Novel Thermally and Mechanically Stable Hydrogel for Enzyme Immobilization of Penicillin G Acylase via Covalent Technique

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Received 8 August 2007; accepted 4 March 2008
DOI 10.1002/app.28379
Published online 11 June 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: κ-Carrageenan hydrogel crosslinked with protonated polyethyleneimine (PEI^+) and glutaraldehyde (GA) was prepared and evaluated as a novel biocatalytic support for covalent immobilization of penicillin G acylase (PGA). The method of modification of the carrageenan biopolymer is clearly illustrated using a schematic diagram and was verified by FTIR, elemental analysis, DSC, and INSTRON using the compression mode. Results showed that the gels' mechanical strength was greatly enhanced from 3.9 kg/cm^2 to 16.8 kg/cm^2 with an outstanding improvement in the gels thermal stability. It was proven that, the control gels were completely dissolved at 35°C, whereas the modified gels remained intact at 90°C. The DSC thermogram revealed a shift in the endothermic band of water from 62 to 93°C showing more gel-crosslinking. FTIR revealed the presence of the new functionality, aldehydic carbonyl group, at 1710 cm^{-1} for covalent PGA immobilization. PGA was successfully immobilized as a model industrial enzyme retaining 71% of its activity. The enzyme loading increased from 2.2 U/g (control gel) to 10 U/g using the covalent technique. The operational stability showed no loss of activity after 20 cycles. The present support could be a good candidate for the immobilization of industrial enzymes rich in amino groups, especially the thermophilic ones. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 4105–4111, 2008

Key words: carrageenan; biopolymer; covalent immobilization; penicillin G acylase; hydrogel

INTRODUCTION

Industrial applications of immobilized biocatalysts have been gaining importance in recent decades as they are widely used in pharmaceutical and food biotransformations.[1,2] For example, penicillin G acylase, β-galactosidase, and pectinase are among the fewest industrially used enzymes.[3] Penicillin G acylase (PGA) is considered to be one of the most importantly used enzymes in drug production. PGA is catalyzing the hydrolysis of penicillin G (PG) to produce 6-aminopenicillianic acid (6-APA), which is a precursor for manufacturing many semisynthetic antibiotics such as amoxicillin and ampicillin, representing 19% of the estimated worldwide antibiotic market.[4]

Many carriers, both organic and inorganic, have been used for the immobilization of PGA.[5] However, organic polymeric carriers are most widely used for the immobilization of enzymes.[6] Organic carriers were previously classified into synthetic and natural carriers.[7] Synthetic organic carriers such as Eupergit C and oxirane acrylic enjoy high thermal and mechanical stability as well as resistance to wide pH ranges and buffers. They have been used as enzyme carriers due to the mild reaction conditions in the immobilization process.[8] However, they have a disposal problem in addition to being very expensive.

On the other hand, organic carriers based on natural polymers such as biopolymers: alginate, agarose, cellulose, carrageenan, and gelatin have the advantage of being generally inexpensive, of hydrophilic nature, environmentally friendly, and porous, which allows enhanced enzyme loading. For example, Boadi et al., 2001[9] used alginate and carrageenan to entrap tannase then crosslinked the gel beads with chitosan followed by glutaraldehyde. Whereas, Shukla et al., 2004[10] used κ-carrageenan gel to entrap horseradish peroxidase and crosslinked the gel beads using KCl and PEI. However, these supports suffer from one or more of the following problems: (a) poor thermal stability, (b) poor mechanical stability, (c) poor stability towards microbial attacks, d) they use the entrapment technique, which limits their industrial use as supports for enzyme immobilization due to enzyme leakage. So, efforts to immobilize

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Contract grant sponsor: Laboratory of Advanced Materials and Nanotechnology, National Research Centre.

enzymes on newer type of carriers, especially with covalent bonds are still underway in many laboratories.[11–14] The covalent technique has the draw back of loosing some of the bound enzyme activity compared with the adsorption and entrapment ones. On the other hand, it keeps the enzyme so well bound to the support that the enzyme does not leak, which is very useful in reuse.

As an example of a naturally abundant biopolymer, carrageenan (a water-soluble sulfate galactans) is isolated from red seaweed polysaccharides. There are three forms of carrageenans: κ-carrageenan (carrageenose 4′-sulfate), ι-carrageenan (carrageenose 2,4′-sulfate), and λ-carrageenan (carrageenose 2,6,2′-sulfate)[15] and they differ in their ratios of sulfates to hydroxyl groups. The number of sulfate groups decreases from λ-carrageenan to ι-carrageenan to κ-carrageenan. It is well known that the attachment of the $-SO_4^-$ substituent can convert organic compounds (even hydrocarbons) to water-soluble derivatives. Thus λ-carrageenan does not form a gel, whereas ι- and κ-carrageenans do. κ-Carrageenan was found to form a harder gel than ι-carrageenan since the latter contains more sulfate groups than κ-carrageenan.

κ-Carrageenan gel is one of the main supports used for cell and enzyme immobilization via entrapment.[16–18] However a drawback of unmodified carrageenan is its poor mechanical and thermal stability,[19] in addition, it has a lack of active functional groups to immobilize enzymes covalently.

The biotechnology of red seaweed polysaccharides and in particular that of carrageenans, is still in its infancy compared with other polysaccharides such as alginates and agaroses. Chao et al., 1986[20] studied the alterations to the carrageenan-gel mechanical and thermal stability brought about by KCl and a range of amino compounds. They concluded that all amino compounds improved the gels mechanical strength, but only polyamines substantially improved the gels thermal stability (for example, hexamethylendiamine did not improve the gels thermal stability). Hardening of the gel was not optimized and use of these amino compounds was restricted to the hardening of already entrapped enzymes and cells. However, to our knowledge, there are no reports on the use of κ-carrageenan for the immobilization of enzymes using covalent technique via PEI^+ and GA. In addition, previous reports on the carrageenan-polyethylenimine system have not to any greater extent dealt with the effect of the variation of charge density on the formation of polyelectrolytes.

Thus, the objectives of the present study are, first to evaluate the effect of PEI^+ at different pHs (pH 7–9), i.e., below the pKa of the PEI at pH $\sim$ 9.5[21] on the mechanical and thermal stability of κ-carrageenan gel. The polyanionic k-carrageenan gel can be strengthened via the formation of an ionic complex (network) between the protonated amino groups (cations) of PEI^+ and the sulfate groups of the gel (anions). Knowing that hardening of hydrogels by polyelectrolyte complexation is an interesting alternative to covalently crosslinked hydrogels.[22] Second, the remaining free amine moieties of PEI^+ were activated using the bi-functional spacer arm, glutaraldehyde, to bind covalently the industrially commercial enzyme, PGA, as a model enzyme. The chemical modification was elucidated by FTIR and DSC and the immobilization capacity and efficiency of the enzyme were proven by the enzymes activity, operational stability and Lowery assay.

MATERIALS

κ-Carrageenan (Mr: 154,000; sulfate ester $\sim$ 25%) and penicillin G acylase enzyme (40 U/mg) were obtained from Fluka. Polyethyleneimine was obtained from Sigma. Other chemicals were of Analar or equivalent quality. For mechanical strength measurements, INSTRON instrument (model 5564) was used in compression mode. Parallel plate equipment was made in our laboratory for uniform gel sheets preparation. The gel disks dimensions were measured using a micrometer (Micro 2000, 0–25 mm).

EXPERIMENTAL

Preparation of κ-carrageenan gel disks

κ-Carrageenan gels were prepared at a concentration of 2% (w/v) carrageenan in distilled water at 70°C and 0.002% (w/v) NaN_3 was added as antibacterial. The gel solutions were mixed thoroughly using an overhead mechanical stirrer until complete dissolution had occurred. Glass parallel plates equipment designed by Elnashar et al., 2005[23] with 10-mm gaps were then immersed into the hot gel to produce homogeneous gel sheets. The 10-mm thick gel-sheets were cut into disks using cork borers for enzyme immobilization and for mechanical strength measurements. Typically, 3-mm diameter gel disks of average weight 90 mg were produced for immobilization, while 10-mm diameter gel disks were cut out for mechanical strength measurements.

Hardening of the gel disks and Schiff's base formation

The gel disks were hardened using 0.3*M* KCl for 3 h as a control[17] and with a series of 1–7% (v/v) polyethylenimine, pH 8 for 2 h to evaluate the optimum PEI^+ concentration. Then, the optimum PEI^+ concentration was used at different time, 10 min to 12 h to harden the gel. Finally, the gel disks were soaked for

8 h in a series of PEI^+ of pH 7–9, which is below the pKa of PEI (~ 9–9.5) to create some protonated amino groups. We aimed to have a partially protonated PEI so that the protonated part will stick to the carrageenan gel forming a polyelectrolyte complex to improve the thermal and mechanical properties of the gel.[20] While the unprotonated part, free amino groups, will react with the glutaraldehyde forming Schiff's base and creating a free aldehyde terminal group to bind the enzyme with a covalent bond. The modified gel disks were thoroughly washed with distilled water and were soaked in 2% (v/v) glutaraldehyde solution for 2 h. In all above experiments, the gel disks with the highest mechanical strength were chosen for further experiments as an optimum formulation.

Mechanical strength measurement

Gel-disks of 10-mm height and 14-mm diameter were subjected to compression using an INSTRON model 5564 mechanical strength testing machine. The gel strength was measured as the critical compression force, the force required to rupture the gel disk. The test pieces were placed on the INSTRON bottom plate and compressed at a speed of 10 mm/min under a load of 1 *N*. As the disk was compressed (strained), it exerted an increasingly higher reaction force against the compression. Beyond a certain deformation (stress), the material can no longer resist and fractures. The mechanical strength of the sample was calculated from load per unit area of the disk (kg/cm^2) at the fracture point. Samples were used in triplicates and data are means ± SD ($n = 3$).

Thermal stability of carrageenan gel formulations

Three formulations of gel disks were used for this test: blank gel of 2% (w/v) κ-carrageenan, control (blank/KCl) and modified formula of 2% (w/v) κ-carrageenan soaked in 4% (w/v) PEI^+ at pH 7.0 for 8 h followed by 2% (v/v) glutaraldehyde for 2 h. The gel disks were incubated for 1 h in distilled water at 25–90°C. The appearance of the gel was then inspected visually to check whether the gel disks remains solid or dissolves.

Elucidation of the modified gel using FTIR, elemental analysis and DSC

Fourier transform infrared spectroscopy

IR transmission spectra were obtained using a FTIR spectrophotometer (FTIR-8300, Shimadzu, Japan). The test is aiming to prove the presence of the new functional group, carbonyl group, after carrageenan gel modification with PEI^+ followed by glutaraldehyde. A total of 2% (w/w) of the sample, with respect to the potassium bromide (KBr; S. D. Fine Chem) disk, was mixed with dry KBr. The mixture was ground into a fine powder using an agate mortar before it was compressed into a KBr disk under a hydraulic press at 10,000 psi. Each KBr disk was scanned 16 times at 4 mm/s at a resolution of 2/cm over a wavenumber range of 400–4000/cm, using Happ-Genzel apodization. The characteristic peaks were recorded.

Elemental analysis

The elemental analysis of pure carrageenan powder and the modified dry gel, 2% (w/v) carrageenan followed by protonated PEI at pH 7, has been performed to prove the incorporation of the PEI to the carrageenan gel. The analysis was made for 5–10 mg of carrageenan powder and the dry gel using the ''Vario El Elementar''apparatus.

Differential scanning calorimetry

The differential scanning calorimetric analysis was used to characterize the thermal behavior of the gel-PEI^+ polyelectrolyte complex to prove the gel-hardening process. Two samples were analyzed, the blank carrageenan gel and the gel modified with PEI^+ at pH 7 followed by GA. DSC thermograms were obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan). The temperature calibration was performed using indium as the standard. Samples were crimped in a standard aluminum pan and heated from 0 to 200°C at a heating rate of 10°C/min under constant purging of dry nitrogen at 30 mL/min. An empty pan, sealed in the same way as the sample, was used as the reference.

PGA immobilization and soluble protein determination

For immobilization of PGA, four disks of modified gel (10-mm height × 3-mm diameter) were washed thoroughly with distilled water and soaked in 2 mL of 7.5 U/mL PGA for 30 min. The gel disks were thoroughly washed with 0.1*M* phosphate buffer, pH 7.5 and the supernatant and the wash were kept for soluble protein assay via Lowery assay using bovine serum albumin (BSA) as a standard protein. The protein immobilized on and into the carrier *Pg* (mg/g) was calculated using the following equation:

$$Pg = \frac{CoVo\_C_fV_f}{w} \quad (1)$$

where *Co* is the initial protein concentration (mg/mL), C_f the protein concentration of the filtrate (mg/mL), *Vo* the initial volume of the enzyme solution

Scheme 1 Hydrolysis of penicillin G by penicillin G acylase.

(mL), V_f the volume of filtrate (mL) and w the weight of carrier material used (g).

Free and immobilized penicillin G acylase assays

PGA was assayed using its native substrate, penicillin G (PG). Assays were carried out in 0.1*M* NaH_2PO_4 pH 8.2, 15 m*M* penicillin G, 0.002% (w/v) phenol red indicator at 25°C in a 4 mL assay volume. The assay was prepared as a mixture containing the NaH_2PO_4, PG, and phenol red as shown in Scheme 1.

In the case of immobilized PGA, two gel disks were washed thoroughly to get rid of any unbound enzyme and the covalently immobilized enzyme on and into the gel's pores were stored in phosphate buffer solution at 4°C for enzyme assay. Free or immobilized PGA was added to the assay mixture after which the absorbance at 430 nm was monitored and it was proportional to the concentration of the produced phenyl acetic acid according to the Beer–Lambert Law. One unit of activity is defined as the amount of enzyme that will produce 1 μmol of phenyl acetic acid per min under the assay conditions.[24] All experiments were carried out in triplicate and data are means ± SD (n = 3).

Operational stability of immobilized PGA

The reusability of immobilized PGA was studied using the control and modified gel. The control is carrageenan gel hardened with 0.3*M* KCl for 3 h, whereas the modified gel formulation is carrageenan gel hardened with 4% (w/v) PEI^+ at pH 7 for 8 h followed by 2% (v/v) GA for 2 h. Both formulations were then soaked in 7.5 U/mL PGA for 30 min. Two disks of immobilized PGA, control and modified gel containing 1.4 and 2.9 U respectively, were immersed in 2 mL containing 15 m*M* PG and 0.1 m*M* NaH_2PO_4, pH 8.2 were shaken vigorously for 5 min after which the absorbance at 430 nm was measured to estimate PGA activity. The same gel-disks were then washed with a buffer of 0.1*M* NaH_2PO_4, pH 7.5 and re-assayed as above; this procedure was repeated 20 times and the starting operational activity was considered as 100% relative activity.

RESULTS AND DISCUSSION

Optimization of PEI^+ concentration, soaking time and pHs

Optimization of the blank formulation of 2% (w/v) carrageenan has been achieved through PEI^+ at different concentrations, pHs and soaking time and the final results were shown in Figure 1(a–c). Figure 1(a) revealed that the blank formulation has a mechanical strength of 2 kg/cm^2, whereas the control one using 0.3*M* KCl for 3 h showed an increase to 3.9 kg/cm^2. The blank gel after being treated with 1–7% PEI^+ at pH 8 for 2 h, revealed a maximum mechanical strength of 7.3 kg/cm^2 with an optimum PEI^+ concentration range of 3–5% (v/v). The outstanding increase in the mechanical strength using PEI^+ compared with the control (3.9 kg/cm^2) may be attributed to the fact that KCl is a monovalent cation, whereas PEI^+ is a polycation, which could form a gel network through many points.[25] A PEI^+ concentration of 4% (v/v) has been chosen for optimization of the soaking time as shown in Figure 1(b). The gel mechanical strength increased by increasing the soaking time reaching its maximum value of 9.5 kg/cm^2 after 8 h. By decreasing the pH of PEI^+, from pH 9–7 as shown in Figure 1(c), the gel mechanical strength revealed a gradual increase from 9.5 to 12.5 kg/cm^2. This may be due to the increase in number of protonated amino groups of PEI with the decrease of pH, so more ionic interaction between protonated amino groups and sulfate groups in carrageenan forming stronger polyelectrolyte complex. For future studies, measuring zeta potential of the modified gel could give quantitative results on the degree of gel protonation.

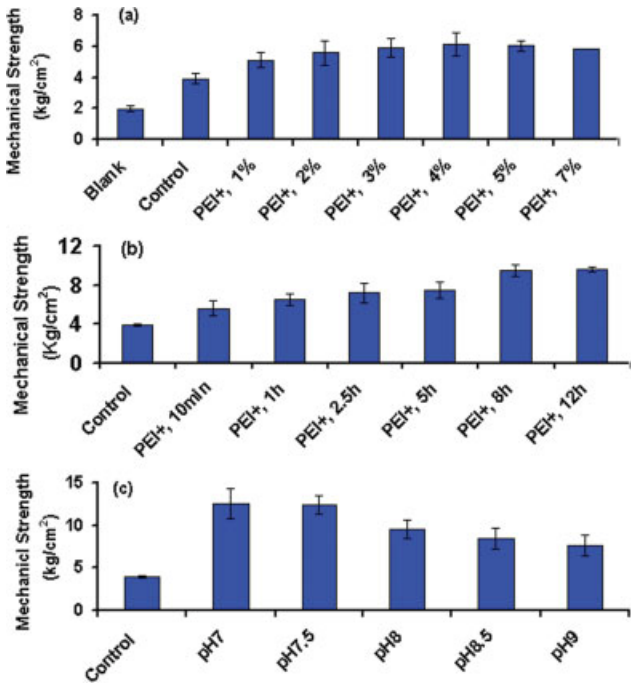

Figure 1 (a–c). Effect of soaking carrageenan gel (a) for 3 h in 0.3*M* KCl (control) and in 1–7% (v/v) PEI^+ at pH 8; (b) for 10 min to 12 h in 4% (v/v) PEI^+ at pH 8; (c) for 8 h in 4% (v/v) PEI^+ at pH 7–9 on the gel mechanical strength. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

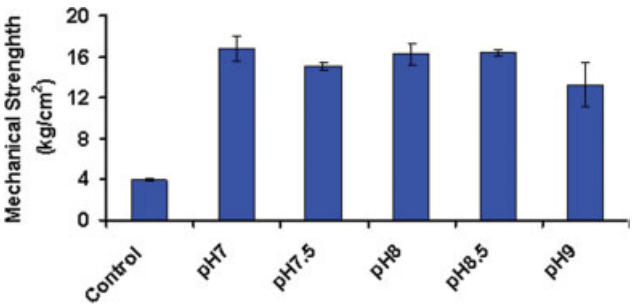

Figure 2 Effect of soaking carrageenan gel in 4% (v/v) PEI^+ at pH 7–9, then in 2% (v/v) GA for 2 h on the gel mechanical strength. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Schiff's base formation and gel thermal stability

The gel formulations using 4% (v/v) PEI^+ at pH 7 to pH 9 for 8 h have been further modified with the bifunctional spacer arm, glutaraldehyde of 2% (v/v) for 2 h to form Schiff's base (visually characterized) with the free amino groups of PEI^+[26] and the unreacted GA side created a free aldehyde group (proved by FTIR) for covalent immobilization of PGA. The mechanical strength of the new carrageenan/PEI^+/GA showed an improvement over all of the corresponding carrageenan/PEI^+ formulations. The maximum mechanical strength for carrageenan/PEI^+/GA was achieved at 16.8 kg/cm^2, as shown in Figure 2, which could be attributed to the crosslinking of the PEI's free amino groups with glutaraldehyde. The thermal stability of the PEI^+ formulations revealed greater stability over the control gel, where the control gel dissolved at 35°C, all modified gel formulations remained intact at 90°C. This fact has been supported by Chao et al., 1986,[20] who proved that only polyamines substantially improved the gels thermal stability. The outstanding improvement in the modified gels thermal stability could be regarded to the strong polyelectrolyte complexation between the polyanion, carrageenan gel, and the polycation, PEI^+.[27]

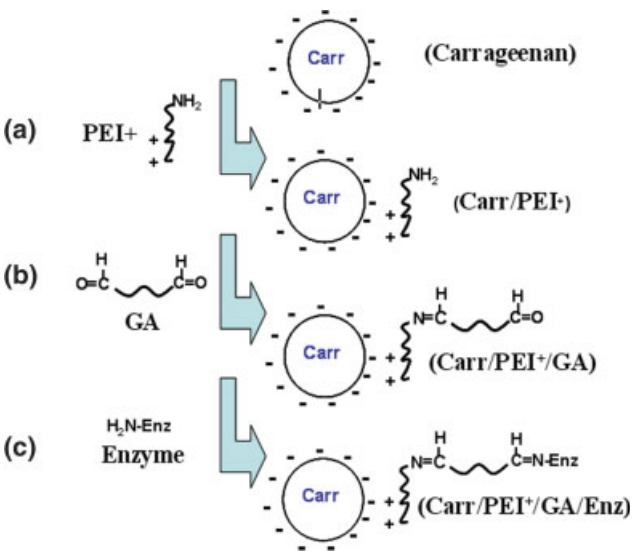

Figure 3 Schematic representation of gel modification and enzyme immobilization. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Schematic representation of gel modification and enzyme immobilization

The whole process of gel modification, Schiff's base formation and covalent immobilization of PGA has been explained and illustrated in a simple schematic diagram as shown in Figure 3.

Figure 3(a) is showing the hardening of the carrageenan gel with PEI^+, to form Carrageenan-PEI^+ polyelectrolyte crosslinking. In Figure 3(b), the free amino groups of PEI^+, have been used to react with the bifunctional spacer arm, glutaraldehyde, to form Schiff's base and to create a new functional group, aldehyde group, on the modified carrageenan gel. In Figure 3(c), the gel free aldehyde group is coupled with the lysine amino groups of the PGA to form covalent immobilization.

FTIR, elemental analysis and DSC

The FTIR spectroscopy (Fig. 4) of the unmodified and the modified gel (carrageenan/PEI^+/GA)

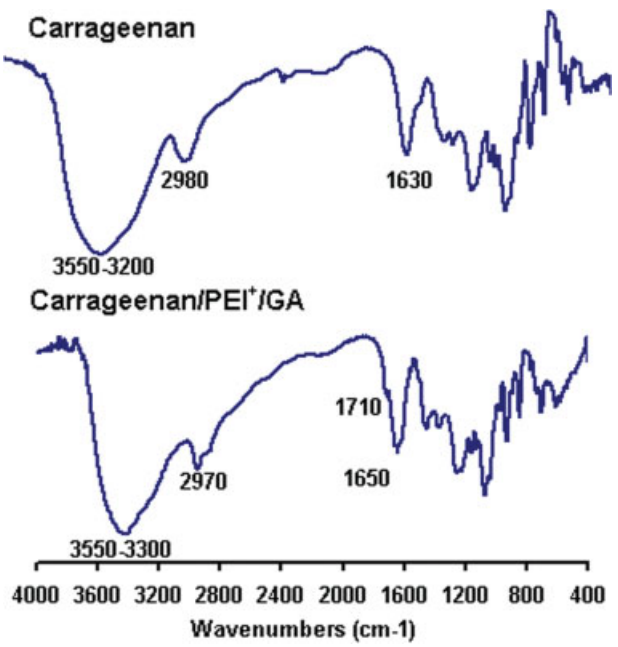

Figure 4 FTIR of carrageenan and modified carrageenan with protonated polyethylenimine (PEI^+) and glutaraldehyde (GA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

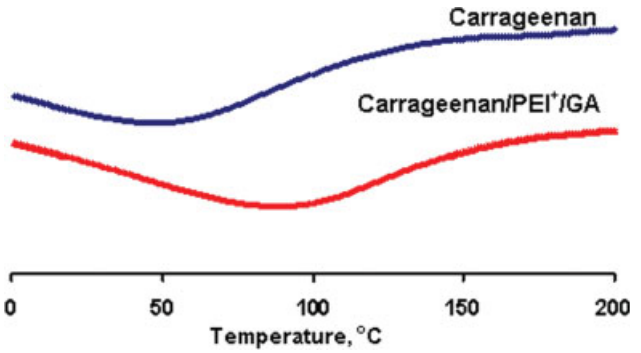

Figure 5 DSC thermogram of carrageenan and modified carrageenan with protonated polyethylenimine (PEI^+) and glutaraldehyde (GA). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proved the presence of a new gel functional group using only the modified gel, aldehydic carbonyl group, at 1710 cm^{-1}. Although the aldehydic carbonyl band is small, it has been proved by the Schiff's base color formation, which showed an increase in color from pale yellow to brown red color by increasing the PEI protonation from pH 9–7, whereas the unmodified gel was colorless. Schiff's base band could also be part of the shoulder at 1610–1670 cm^{-1}. The FTIR bands of carrageenan after the gel-complex formation have been perturbed. That could be due to the interaction between carrageenan, PEI^+ and GA derived from hydrogen bonds formation between the hydroxyl, carbonyl and amino groups.[28] The elemental analysis data for the pure carrageenan was 20.319% (C), 3.232% (H), 0% (N), 3.167% (S) and 73.282% (O), whereas for the modified gel with PEI^+ at pH 7 it was 33.455% (C), 9.671% (H), 15.059% (N), 1.61% (S), and 40.205% (O). The absence of the nitrogen in the pure carrageenan and its presence in high concentration in the modified gel proved the presence of PEI in contact with the carrageenan gel as shown in Figure 3(a). The DSC thermogram of modified and unmodified carrageenan gels was shown in Figure 5. The results were revealing a broad endothermic peak at about 62°C due to evaporation of absorbed water molecules in the thermogram of κ-carrageenan gel.[29] This band has been shifted towards a higher temperature (93°C) in the modified gel, which could be regarded to an increase in the gel thermal stability leading to gel dehydration and loss of water molecules at higher temperature.

PGA immobilization via adsorption and covalent technique

PGA immobilization was made using all the formulations used in ''Schiff's base formation and gel thermal stability'' section due to the fact that all formulations showed high and comparable mechanical strength as shown in Figure 2. The data shown in Table I are revealing a maximum PGA loading and percent enzyme efficiency of 10 U/g wet gel and 71%, respectively, in case of PEI^+ at pH 7 compared to 4.8 U/g wet gel and 21% in case of the control. It is likely that the PGA loading was maximum using the lowest pH of PEI^+, pH 7, which showed the maximum gel mechanical strength. This could be regarded to more protonation of PEI^+ at pH 7 than other pHs (pH 7.5–9) and consequently more chances of forming carrageenan/PEI^+ polyelectrolyte interaction. Thus we expect more aldehyde groups to bind to the gel's free amino groups of PEI^+, pH 7 and consequently more PGA loading.

TABLE I
Immobilized PGA activity and loading. Four wet gel disks of modified gel (Carr/PEI^+(pH 7–9)/GA) and control (Carr/KCl) were soaked for 30 min in 2 mL buffer containing 15 units of free PGA.

Gel samples	Theoretical activity of immobilized PGA (U)	Actual activity of immobilized PGA (U)	% Efficiency of immobilized PGA (actual/ theoretical) *100	Pg(U/g wet gel)
Control	13.3	2.8	21	4.8
pH 7	8.1	5.8	71	10
pH 7.5	9.5	4.3	45.6	7.4
pH 8.0	10.4	4.4	41.9	7.5
pH 8.5	9.5	5.5	57.4	9.4
pH 9.0	10.5	5.1	48.4	8.8

Operational stability of immobilized PGA

The data shown in Figure 6 are showing no loss of PGA activity by reuses and instead, the relative activity increased to 125% after 20 reuses, whereas that of the control gel decreased to 30% after 11 reuses

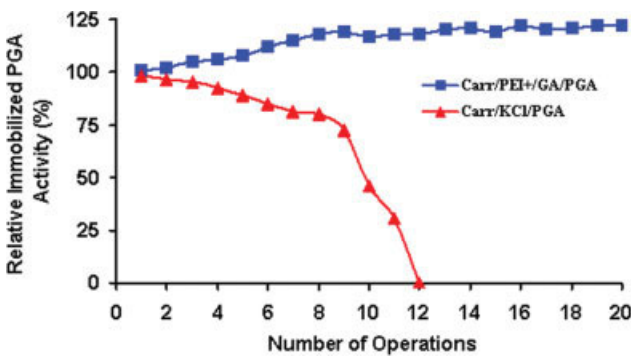

Figure 6 Operational stability of immobilized PGA. Using modified gel (Carr/PEI^+/GA) and control gel (Carr/KCl) for immobilization of PGA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and the gel disks have been destroyed completely after 12 reuses. The reason for the increase of the relative activity by the reuses in case of the modified gel could be regarded to: first, the technique of immobilization is covalent, so we do not expect any leakage of PGA from the gel; second, there are hydrogen bond interactions between the modified gel (polysaccharide) containing —OH, —NH_2 and —C=O groups and the substrate, penicillin G, containing —COOH and —NH groups. These H-bonding interactions could also increase the PG concentration surrounding the gel surface more than the bulk solution and thus the activity of the immobilized PGA increases till reaching saturation of the gel surface with PG (Remark: the data does not show PGA inhibition by the substrate/product); or may be the immobilized PGA requires a relaxation time in contact with its substrate to reach its maximum efficiency (hypothesis). Whereas, in case of the control gel, the relative activity decreases by time due to the weak interaction of PGA to the gel surface and enzyme leakage using the adsorption immobilization technique.

CONCLUSIONS

In conclusion, novel polysaccharide, carrageenan gel has been thermally and mechanically stabilized to be used for covalent immobilization of enzymes after being treated with PEI^+ and glutaraldehyde. The method of modification is clearly illustrated using a schematic diagram and being proved by FTIR, elemental analysis, DSC, and INSTRON. Penicillin G acylase has been covalently successfully immobilized as a model enzyme and the enzyme retained 71% of its activity. The operational stability showed no loss of activity after 20 cycles. The present support could be a good candidate for immobilization of industrial enzymes rich in amino groups. It could also be a suitable carrier for thermophilic enzymes knowing that the gel was thermally stable at 90°C.

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