was about 5-15% lower than that of the Sepharose beads for albumin, and 15-30% lower for thyroglobulin. In general, the almost equivalent efficiency of carrageenan and agarose beads in protein separation was to be expected since the two polysaccharides are similar in chemical structure and gel properties.

Conclusions

The porosity and the pore structure of the carageenan beads was dictated by the toluene content in the first emulsion. The high toluene content created more pores in the beads and greatly increased the bead surface area. When the toluene content was over 30% (v/v), channels were produced by rupture of various discrete compartments or isolated pores in the beads. These channels could possibly form continuous macropores to allow for convection passing through the beads. The swelling of the beads in solution was also found to be controlled by the heat treatment. Heat treatment results in more tightly cross-linked gel matrices, which cannot swell as much in aqueous

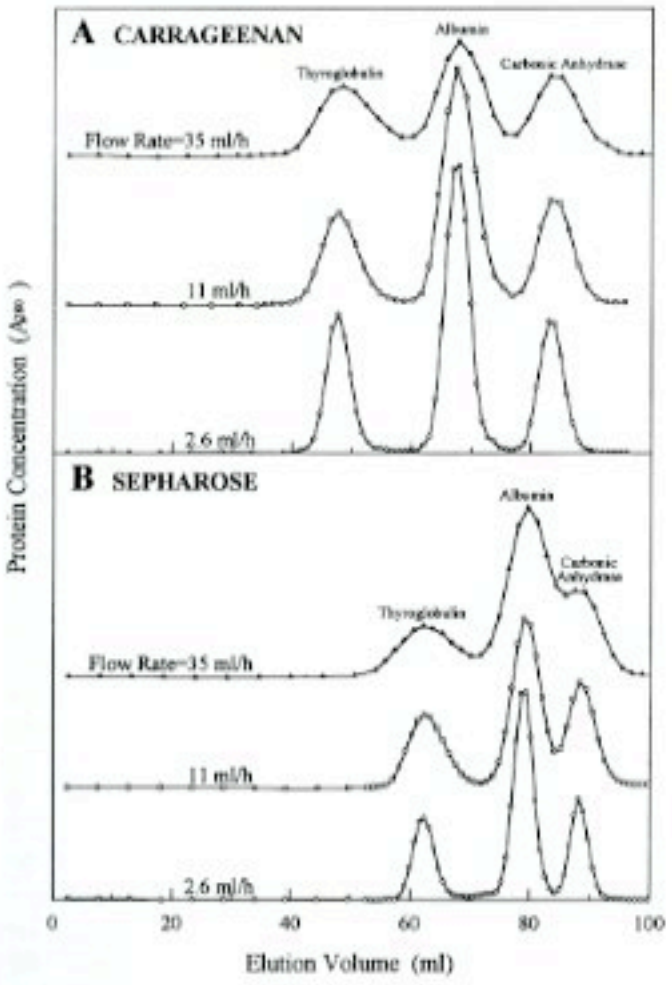

Figure 11. Elution of thyroglobulin, albumin and carbonic anhydrase in 0.1 M phosphate buffer with 0.1 M KCl at pH 7 from A: carrageenan column (51.7X1.6 cm I.D.) and B: Sepharose CL-6B column (54X1.6 cm I.D.).

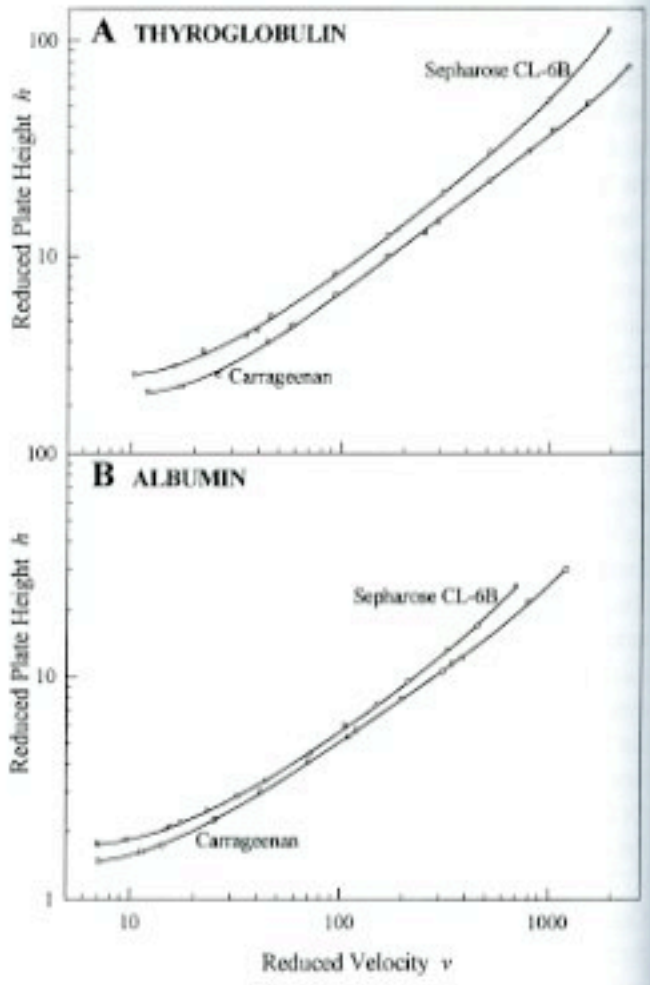

Figure 12. Column efficiency versus reduced velocity for a carrageenan column and a Sepharose column by running a non-retained protein. A: thyroglobulin and B: albumin in 0.1 M phosphate buffer with 0.1 M KCl at PH 7 were run through each column.

solution. The hot air treatment showed no change in the bead surface area, whereas, the steam treatment decreased the surface area suggesting the presence of collapsed pores.

Crosslinked carrageenan beads showed good stability in solutions of varying pH. At low ionic strength, however, the size of the crosslinked hydrogel beads increased . The bead size decreased with an increase in ionic strength. Gelling ions, such as potassium, were more effective in decreasing the bead size than sodium ions due to the formation of gels. The ionic strength also affected the pore structure of the beads. The elution volume of non-adsorbed proteins in a carrageenan column decreased with a decrease in eluent ionic strength. This increased the difference between elution volumes of low molecular weight proteins, which resulted in a better separation of the small proteins than that which could be achieved with a Sepharose CL-6B column. Overall, the separation efficiency of carrageenan beads was slightly better than that of Sepharose beads. However, in all fairness, the gel filtration experiments with sepharose may not have been run under "best case conditions". One disadvantage of the present carrageenan beads was high protein adsorption at low ionic strength with certain proteins. This adsorption could be caused by residues of sulphate groups in the carrageenan beads. Further treatment with lithium aluminum hydride in dioxane may effectively reduce the adsorption capacity of carrageenan beads and make them more suitable for gel filtration chromatography.

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