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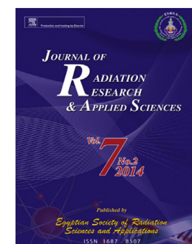

Immobilization of glucose isomerase onto radiation synthesized P(AA-co-AMPS) hydrogel and its application

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ABSTRACT

Isomerization of glucose to fructose was carried out using Glucose isomerase (GI) that immobilized by entrapment into Poly(acrylic acid) P(AA) and Poly(acrylic acid-co-2-Acrylamido 2-methyl Propane sulfonic acid) P(AA-co-AMPS) polymer networks, the enzyme carriers were prepared by radiation induced copolymerization in the presence of (Methylene-bisacrylamide) (MBAA) as a crosslinking agent. The maximum gel fraction of pure P(AA) and P(AA-co-AMPS) hydrogel was found to be 95.2% and 89.6% for P(AA) and P(AA-co-AMPS), respectively at a total dose of 20 kGy. Effects of immobilization conditions such as radiation dose, MBAA concentration, comonomer composition and amount of GI were investigated. The influence of reaction conditions on the activity of immobilized GI were studied, the optimum pH value of the reaction solution is 7.5 and reaction temperature is 65 °C. The immobilized GI into P(AA-co-AMPS) and P(AA) polymer networks retained 81% and 69%, respectively of its initial activity after recycled for 15 times while it retained 87% and 71%, respectively of its initial activity after stored at 4 °C for 48 days. The Km values of free and immobilized GI onto P(AA-co-AMPS) and onto P(AA) matrices were found to be 34, 29.2 and 14.5 mg/mL, respectively while the V_{max} Values calculated to be 3.87, 1.6 and 0.79 mg/mL min, respectively. GI entrapped into P(AA-co-AMPS) hydrogel show promising behavior that may be useful as the newly glucose isomerase reactor in biomedical applications.

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1. Introduction

The increasing use of immobilized enzymes as catalysts in various industrial processes is mainly because of the advantages they confer over their soluble counterparts. These include increased enzymatic stability in extreme conditions of temperature, pH and organic solvents; recovery, reuses of the enzyme, and reduce the cost (Xiao Hua et al., 2013). However, in most of the industrial, analytical, and clinical processes; enzymes are mixed in a solution with substrates and cannot be economically isolated after the exhaustion of the substrates or the completion of a reaction. This single use is wasteful when the cost of enzymes is considering. However, there is an incentive to enzymes in an immobilized or in solubilized form so that they may be reused in a biochemical reactor to catalyze further the subsequent feed (Ashwani Mittal, Khurana, Singh, & Kamboj, 2005).

Various techniques have been developed for enzyme immobilization, including adsorption to insoluble materials (Hanover & White, 1993), entrapment into polymeric gels (Seyhan Tükel & Dilek Alagöz, 2008), and encapsulation in membranes, crosslinking with bifunctional reagents (Hanefeld, Gardossi, & Magner, 2009) or covalent linking onto insoluble carriers (Hasan Atiyeh & Zdravko Duvnjak, 2002). Hence, immobilization can be achieved in many ways, but it always affects, enzyme activity to some extent. The entrapping method with a hydrophilic polymer carrier is advantageous for its relatively high activity yield owing to its mild immobilization conditions. It may be a good choice owing to a relatively inert aqueous environment within the matrix and causing relatively little damage to the structure of the native enzyme (Xiaosen Ouyang et al., 2008). It is also useful in its applicability to a general entrapping of bifunctional components other than enzymes.

Entrapment can be defined as a physical restriction of enzyme within a confined space or network. In this method, enzymes do not chemically bond to polymeric matrices. So, the three-dimensional structure of the enzymes may not affected upon immobilization procedure and the optimum parameters (incubation time, enzyme concentration, pH and temperature) of the immobilized enzyme can be observed (Fusheng Pan et al., 2008). Gelation of poly anionic or poly cationic polymers by the addition of multivalent counter ions is a simple and common method of enzyme entrapment (Dolan, Potter, & Burdock, 2009).

GI obtained from different sources has been immobilized on different support materials such as diethylaminoethyl cellulose (DEAE-C) (Anjuman Gul, Rahman, & Hasnain, 2009) polyacrylamide gel (Bhosale, Rao, & Deshpande, 1996), and alginate beads (Yan Bao, Ma, & Li, 2011). Because of the commercial interest in using glucose isomerase, the enzyme was immobilized by a wide range of the known techniques. According to Wiseman, GI may be the most important of all industrial enzymes of the future (Street, 1977). It catalyzes the reversible isomerization of D-glucose to D-fructose (Moez Rhimi, Messaoud, Borgi, khadra, & Bejar, 2007). The enzymatic conversion of D-glucose to D-fructose is an important industrial process, especially in the production of high fructose corn syrup (HFCS) (Zhiping Yu, Lowndes, & Rippe, 2013). Glucose can be partly converted to fructose at high pH by an alkaline isomerization process. However, this process generates several undesirable byproducts, and therefore it has never been considered to be commercially attractive (Demirel et al., 2006). Besides, GI has received increased attention by industries for its potential application in the production of ethanol from hemicelluloses (Hua Jiang Huang, Ramaswamy, Al-Dajani, & Tschirner, 2010). The enzymatic isomerization of glucose to fructose has attracted considerable interest, since it is more efficient and highly selective, involves less energy consumption, and produces less side products (Yu Wang et al., 2012). Fructose is widely used in the food industry as a sweetener, a diuretic or a special food for diabetics (Bajpai & Anjali Giri, 2003) because it is only slowly reabsorbed by the stomach and doesn't influence the glucose level in blood (Virginie Pasquet et al., 2011).

The work aims to investigate the use of P(AA) and P(AA-co-AMPS) hydrogels as supports for GI immobilization by entrapment using gamma irradiation technique. For this purpose, the effect of pH, temperature, reuses numbers and storage stability on the activity of GI for the free and entrapped enzyme was investigated. The monomers AA and AMPS were chosen due to the following reasons: AA is anionic monomer, cheap common monomer for preparing superabsorbent materials and it can be used to improve the property of gel strength of the prepared copolymer. AMPS is a hydrophilic monomer containing nonionic and anionic groups. AMPS was added to the AA monomer to increase the number of ionic groups in the hydrogel leading to increase their swelling capacity and the nonionic groups can improve their salt tolerance (Tara V. Bright, Clark, O'Brien, & Murphy, 2011).

A comparison between radiation synthesized P(AA) and P(AA-co-AMPS) as enzyme carriers on the enzymatic catalyzed isomerization of glucose to fructose is conducted.

2. Materials and methods

2.1. Materials

Glucose isomerase (E.C.5.3.1.5) was purchased from Genencor International Company, Germany. 2-Acrylamido-2-methylpropane-sulphonic acid (AMPS), acrylic acid (AA) purity 99%, D-glucose and Sodium phosphates dibasic and sodium phosphate monobasic were purchased from Sigma–Aldrich, USA. N, N′-Methylene-diacrylamide (MBAA) was provided by Merck Chemicals, Germany. Magnesium sulfate was purchased from ADWIC, Egypt. The other chemicals such as, solvents, inorganic salts, acids and other reagents were reagent grade and used without further purification.

2.1.1. Gamma radiation source

Gamma radiation from a ^{60}Co source at a dose rate about 5.5 kGy/h using a gamma cell constructed in the National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority (AEA), Nasr City, Egypt. The irradiation process was performed in the air, at room temperature.

2.1.2. Preparation of P(AA-co-AMPS) and P(AA) hydrogels

Radiation induced copolymerization of 20 weight% aqueous comonomer solutions of different compositions. The

solutions were mixed well, transferred into small glass vials then irradiated from a ^{60}Co source at a dose rate about 5.5 kGy/hour and variable total dose that ranged from 5 to 50 kGy. The irradiation process was performed in the air, at room temperature. After copolymerization, the vials were broken and the formed hydrogel cylinders were removed and cut into disks of 2 mm thickness and 5 mm diameter. All samples were washed with excess water and soaked overnight in distilled water to remove the unreacted components, then air dried at room temperature up to constant weight.

2.1.3. *Gel determination of P(AA) and P(AA-co-AMPS) hydrogels*

The gel content (fraction) in the dried samples was estimated by measuring its insoluble part after extraction in distilled water for 18 h at 80 °C to remove the soluble part, and then they were taken out, dried and weighed. The ratio of the remaining mass of insoluble material to original mass was defined as Gel (%) as follows:

$$\text{Gel}\,\% = \frac{W_o - W_g}{W_o} * 100$$

where W_o, W_g are the weights of the dry gel before and after the extraction, respectively.

2.1.4. *Swelling studies of P(AA) and P(AA-co-AMPS) hydrogels*

Water inside the hydrogel allows free diffusion of some solute molecules, while the polymer serves as a matrix to hold water together. The water uptake of the known weight of P(AA) and P(AA-co-AMPS) hydrogels in distilled water at room temperature ~ 25 °C was measured by immersing the samples in distilled water for 24 h after wiping with filter paper, the samples were weighed as quickly as possible. The water uptake percent was calculated from the equation:

$$\text{Swelling degree}\,(\%) = \frac{W_s - W_o}{W_o} * 100$$

where W_o and W_s are the weights of dry and wet hydrogels, respectively.

2.1.5. *Immobilization of GI into P(AA) and P(AA-co-AMPS) hydrogels*

Briefly immobilization procedure, 20 mg/mL GI and 0.3 weight % of MBAA as a crosslinking agent were added separately to 20 mL of AA and 20 mL of AA-co-AMPS 20 weight% in phosphate buffer solution at pH value 7.5 to prepare P(AA-co-AMPS) as a carrier for GI.The mixtures were subjected to 60Co γ-rays then the gel was cut into equal size discs. The immobilized enzyme into P(AA) and P(AA-co-AMPS) were then washed with sodium phosphate buffer and recovered by filtration, dried and then stored at 4 °C.

2.1.6. *Determination of GI activity*

The activity of immobilized glucose isomerase was assessed by measuring the concentration of fructose produced from glucose. The isomerization of glucose to fructose was carried out in a neck flask at the stirring rate of 150 rpm, in which 0.4 g immobilized GI or 1 mL of free GI solution (20 mg GI/mL) was added into 20 mL reaction mixtures composed of 5 mL glucose solution (0.8 M) and 14 mL phosphate buffer of 0.2 Mol/L at pH 7.5, 0.5 mL MgSO4 of 0.5 Mol/L, and 0.5 mL $CoCl_2$ of 0.01 Mol/L. The enzyme reaction was conducted at 65 °C and 150 RPM for 80 min, after which 2 mL of 20% (v/v) HCl was added to the reaction mixture to stop the reaction. The fructose concentration was then measured by the HCl-resorcinol method, in which resorcinol forms a red-colored complex with D-fructose, which has a maximal absorbance at 520 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of glucose into fructose per minute under the assay conditions described. Activity recovery was calculated as the ratio of the activity of immobilized enzyme to the activity of free enzyme, and was expressed as a percentage (Xiangrui Zhu, Junliang Xu 2002).

2.1.7. *Ultraviolet spectrophotometer (UV–Vis)*

Analysis by UV spectrophotometer with determination of the released amounts of protein (the enzyme) and produced amounts of fructose was carried out at 480 nm and 520 nm, respectively using a JASCO V560 Spectrophotometer (Japan) in the range from 200 nm to 900 nm.

2.1.8. *pH Measurement*

The effect of pH was studied at optimum temperature for free and immobilized enzyme by varying the pH in the range of 5–9 in order determine pH stability 1 mL of free enzyme solution equivalent to 0.4 gm immobilized enzyme was mixed with 5 mL phosphate and acetate buffer of various pH values and allowed to pre-incubated at 65 °C. The effect of pH on the activities of free and immobilized GIs was assayed using a 0.8 M glucose solution in 20 mL buffer solution.

2.1.9. *Reusability of immobilized GI*

Reusability of immobilized glucose isomerase was investigated by using the immobilized glucose isomerase sample which puts in 20 mL of the reaction mixture at pH 7.5 and at 65 °C and the reaction was allowed to continue for 80 min. The immobilized enzyme was removed immediately from the reaction mixture and fructose amount was determined. The same experiment using the same immobilized glucose isomerase sample was repeated 15 times within 15 days.

2.1.10. *Storage stability of free and immobilized GI*

The residual enzyme activity on storage was measured by storing free and immobilized enzyme at 4 °C and their residual activities were measured periodically for five weeks.

3. Results and discussion

3.1. *Effect of irradiation dose and AA concentration on gel content of P(AA) and P(AA-co-AMPS) hydrogels*

AA is one of the monomers which are able to form a hydrogel by crosslinking during irradiation with gamma rays. To improve the gel properties to meet the demands for many practical applications AMPS was added to prepare P(AA-co-AMPS) as a carrier for GI and a comparison between PAA and P(AA-co-AMPS) as carriers for GI is conducted.

The effect of irradiation dose on the gel content of pure P(AA) hydrogel and P(AA-co-AMPS) copolymer hydrogel of composition (75/25) weight% was investigated and shown in Fig. 1. It can be seen that, the gel content increase with increasing irradiation dose for both enzyme carriers, P(AA) and P(AA-co-AMPS) the higher irradiation dose, the larger the effective crosslinker density of the hydrogels, then reaches a plateau value at lower than 90%. This is may be due to chain scission more significant than crosslinking during the final stage of the reaction and the ratio between the yields of scission and crosslinking will possibly equals to one and this explains the plateau values. The maximum gel fraction of pure P(AA) and P(AA-co-AMPS) hydrogel was found to be 95.2% and 89.6% for P(AA) and P(AA-co-AMPS), respectively at a total dose of 20 kGy.

The high gel fraction, caused by increased irradiation dose as a result of the high degree of crosslinking into the polymer network.

3.2. *Effect of irradiation dose on swelling degree of enzyme carriers*

Fig. 2 shows the influence of irradiation dose of the swelling characteristics of the P(AA) and P(AA-co-AMPS) hydrogels in distilled water at room temperature ~ 25 °C. It can be seen that the irradiation dose affects the swelling behavior of the hydrogels and considerable variation in the swelling capacity of hydrogels was noted when P(AMPS) is incorporated with P(AA). It is obvious that at a given irradiation dose, the order of swelling capacity values was P(AA- co-AMPS) hydrogel > P(AA) hydrogel.

3.3. *Effect of MBAA as a crosslinking agent concentration on protein release (enzyme) and GI activity*

Figs. 3 and 4 show the influence of different MBAA concentrations on the properties of the enzyme carriers. As can be seen from Fig. 3 the protein release from the hydrogel decrease with increasing the concentration of the MBAA. When large amounts of MBAA 0.5 weight% are used, the protein release decreases to a great extent which is because the increase in the crosslinking content, i.e. the hydrogel of the high crosslinking content is obtained with large MBAA content. MBAA not only determine the crosslinking content but also offer to the pore size. In the meanwhile, the diffusion of the substrate and activity of the immobilized GI depends mainly on the pore size and crosslinking content.

Considering the diffusion of the substrate, the results suggest that the best property of the hydrogel is obtained when 0.3 weight% of MBAA used in the reaction mixture. The effect of MBAA concentration on the activity of immobilized enzyme was studied and shown in Fig. 4. It can be seen that as the MBAA concentration increases the activity of enzyme GI increases to reach its maximum value at 0.3 weight% of MBAA then it decreases.

These results can be explained according to the following:-

At low MBAA concentrations, the amount of crosslinking network structure formed in the gel is low, therefore, when the

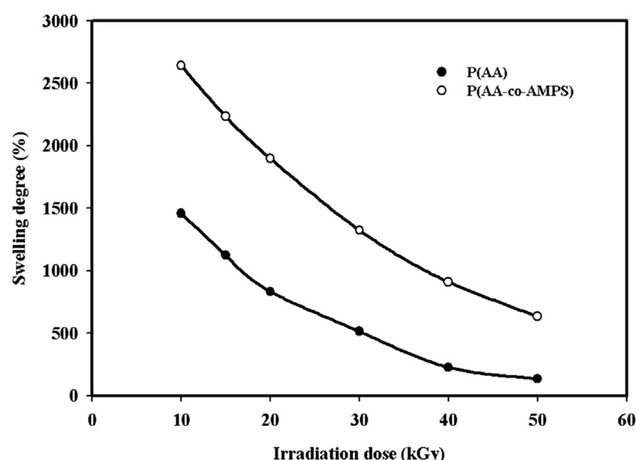

Fig. 2 – Effect of irradiation dose on the swelling degree of P(AA) and P(AA-co-AMPS) in distilled water at room temperature, comonomer concentration; 20 weight%, MBAA concentration; 0.3 weight%.

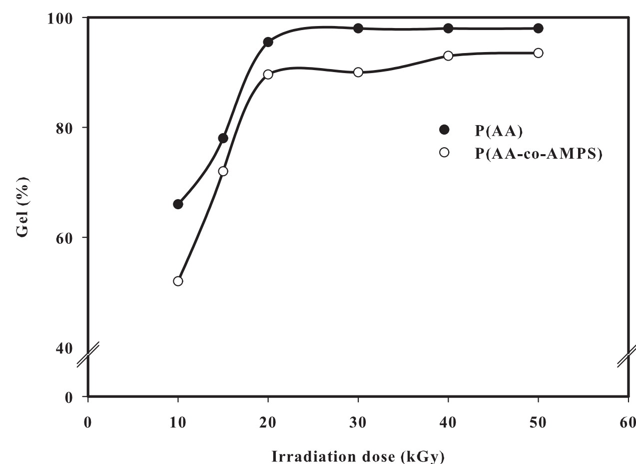

Fig. 1 – Effect of irradiation dose on gel (%) for P(AA) and P(AA-co-AMPS) of composition (75/25) weight%, comonomer concentration; 20 weight% MBAA concentration; 0.3 weight%.

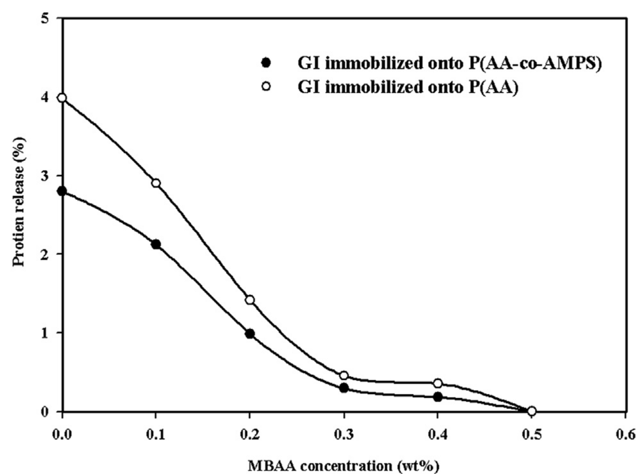

Fig. 3 – Effect of MBAA concentration on protein release at 25 °C in phosphate buffer at pH; 7.5. Irradiation dose; 20 kGy. GI concentration; 20 mg/mL.

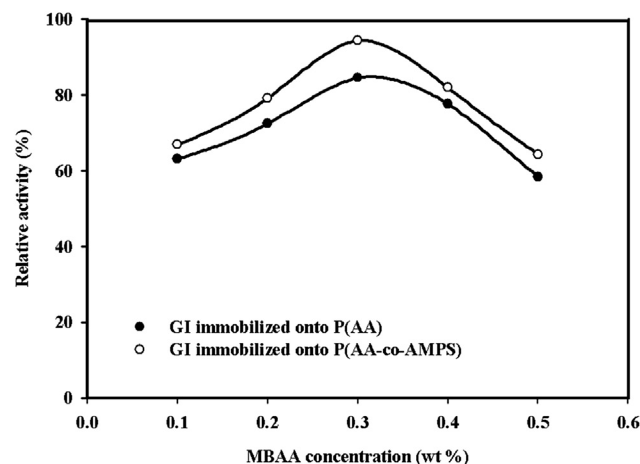

Fig. 4 – Effect of MBAA concentration weight% on the activity of GI immobilized onto P(AA) and P(AA-co-AMPS), at 65 °C in phosphate buffer at pH; 7.5.

hydrogel washed with water the entrapped enzyme escape from the hydrogel resulting in reducing the enzyme concentration and consequently the activity of enzyme is reduced.

At MBAA concentration higher than 0.3 weight% the crosslinking content may become so high that the polymer network may prevent the diffusion of the substrate and product through the network structure. These effects may cause a drop in the enzyme activity.

3.4. *Effect of immobilized GI amount on the enzyme activity*

The relationship between the amount of GI immobilized and the relative activities of the entrapped GI into P(AA-co-AMPS) polymer network was studied and the results are shown in Fig. 5. Different concentrations that ranged from 5 mg/mL to 40 mg/mL of glucose isomerase solutions at pH 7.5 then added to the reaction mixture in which AA/AMPS of composition (75/25) weight%, comonomer concentration of 20 weight% and MBAA 0.3 weight% and Then the mixtures irradiated with a total dose of 20 kGy.

As can be seen from the figure that the relative activity increase as the amount of the immobilized GI increase to reach a maximum value at 20 mg/ml thereafter it decreases. With an increase in GI amount, more GI molecules were entrapped into the polymer network and thus the enzyme activity increased. However, when GI amount was greater than 20 mg/mL the enzyme activity decreased significantly due to the steric hindrance and distortion effects that change the steric configuration of GI molecules thus decrease in relative activity.

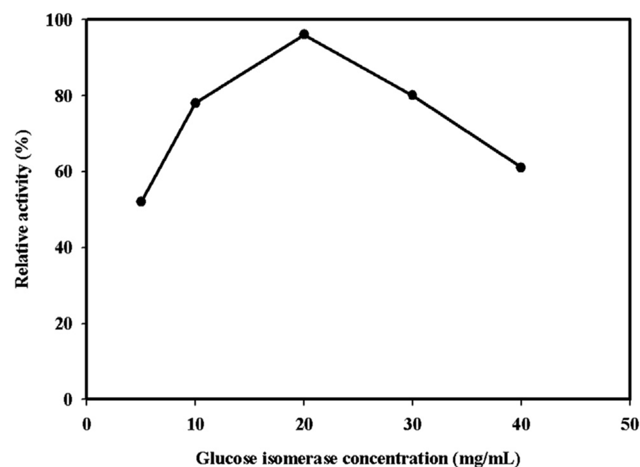

Fig. 5 – Effect of GI amount on the activity of immobilized GI at 65 °C in phosphate buffer at pH; 7.5.

3.5. *Effect of irradiation dose on the activity of free and immobilized GI*

The change of GI activity in the free and immobilized form with irradiation dose was studied and shown in Fig 6. It is obvious that the activity of free GI decreases with increasing irradiation doses, however, the activity of the immobilized enzyme by the entrapment increase with the irradiation dose to reach a maximum value at 20 kGy then it decreases. Such behavior is observed for the enzyme entrapped into P(AA) hydrogel and that entrapped into P(AA-co-AMPS) hydrogel. The low activity of the entrapped enzyme at low doses may be attributed to escaping of enzyme through the loosely cross-linked hydrogels and consequent decrease in the enzyme amount inside the hydrogel network results in a decrease in the overall activity.

On the other hand, the decrease in activity of immobilized GI with the increase of absorbed doses greater than 20 kGy may be due to the increase in crosslinking content that lowers the substrate and product diffusion. Also, The reduction of the enzyme activity may be due to the interaction of enzyme with γ-rays at high doses which may lead to denaturation of the protein molecules so that 20 kGy was chosen as a suitable irradiation dose for entrapment of GI into P(AA) and P(AA-co-AMPS) hydrogels as enzyme carriers.

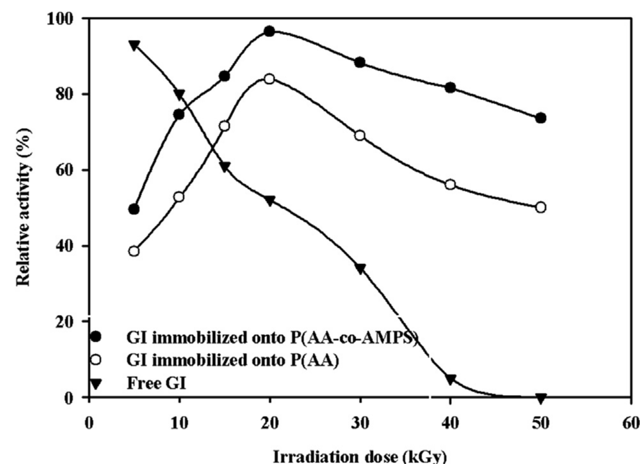

Fig. 6 – Effect of irradiation dose on the relative activity of free and immobilized GI enzyme at 65 °C, at pH; 7.5, free GI concentration 20 mg/mL and the immobilized hydrogels contain the equivalent amount of the enzyme, glucose concentration 0.8 M.

3.6. Effect of reaction conditions on the enzyme activity

Enzymes exhibit their maximum activities under certain conditions. A small change in pH, temperature and ionic strength of the medium may cause denaturation and loss of activity of the enzyme (Tara V. Bright et al., 2011).

3.6.1. Effect of pH value of reaction solution on the activity of free and immobilized GI

The effects of pH value of reaction solution on the activities of free and immobilized GI by entrapment into various hydrogels such as P(AA) and P(AA-co-AMPS) polymer networks were examined at variable pH in phosphate -acetate buffer solutions as presented in Fig 7.

As can be seen, the optimum pH value of the reaction solution for free and immobilized GI was found to be 7.5. And it is not affected by immobilization. At low pH values, the enzyme exhibited very low activities due to the protonation of various amino groups in enzyme molecules which is an important factor in the stability of enzyme structure. It is observed that GI immobilized onto P(AA-co-AMPS) had a wider application range of pH value of reaction solution than GI immobilized onto P(AA) since it retained 82% of its maximum activity at pH values of 6 and 79% of its maximum activity at pH values of 8. However, GI immobilized onto P(AA) retained 74% of its maximum activity at pH values of 6 and 70.6 at pH values of 8. It is also observed that at high pH values that ranging from 8 to 9 the retained activities of GI immobilized onto P(AA-co-AMPS) is higher than that of free enzyme indicating higher stability of GI entrapped into P(AA-co-AMPS) hydrogel.

3.6.2. Effect of reaction temperature on the activities of free and immobilized GI

Effect of reaction temperature on the activities of free and immobilized GI by entrapment into P(AA) and P(AA-co-AMPS) hydrogels was investigated by measuring the relative activity in the temperature range of 40–90 °C and the data are shown in Fig 8. It can be seen that the activity increased significantly when the temperature increased until 65 °C then the enzyme activity decreased. The optimum reaction temperatures for free and immobilized GI were all found to be 65 °C. The relative activity of GI immobilized onto P(AA-co-AMPS) was higher than that of GI immobilized onto P(AA) in the tested temperature range.

The results suggested that GI stabilized by immobilization. GI immobilized onto P(AA-co-AMPS) had a wider application range of reaction temperature than that immobilized onto P(AA) hydrogel. Immobilized GI onto P(AA-co-AMPS) retained 64.6% and 85.3% of its maximum activity at 50 °C and 80 °C, however, GI immobilized onto P(AA) retained 59% and 74.4% of its maximum activity at 50 °C and 80 °C, respectively. It was also obvious that the immobilization of GI onto P(AA-co-AMPS) enhances the thermal stability of the enzyme at elevated temperature that ranged from 70 to 90 °C compared to free enzyme which is advantageous for industrial application. This could be attributed to the increased stability of the immobilized enzyme, where the conformational change was restricted by the entrapment.

3.6.3. The effect of ionic strength on the activity of free and immobilized GI

The effect of additional amount of NaCl on activity of free and immobilized GI was studied with a concentration range from 0 to 1.0 mol/L at the optimum pH 7.5 and optimum temperature 65 °C. As shown in Fig 9. It can be seen that the activity of free and immobilized GI decreased with increasing NaCl concentration in the substrate solution. However, at a given NaCl concentration i.e. ionic strength, the activity of GI immobilized onto P(AA-co-AMPS) is higher than that of the free form indicating higher stability of GI entrapped into P(AA-co-AMPS) copolymer network .Salt has positive effect for the denaturation of enzyme molecules by facilitating the protonation as the ionic strength increases, NaCl concentration increases and more NaCl ions in the substrate solution can screen the electrostatic interaction between the carrier and

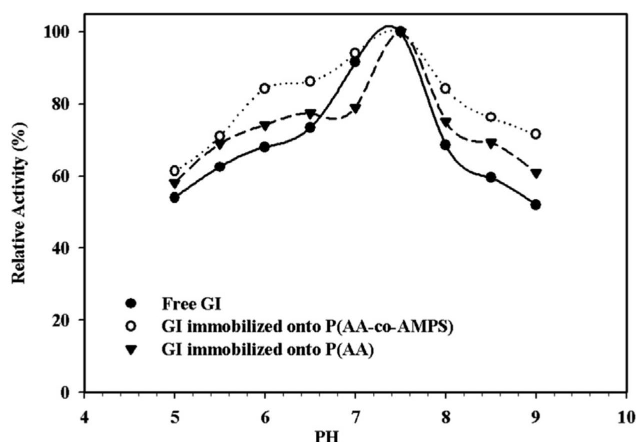

Fig. 7 – Effect of pH of the reaction solution on the activity of Free and immobilized GI at 65 °C, free GI concentration 20 mg/mL and the immobilized hydrogels contain the equivalent amount of the enzyme, glucose concentration 0.8 M.

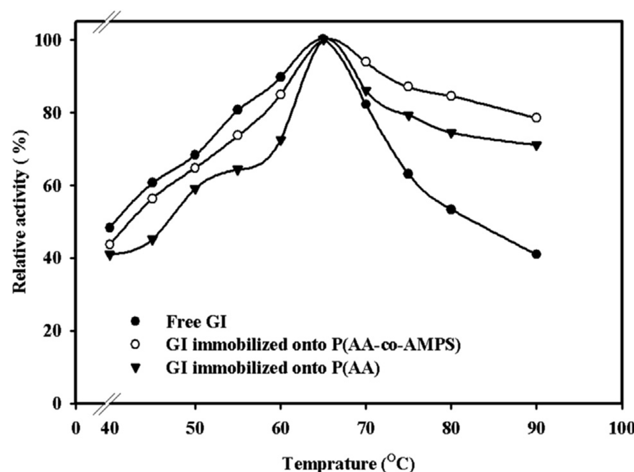

Fig. 8 – Effect of reaction temperature on the activities of free and immobilized GI at pH; 7.5, free GI concentration 20 mg/mL and the immobilized hydrogels contain the equivalent amount of the enzyme, glucose concentration 0.8 M.

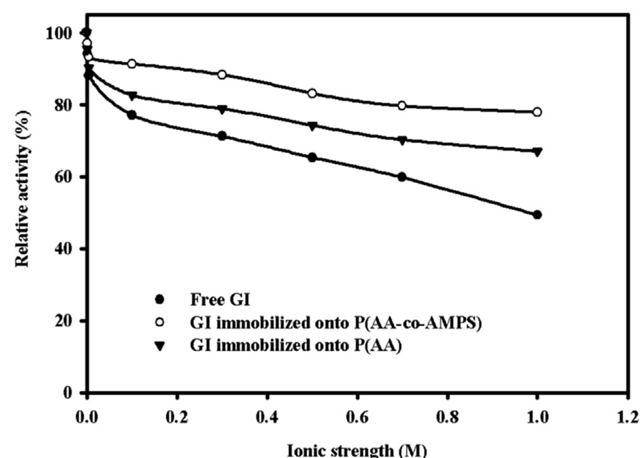

Fig. 9 – Effect of ionic strength on the activity of free and immobilized GI in phosphate buffer of pH; 7.5, temperature; 65 °C.

the enzyme molecules and makes the activity for GI decreases with increasing NaCl concentration.

On the other hand, according to Donnan osmotic pressure equilibrium, the increase in the movable counter ions concentration in the solution leads to the decrease in the osmotic pressure between the hydrogel networks and the external solution (Yan Bao et al. 2011), and affects their swelling ability. Fig. 10 shows the relationship between the ionic strength of the solution and the equilibrium swelling of the prepared hydrogels, it is obvious that the equilibrium swelling is highly dependent on the ionic strength of the medium. The increase in the ionic strength leads to a decrease in the swelling degree of the hydrogel consequently decreases its ability toward the substrate.

Also, the ionic strength may lead to a denaturation process of enzyme molecules, thereby reducing electrostatic forces in the presence of high concentration of Na^+ and Cl^- ions. The obtained results gave an indication that the activity of enzyme is mainly governed by the ionic strength effect.

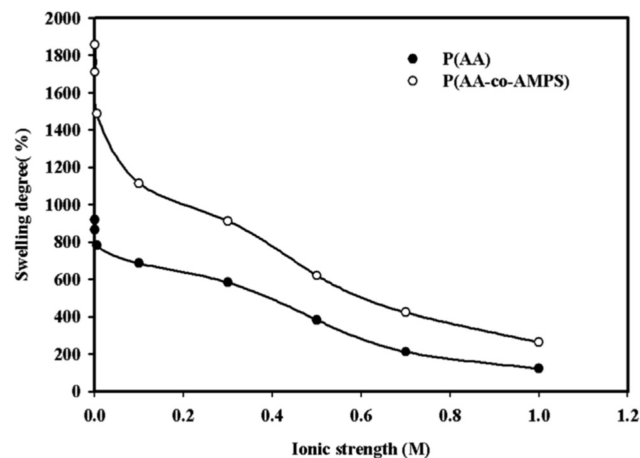

Fig. 10 – Effect of ionic strength (M) on the swelling degree of P(AA) and P(AA-co-AMPS) of composition (75/25) weight %, at 25 °C, irradiation dose; 20 kGy.

3.7. *Reusability of the immobilized GI*

It is important to note that the hydrogel was separated from the reaction mixture by simple filtration,washed with water, dried and reused in the next cycle therefore the hydrogels were repeatedly used 15 times in the reaction media under similar conditions. The change of enzyme activity of repeated use was investigated and shown in Fig. 11. It can be seen that the activity of the immobilized GI decreases, after each cycle. The decrease in enzyme activity may be due to the gradual protein denaturation and release of protein from the polymer network these effects may cause a drop in enzyme activity.

It was observed that after 15 the use enzymes entrapped in P(AA-co-AMPS) hydrogel retained 81% of its initial activity, however, the retained activity for into P(AA) hydrogels were found to be 69%. The results suggest that GI entrapped into P(AA-co-AMPS) has better operational stability, this behavior could be attributed to the diffusion effects.

3.8. *Storage stability*

In order to examine the storage stability, the free and immobilized GI forms were stored at 4 °C and the activities were measured periodically over duration of 48 days. It is obvious that upon 48 days of storage the retained activity of the free enzyme was 52%, while the enzyme entrapped into P(AA-co-AMPS) and P(AA) retained about 87% and 71% of their initial activities, respectively. It is well known that the stability of enzymes can be improved by immobilization because of diffusional resistance. P(AA-co-AMPS) and P(AA) supports should provide a stabilizing effect, minimizing possible distortion effects which might be imposed from aqueous medium on the active site of the immobilized enzyme. The generated multipoint ionic interactions between enzyme and matrix should also convey a higher conformational stability of the immobilized enzyme.

The decrease in the activities of GI entrapped into P(AA-co-AMPS) and P(AA) after the storage time can be explained by the existence of (COOH, SO_3H) groups in the polymer network. Fig 12

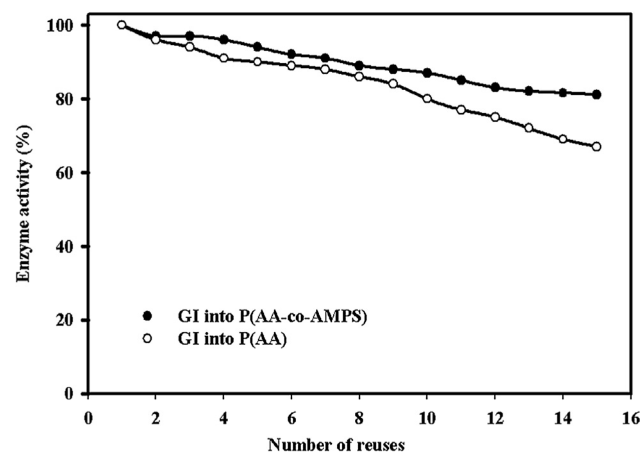

Fig. 11 – Reusability stability of immobilized GI at 65 °C in phosphate buffer at pH; 7.5.

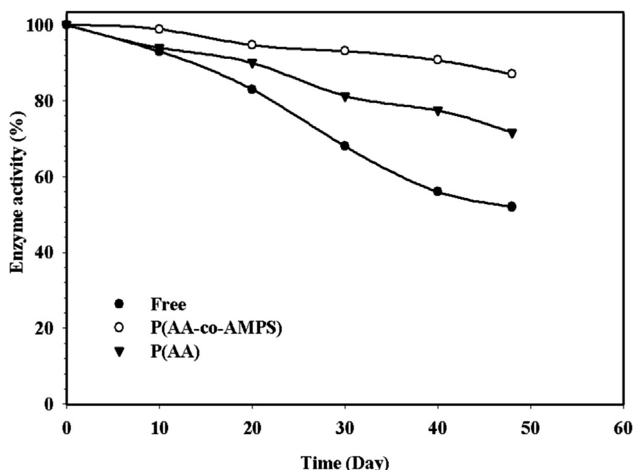

Fig. 12 – Effect of storage stability on the activity of free and immobilized GI in phosphate buffer solution of pH value 7.5 at 65 °C.

3.9. *Kinetic studies of free and immobilized GI*

In order to study the influence of the substrate concentration on the catalytic activity of the immobilized enzymes as well as to elucidate the kinetic effect of immobilization, Michaelis–Menten constant (Km) and the maximum reaction rate (V_{max}) were determined from the classical Michaelis–Menten kinetics and Lineweaver–Burk plots by measuring the initial reaction rates with different glucose concentration solutions that ranged from (5 up to 200 mg/mL), the rates of glucose isomerization observe the change of enzyme activity in a fixed period of time; 5 min. Km values of free and immobilized GI onto P(AA-co-AMPS) and into P(AA) were found to be 34, 29.2 and 14.5 mg/mL, respectively. The Vmax values of free and entrapped enzyme into P(AA-co-AMPS) and P(AA) were found to be 3.87, 1.6 and 0.79 mg/mL min, respectively Fig 13.

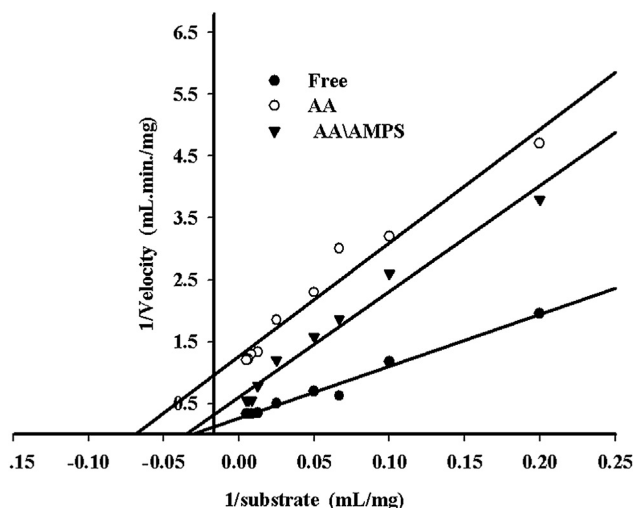

Fig. 13 – Linweaver-Burk plots for the free and entrapped GI into P(AA) and into P(AA-co-AMPS) hydrogels, at 65 °C and pH; 7.5.

Table 1 – Kinetic parameters of the free and immobilized glucose isomerase.

	V_{max} (mg/mL min)	K_m (mg/mL)
Free enzyme	3.87	34
Immobilized onto P(AA-co-AMPS)	1.6	29.2
Immobilized onto P(AA)	0.79	14.5

There is a decrease in K_m values compared to the free glucose isomerase. Similar observations for immobilization of glucose isomerase, have been reported by (Demirel et al., 2006)

On the other hand, K_m and V_{max} values of the free and entrapped enzyme into P(AA-co-AMPS) were quite close to each other. Therefore, the bio conversion of glucose to fructose can be successfully performed by GI entrapped into P(AA-co-AMPS) hydrogel.

3. Conclusion

To enhance the operational stability, storage stability, and reusability, glucose isomerase was immobilized by entrapment into P(AA) and P(AA-co-AMPS) polymer hydrogels for isomerization of glucose to fructose, the influence of the reaction conditions on the activity of immobilized GI was studied to find that the optimum pH value of the reaction solution is 7.5 and reaction temperature is 65 °C. The immobilized enzyme showed good thermal stability and reasonable reusability, in comparison with the free counterpart, the immobilized GI onto P(AA-co-AMPS) and P(AA) retained 81% and 69%,respectively of its initial activity after recycled for 15 times and retained 87% and 71%, respectively of its initial activity after stored at 4 °C for 48 days. The activity of the entrapped GI into P(AA-co-AMPS) was higher than that entrapped into P(AA) polymer hydrogel this was because the high amount of hydrophilic groups in the P(AA–co- AMPS) polymer network increased the enzyme conformational stability. The K_m values of free and immobilized GI onto P(AA-co-AMPS) and onto P(AA) matrices were found to be 34, 29.2, 14.5 mg/mL, respectively while the V_{max} Values calculated to be 3.87, 1.6, 0.79 mg/mL.min, respectively. Therefore the bio conversion of glucose to fructose can be successfully performed by GI entrapped into the prepared hydrogels.Table 1

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