ACTIVITY OF ENZYME TRYPSIN IMMOBILIZED ONTO MACROPOROUS POLY(EPOXY-ACRYLAMIDE) CRYOGEL

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ABSTRACT

Bovine pancreatic trypsin has been covalently immobilized onto monolithic polyacrylamide cryogels with epoxy functionality (Epoxy-MPAAGs). The covalent immobilization of trypsin onto Epoxy-MPAAGs was achieved through incorporation of ethylenediamine-glutaraldehyde spacers on the cryogels' surfaces. The immobilization yield and the influence of pH and temperature conditions on the activity of the immobilized trypsin were assessed by using the hydrolysis of a low molecular weight substrate, N-α-benzoyl-D, L-arginine-p-nitroanilide (BAPNA). Optimal pH and temperature values of 9 and 50 °C, respectively, were established for both the immobilized trypsin and the free trypsin. Activity measurements from the native trypsin before immobilization and the residual free trypsin after immobilization by hydrolysis of BAPNA demonstrated activity recovery (activity based immobilization yield) of about 60 – 70% for the Epoxy-MPAAGs cryogel immobilized trypsin. The Epoxy-MPAAGs cryogels immobilized trypsin was stable up to 65 days in aqueous storage conditions, at 4 °C. Apparently the Epoxy-MPAAGs cryogels immobilized trypsin showed improved catalytic activity above 50 °C.

Key words: Enzyme trypsin, immobilized trypsin, polyacrylamide cryogels

INTRODUCTION

Cryogels (from the Greek *kryos* meaning frost or ice) are macroporous heterophase gels in which polycrystals of frozen solvent act as porogens during the gel formation (Lozinsky *et al*. 2002). Cryogels are characterized by interconnected systems of macropores and the unique sponge-like morphology that can permit unhindered diffusion of solutes of practically any size (Lozinsky *et al*. 2002). Cryogels have found different applications in medical, biotechnological and pharmaceutical areas (Lozinsky *et al*. 2002, Plieva *et al*. 2005, 2006). In general, monolithic cryogels have adequate osmotic, chemical and mechanical stabilities that make them attractive matrices for biocatalysts and chromatography of large entities such as protein aggregates, membrane fragments and viruses.

Previously, hydrophilic macroporous polyacrylamide monolithic cryogels with epoxy functionality (Epoxy-MPAAGs) have been synthesized (Plieva *et al*. 2008). These supermacroporous polymeric materials have been used successfully to immobilize covalently bovine pancreatic trypsin through ethylenediamine-glutaraldehyde spacer arm (Johnson *et al*. 2011a). Trypsin, which is a relatively small spherical enzyme with molecular diameter about 38 Å and molecular mass about 23.8 kDa (Diaz and Balkus 1996, Stroud *et al*. 1974), demonstrates well-defined activity and selectivity (Goradia *et al*. 2005).and can be easily quantified spectrophotometrically

The term immobilized enzyme refers to an enzyme that is physically confined to or localized within a support with retention of its catalytic activity, thus can be used repeatedly and continuously (Worsfold 1995). However, upon covalent immobilization several changes can occur in the properties of an enzyme as a result of activation and coupling procedures. Those changes can be either advantageous or disadvantageous on the enzymatic kinetics depending on the type of the support, the method of activation and the intended use of the finished preparation (Adlercreutz *et al*. 2007). Strong activation might lead to the multipoint attachment that makes the enzyme molecule more rigid and more stable against thermal denaturation and other inactivating agents (Maurich *et al*. 2008). Moreover, the activated matrix may introduce ion exchange groups in the immobilized enzyme that might change the native properties of the enzyme. Trypsin as a serine protease has an active site made up by aspartic acid, histidine and serine residues with the surface composed of disulfide, amino and thiol groups (Adlercreutz *et al*. 2007). Thus the external charges/ions from the immobilizing support or pH changes in the reaction medium can obviously affect the activity of the immobilized trypsin.

Theoretical prediction of the pH optimum of enzymes requires knowledge of the active site characteristics of enzymes, which are very difficult to obtain unless determined experimentally (Shuler and Kargi 2006). In addition, the rate of enzyme-catalysed reaction increases with temperature up to a certain limit; but above a certain temperature, the activity decreases with temperature due to enzyme denaturation (Shuler and Kargi 2006). Thus there exists a competition between thermal activation and thermal deactivation or denaturation. This calls for a thorough characterization of the Epoxy-MPAAGs cryogel-immobilized trypsin preparations in order to establish their effective working conditions.

Therefore, this study investigates the catalytic behaviour of trypsin immobilized onto macroporous polyacrylamide cryogels with epoxy functionality (Epoxy-MPAAGs). The activity-based immobilization yields are determined and the optimum pH and temperature conditions for the immobilized trypsin are established using a low molecular weight substrate - N-μ-benzoyl-*D, L*-arginine-*p*-nitroanilide (BAPNA). Catalytic activities of the immobilized trypsin preparations are compared to those for the free trypsin under similar experimental conditions to assess the consequential effects of covalent immobilization.

EXPERIMENTAL

Materials

Bovine pancreatic trypsin (EC 3.4.21.4) salt free sample was obtained from Novozymes Denmark and immobilized onto previous prepared Epoxy-MPAAGs cryogels. N- μ-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA) ≥ 98 % was purchased from Sigma (St. Louis, USA), tris salt (2-amino-2-(hydroxymethyl)-1,3-propanediol) from Kebolab (Denmark), calcium chloride dihydrate ($CaCl_2.2H_2O$) of analytical grade and ethanol ≥ 95 %, acetic acid 96 %, sodium acetate, sodium phosphate (Na_3PO_4), sodium bicarbonate ($NaHCO_3$) and sodium hydroxide (NaOH) were from Merck (Darmstadt, Germany).

Equipment

For trypsin activity assays, a computer assisted UV-Spectrophotometer (Shimadzu UV-1650 PC) connected to a thermal control unit (Julabo 13) was used. A Watson-Marlow peristaltic pump (0-99 rpm) was used for flow-through activity assays.

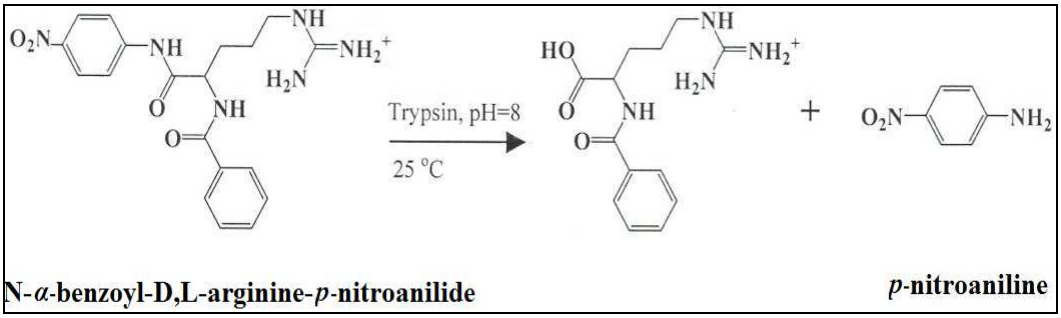
Methods

Preparation of Poly(epoxy-acrylamide) Cryogels and Trypsin Immobilization.
Epoxy-MPAAGs monolithic cryogels (approx. 0.5 mL in volume) were prepared by polymerization of the co-monomers AAm, MBAAm and AGE using cryogelation technique. Total monomer concentrations of 5, 10 and 15 % (w/v) were used in the reaction mixtures. A detailed procedure for the preparation of the cryogels including their characteristic properties has been documented by the authors (Johnson *et al*. 2011(a)). Bovine pancreatic trypsin was immobilized onto the cryogels by using a procedure previously reported by the authors (Johnson *et al*. 2011(b)).

Determination of the Activity Based Immobilization Yield
The initial rate of hydrolysis of the substrate, N-*α*-benzoyl-D, L-arginine-*p*-nitroanilide (BAPNA) (Scheme. 1) was used for the determination of trypsin activity. Fresh, free trypsin solution of approximately 4 mg/mL and residual free trypsin solution (after immobilization) from each of the immobilization preparation were diluted (n = 4) with 0.1 M Na_3PO_4/HCl pH 7.2 buffer while 6.0 mg BAPNA were dissolved into 6.0 mL of 50% aqueous ethanol. The reactant solutions were separately thermally equilibrated at 40 °C for about 25 minutes prior to reactions in thermally controlled cuvettes set at 40 °C for 3 min. The reaction mixture in the cuvette contained 0.28 mL BAPNA solution, 0.52 mL of 0.1 M Na_3PO_4/HCl pH 7.2 buffer and 0.2 mL of the diluted trypsin solution. Two blank solutions comprising of only the BAPNA solution and the 0.1 M Na_3PO_4/HCl buffer were also prepared. The absorbances were recorded at 400 nm using a Shimadzu UV-1650 PC spectrophotometer. Activity based immobilization yield (IY) was determined as the percentage of the activity units of the actually immobilized trypsin (AI) to the activity units for the fresh free trypsin utilised for the immobilization (TI), i.e.,

$$IY = \frac{AI}{TI} \times 100 = \frac{TI - FI}{TI} \times 100\ \%,$$

where FI is the activity units of the residual free trypsin.



Scheme 1: The enzymatic activity assay of trypsin using BAPNA: The chemical equation for the trypsin catalyzed hydrolysis of BAPNA.

pH and Temperature Optimization for the Epoxy-MPAAGs Cryogel Immobilized Trypsin
The activities of trypsin immobilized onto the 5 and 10 % (w/v) Epoxy-MPAAGs cryogels were studied over the pH and temperature profiles. The influence of pH was investigated at a constant temperature of 30 °C using different buffers depending on the pH range of interest, i.e., pH 5-6 acetic

acid/sodium acetate, pH 7-9 tris-HCl and pH 10-11 $NaHCO_3$/NaOH. The effect of temperature was investigated in the temperature range of 23 to 80 °C at a constant pH of 7.6. All buffers used had a concentration of 0.3 M and their ionic strengths were enhanced by 0.1mM $CaCl_2$.

The substrate, BAPNA of 6.5 x 10^{-4} M, was prepared by first dissolving 10 mg BAPNA into 10 mL of 50 % (v/v) ethanol and then in the buffer of choice under the volume ratio 1:2.5 BAPNA solution:buffer, respectively. The monolithic cryogel with immobilized trypsin was packed in a rubber stoppered glass column (i. d. 0.7 cm, length 4 cm) immersed into water bath. Using syringe, both ends of the cryogel immobilized trypsin packed glass column were connected with rubber tubings through which the BAPNA solution was passed to saturate the enzyme.

pH and Temperature Optimization for the Free Trypsin

The influences of pH and temperature on the activity of native free trypsin were studied by incubation under similar conditions to those applied for pH and temperature optimization of the immobilized trypsin under the flow-through condition. Same buffer systems and temperature ranges were applied as for the immobilized trypsin. For the activity assays, BAPNA and trypsin solutions were equilibrated at the particular test temperature (23-80 °C) for a minimum of 10 minutes. The solutions were then mixed in appropriate portions to give [BAPNA] of 6.5 x 10^{-4} M and [trypsin] of 0.2 mg/ml in the reaction cuvette. Replicate absorbance readings at 400 nm were recorded against similarly treated BAPNA blank.

Storage stability and reusability of epoxy-MPAAGs immobilized trypsin

The epoxy-MPAAGs immobilized trypsin was stored in aqueous medium at 4 °C. The storage stability of the enzyme was studied by determining residual activities and pH profiles after 50 and 65 days storage, respectively, under similar incubation conditions by using BAPNA activity assays ([BAPNA] = 6.5 x 10^{-4} M, temp = 30 °C). To test for reusability efficiency, a single batch of immobilized trypsin was re-used for the BAPNA activity assay (pH = 7.6) for about 5 – 7 times. After each assay, the immobilized trypsin was thoroughly washed with de-ionized distilled water and stored at 4 °C for the next analysis.

RESULTS AND DISCUSSION

Immobilization of Trypsin onto the Monolithic Epoxy-MPAAGs Cryogels

Epoxy-MPAAGs cryogel monoliths are macroporous polymeric materials of interconnected pore structure (Fig. 1), with pore size range of about 10 to 150 µm. During the process of trypsin immobilization, the white coloured monolithic cryogels turned dark brown on treatment with glutaraldehyde and persistent pale-yellow at the end of the $NaBH_4$ reduction stage (Scheme 2). The colour change could be attributed to the cryogels' chromophore structural conformation with each stage of trypsin immobilization.

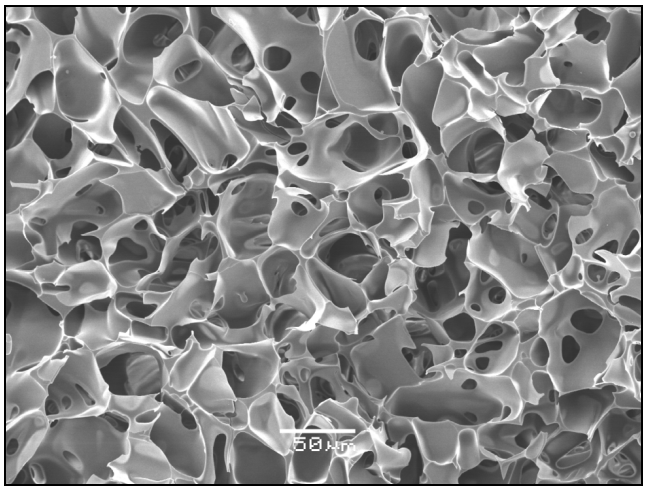
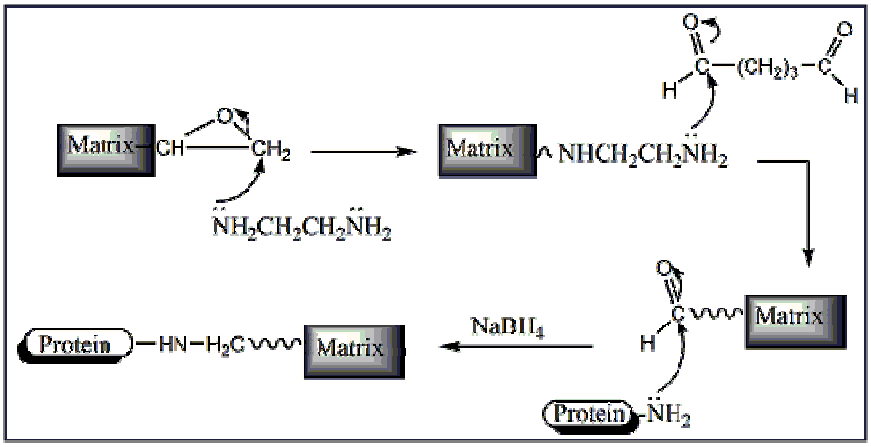


Figure 1: SEM image for 10 % poly(epoxyacrylamide) cryogel.

The enzyme trypsin was covalently attached onto the surfaces of the Epoxy-MPAAGs cryogels using ethylenediamine-glutaraldehyde as spacer arm (reaction scheme 2). This Schiff base-glutaraldehyde covalent immobilization has been reported to be suitable for the immobilization of trypsin (Petro *et al*. 1996). Although the immobilization reaction involves many steps and hence time consuming, linking of ethylenediamine on epoxy groups leads to a longer spacer placed between the support and the ligand which is useful in avoiding steric hindrance effects with small ligands (Mallik and Hage 2006).



Scheme 2: Reaction Scheme for Covalent Attachment of Trypsin onto the Epoxy-MPAAGs Cryogels using Ethylenediamine and Glutaraldehyde; Followed by Reduction of the Schiff Base by Sodium Borohydride (Petro *et al*. 1996).

The relatively long spacer arm resulting from the chemical coupling of ethylenediamine between the surface groups of the cryogel and glutaraldehyde leads to the easy accessibility and attachment of trypsin macromolecules. Moreover, multiple polar functionalities on the Epoxy-MPAAGs cryogel could enhance its hydrophilicity thus providing for a relatively more favourable condition for enzyme trypsin immobilization (Liang and Ruckenstein 1995).

Determination of the Activity Based Immobilization Yield

The graphs for the initial rates of hydrolysis of BAPNA for both free trypsin before immobilization and residual free trypsin (after immobilization by the 10 and 15% Epoxy-MPAAGs cryogels) are presented in Figs. 2 and 3. The slopes of these linear graphs represent the activities of free trypsin before immobilization and those of residual free trypsin after immobilization. In these figures, the differences in activities (slopes of the linear graphs) before and after immobilization are equivalent to the loss of activity of the initial trypsin due to immobilization. This activity loss is considered as the limiting activity that can be recovered from the cryogel-trypsin conjugates. The results suggest that a maximum of about 60% of the original free trypsin activity that disappeared during the immobilization process could be recovered in the 10% Epoxy-MPAAGs cryogel-trypsin conjugates while for the 15% Epoxy-MPAAGs cryogel-trypsin conjugates a maximum of about 70% of the original free trypsin activity could be recovered from the immobilized enzyme.

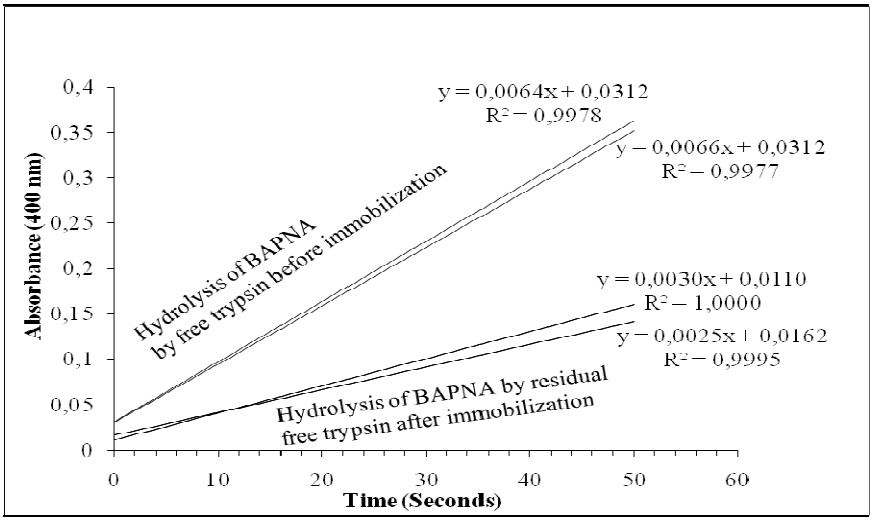


Figure 2: Absorbance v/s Time Graphs: Estimation of Initial Rates of Hydrolysis of BAPNA for the Determination of the Activity Based Immobilization Yield for the 10 % Epoxy-MPAAGs Cryogel-Trypsin Conjugates.

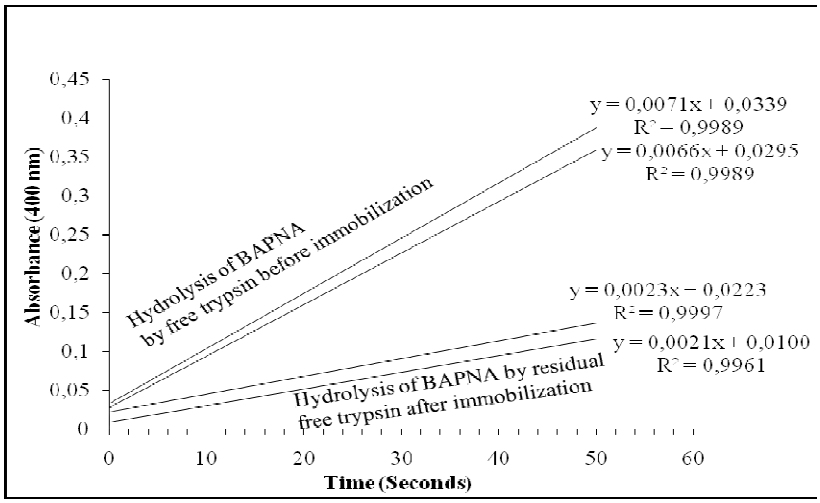


Figure 3: Absorbance v/s Time Graphs: Estimation of Initial Rates of Hydrolysis of BAPNA for the Determination of the Activity Based Immobilization Yield for the 15 % Epoxy-MPAAGs Cryogel-Trypsin Conjugates.

The Influence of pH on Activity of Immobilized and Free Trypsin

The influence of pH on the activity of the epoxy-MPAAGs cryogel immobilized trypsin and the free trypsin is depicted in Fig. 4. The immobilized trypsin and the free trypsin exhibited similar pH profiles at 30 ºC with optimum pH of 9 for both the immobilized and free trypsin. This similarity in pH profiles gives an indication that conformational changes on the enzyme following immobilization are not significant. The pH profiles were seen to have similar trends of increasing relative activity in the pH range 5-9, followed by a drop at pH 10. The profiles show that over 60% of activities were found in the pH range 7-10. At pH 10 BAPNA solution was allowed into the heated immobilized trypsin column to equilibrate without being pre-heated at the set temperature to reduce the risk of self hydrolysis. The immobilized trypsin appears to be slightly more pH stable than the free enzyme probably due to an apparent local change of pH in the enzyme microenvironment (Adlercreutz *et al.* 2007). Upon immobilization, the enzyme is normally localized on a new specially charged microenvironment whose pH could appear to be different from the one in the bulk solution. Thus the kinetics of immobilized enzymes might as well become affected and in a severe case, a new optimum pH might arise. However, the above effects due to the nature of support would become more pronounced for a highly charged support and with diffusion restrictions. These effects are not expected to be prominent in the cryogels since they have almost no diffusion limitations.

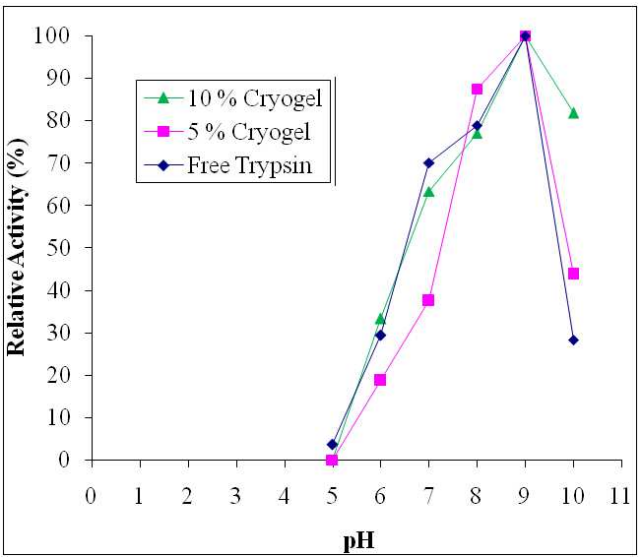


Figure 6: Effect of pH on Activity of Free trypsin and epoxy-MPAAGs Immobilized Trypsin

The Influence of Temperature on Activity of Immobilized and Free Trypsin

The influence of temperature on the activity of the free and the Epoxy-MPAAGs cryogel immobilized trypsin is shown in Fig. 5. The optimum temperature for both free trypsin and trypsin immobilized onto the 10% Epoxy-MPAAGs cryogel was about 50 ºC. Although the optimum temperature for the immobilized trypsin onto the 5% Epoxy-MPAAGs cryogel was about 40 ºC, still over 80% of maximum activity was at 50 ºC. Below 50 ºC, free trypsin was more active than the immobilized trypsin. At room temperature (~ 23 ºC) the free trypsin exhibited over 50% relative activity far above that of immobilized trypsin. The higher activity of the free trypsin compared to the immobilized enzyme at lower temperature could be attributed to substrate diffusion limitation which is absent in free trypsin solution.

Above 50 ºC, 10% Epoxy-MPAAGs immobilized trypsin appeared to be the most thermally stable. For instance, at 60 ºC it had about 80% of relative activity, almost twice the activity of free trypsin and the 5% Epoxy-MPAAGs immobilized trypsin. Generally, since the chemical constitution of the 5% and 10% cryogels is the same, and sincethe method of immobilization of trypsin onto these cryogels was the same, not much difference is expected in terms of stability characteristics. The low temperature stability of the trypsin immobilized onto the 5% cryogels could thus be accounted for by the relatively longer experimental time at the test temperature.

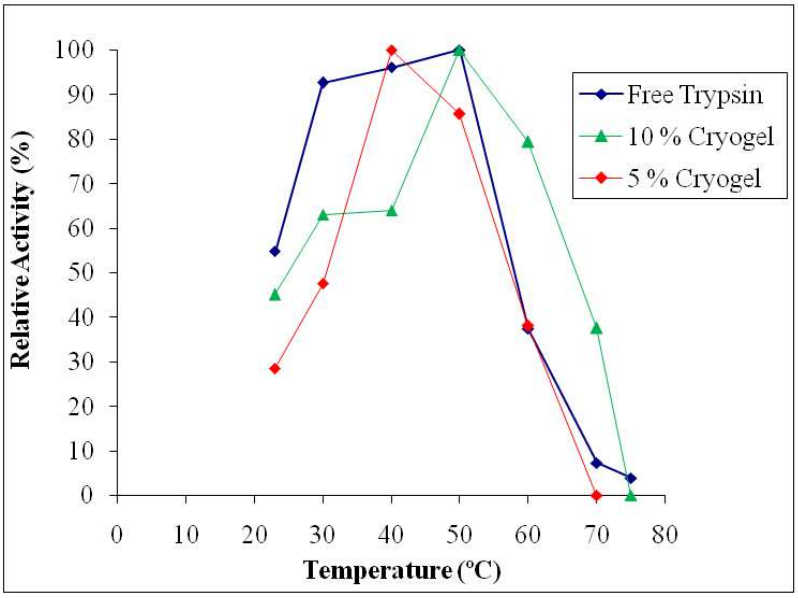


Figure 5: Effect of temperature on activity of free trypsin and epoxy-MPAAG immobilized trypsin

The observed pH- and temperature-activity profiles for epoxy-MPAAG immobilized trypsin were similar to those reported by other workers (Bougatef *et al*. 2007, Klomkloa *et al*. 2007, Bayramoglu *et al*. 2008) for immobilized trypsin from the same or different sources and using different substrates.

Storage Stability and Reusability of Epoxy-MPAAGs Immobilized Trypsin

Results for the effect of storage of the immobilized trypsin in aqueous medium at 4 °C on enzymatic activity are shown in Fig. 6. The results indicate that the cryogels' immobilized trypsin is quite stable at the prescribed storage conditions. Free trypsin solutions exhibit poor storage stability due to self auto-digestion and relatively higher rate of microbial degradation (Goradia *et al*. 2005). Normally the immobilized trypsin is localized in a special microenvironment provided by the support material and could therefore be shielded from microbial attack and oxidation thus enhancing stability against microbial degradation. Yamada *et al*. (2003) observed that trypsin immobilized onto grafted polyethylene plates was more stable than the native trypsin under the same storage conditions. According to Goradia *et al*. (2005) and Maurich *et al*. (2008) immobilization prevents autolysis thus making the immobilized trypsin relatively more stable than the free trypsin under the same storage conditions. The immobilized trypsin could be reused up to seven times without significant loss of activity.

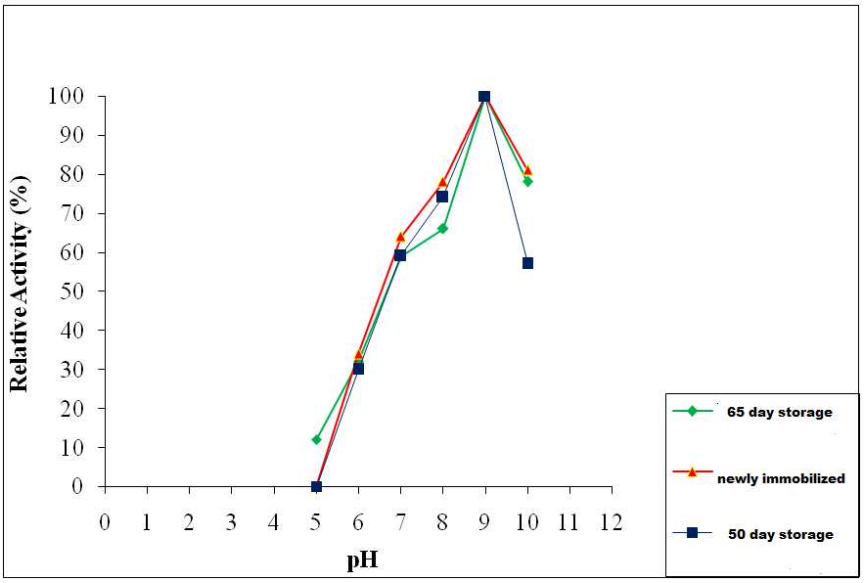


Figure 6: Effect of storage at 4 °C in aqueous condition on pH-activity profile of bovine trypsin immobilized onto 10% Epoxy-MPAAGs cryogel.

CONCLUSION

The chemical constitution and pore structures of the hydrophilic macroporous poly(epoxy-acrylamide) monolithic cryogels (Epoxy-MPAAGs cryogels) coupled with their mechanical stability made them effective matrices for covalent immobilization of bovine trypsin. The immobilization procedure provided for both suitable coupling environment and good recovery of activity. Activity recovery of about 60 to 70% were obtained for bovine pancreatic trypsin immobilized onto the macroporous poly(epoxy-acrylamide) monolithic cryogels. Generally, the covalent immobilization improved the activity of bovine trypsin without significantly affecting the pH and the temperature profiles for the enzyme. The cryogel immobilized trypsin and free trypsin exhibited similar pH and temperature profiles, with their optimum pH and temperature at about 9 and 50 °C respectively. The Epoxy-MPAAGs cryogels immobilized bovine trypsin exhibited good storage stability in aqueous storage conditions at 4 °C. Practical applications of trypsin in biocatalysis and bioreactors can be promoted by appropriate use of the studied Epoxy-MPAAGs cryogel-trypsin conjugates.

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