Covalent Immobilization of Glutathione-S-Transferase onto Magnetic Iron Nanoparticles via Epichlorohydrin Spacer Arm

[1]M. Kemal Caglar, [2]Nuri Gulesci*, [1]Ramazan Bilgin and [1]Guzide Yucebilgic
[1]*Cukurova University, Faculty of Sciences and Letters, Department of Chemistry, 01330, Adana, Turkey*.
[2]*Gumushane University, Faculty of Health Sciences, Department of Occupational Health and Safety, 29100 Gumushane, Turkey*.
nurigulesci@gumushane.edu.tr

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Summary: This study examined the synthesis of Fe_3O_4 magnetic nanoparticles (MNPs) and the covalent immobilization of glutathione-S-transferase (GSTs) onto these nanoparticles using an epichlorohydrin (ECH) spacer arm, as well as the optimal reaction conditions for both free and immobilized enzymes and the reusability of the immobilized enzyme. The optimal pH values for free and immobilized enzymes were established as 7.0 and 6.0, respectively. It was found that the ideal temperature for both free and immobilized enzymes was 37 and 40 °C, respectively. Under the optimum conditions, the values of V_{max} for glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) substrates of free GSTs enzyme were measured as 204.8 U/mg prot., and 194.3 U/mg prot., respectively. The immobilized GSTs enzyme's CDNB and GSH substrates had V_{max} values of 33.29 U/mg prot. and 33.08 U/mg prot., respectively. The free GSTs enzyme's CDNB and GSH substrates have K_m values of 0.201 mM and 0.1873 mM, respectively. GSH and CDNB have K_m values of 0.3042 mM and 0.2523 mM, respectively. At various temperatures, we contrasted the thermal stability of free and immobilized GSTs. The immobilized GSTs enzyme retained 50% of its activity after 20 were reused. After 30 days of storage at 25 °C and 4 °C, it was discovered that 18% and 32% of the free GSTs enzyme activity were preserved, and 30 days of storage at 25 °C and 4 °C resulted in 20% and 41% preserved enzyme activity for the immobilized enzyme, respectively.

Keywords: Characterization, Enzyme, Magnetic Nanoparticles, Synthesis.

Introduction

GSTs are a category of pervasive protein superfamilies produced by multiple genes [1, 2]. In crustaceans, they are crucial detoxification enzymes. [3, 4]. The insects include three main detoxification enzymes: cytochrome P450, carboxyl esterase, and GSTs. [5]. Of the three enzymes that detoxify, GSTs belongs to the dimer cytoplasmic isozyme family. GSTs can catalyze a nucleophilic addition reaction between reduced glutathione and electrophilic compounds and can transport cellular toxins out of vacuoles. GSTs are referred to as phase II metabolic enzymes because it is an essential component in the second phase of the antitoxic mechanism [6]. Furthermore, antioxidant functions, hormone synthesis, metabolism, development, and intracellular transport require GSTs in living things [7]. GSTs can also metabolize the by-products produced during the processing of environmental pollutants, anti-cancer drugs, pesticides, herbicides etc. [8]. Some reports indicate that insect resistance to carbamate, organophosphorus, and pyrethroids pesticides is associated with an increased metabolic detoxification activity of GSTs. This increase is linked to gene amplification, gene expression, and the up-regulation of gene transcription [9].

In this study, GSTs were isolated from sheep liver. GSTs were immobilized onto MNPs via ECH intermediate spacer arm. We examined the maximum activity, and kinetic parameters (K_m, V_{max}) and investigated the use of stability again.

Experimental

Valenzuela *et al*. method was used to make MNPs. Surface the hydroxylation process added reactive -OH groups to MNPs. After that, epichlorohydrin was added, which produces reactive epoxide groups that covalently bind to GST's -NH_2 groups during the immobilization process [10]. GSTs enzyme preparation was obtained for sheep liver by the method of Coskun, *et al*. [11]. GSTs enzymes for the preparation of the sheep liver preparations were used. Glutathione-S-transferase activity measurement according to the method of Habig *et al*. [12]. Protein assay was determined by Bradford *et al*. [13]. GSTs were immobilized by Axen *et al*. [14].

*To whom all correspondence should be addressed.

Characterization of Free and Immobilized Glutathione-S-Transferase

An immobilization was carried out, determining the most suitable immobilization time for 1, 2, 3, 6, 12, and 24 hours. Free and immobilized glutathione-S-transferase (GSTs@MNPs-ECH) activities were measured at pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, for the determination of the optimum pH. Free and GSTs@MNPs-ECH activities were measured at 20, 25 30, 35, 37, 40, 45, and 50 °C to determine the optimum temperature for GSTs activity were measured. To determine the optimal buffer concentration 25, 50, 75, 100, 125, and 150 mM of buffer concentrations were determined.

Free and Immobilized Glutathione-S-Transferase Characterization

The optimum conditions were determined, from 0.3 to 2 mM and the free and GSTs@MNPs-ECH activity in concentrations of 0.3 to 2 mM was measured CDNB. Maximum activity (V_{max}) and Michaelis-Menten constant (K_m) were graphically evaluated by using the Lineweaver Burk plot. For the determination of thermal stability of the free and immobilized glutathione-S-transferases temperature at which they show the maximum activity (37 - 40 °C) and 60 °C were measured residual activity after a certain time on hold. Free and immobilized GSTs in determining the storage stability of glutathione-S-transferase activity in the remaining examples at 40 °C and waiting periodically at certain days at room temperature was measured.

Results and Discussion

pH-related changes of GSTs activities of free and covalently GSTs@MNPs-ECH are shown together in Fig. 1. Three different optimum pH for the free enzyme are 5.7 and 8. pH was determined as 6 for immobilized enzyme.

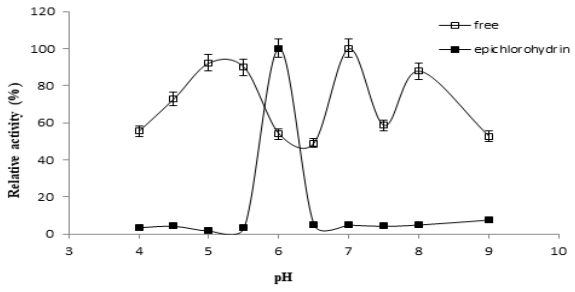

Fig. 1: pH-related changes of GSTs activities for free and covalently immobilized enzymes free enzyme and MNPs via the ECH intermediate arm.

Temperature-related changes of the GSTs activities for the free and covalently immobilized enzyme on to and MNPs via ECH intermediate arm are shown together in Fig. 2. The optimum temperature was determined as 37 ºC for free enzyme, however, two different optimum temperatures as 25 ºC and 40 ºC were determined for immobilized enzyme.

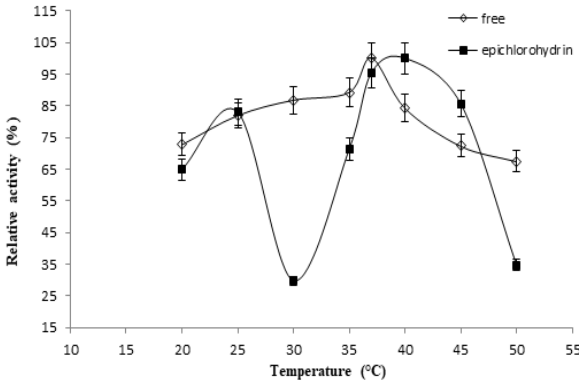

Fig. 2: The Effect of temperature on the activity of GSTs.

The activity of free GSTs was measured at different concentrations of two different substrates under the optimum conditions, Lineweaver-Burk plots were drawn using the Sigma Plot Enzyme Kinetic Module program. (Fig. 3 and Fig. 4). V_{max} and K_m values were calculated for CNDB (194.3 U/mg prot., 0.201 mM respectively), and GSH (204.8 U/mg prot., 0.1873 mM respectively).

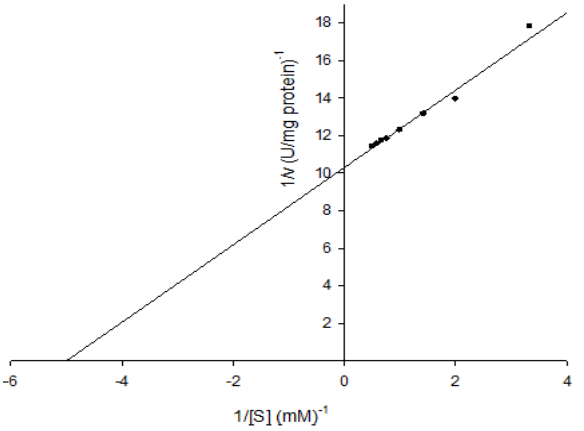

Fig. 3: Lineweaver-Burk graph for free GSTs (GSH).

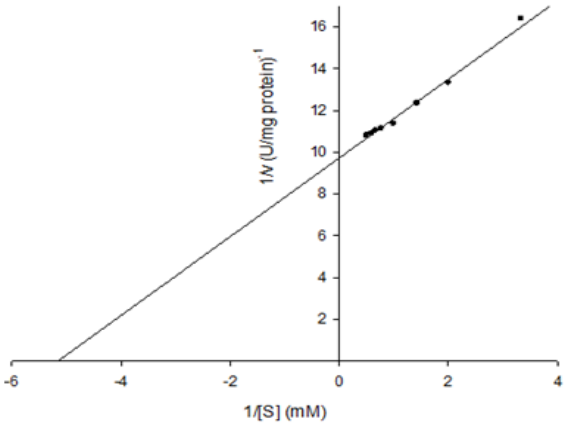

Fig. 4: Lineweaver-Burk graph for free GSTs (CDNB).

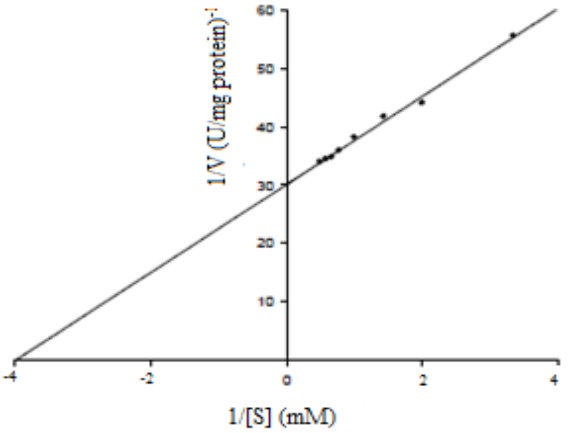

Fig. 5: Lineweaver-Burk graph of GSTs (1mM GSH, 100 mM pH 6.0 citrate buffer, 30 °C) covalently immobilized to MNPs via ECH intermediate arm.

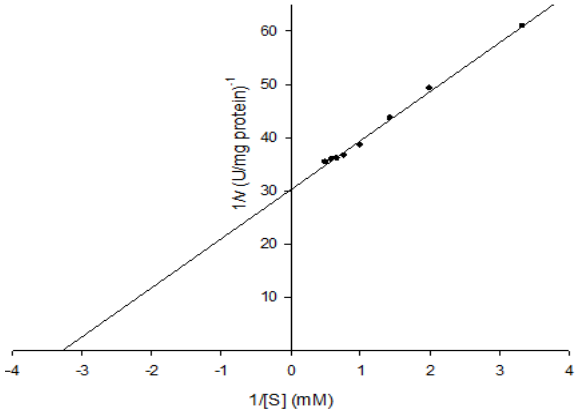

Fig. 6: Lineweaver-Burk graph of GSTs (1mM CDNB, 100 mM pH 6.0 citrate buffer 30 °C) covalently immobilized to MNPs via ECH intermediate arm.

After the initial activities of free GSTs were determined, the remaining activities of these enzymes were measured at certain storage time intervals for 30 days and their relative activities were calculated for 30 days at 4 ° C and room temperature (25 ° C).

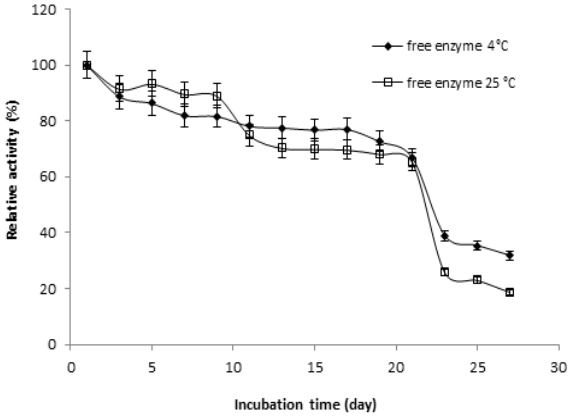

Fig. 7: Changes of the activity of free GSTs at 25 ºC and 4 ºC depending on the storage time.

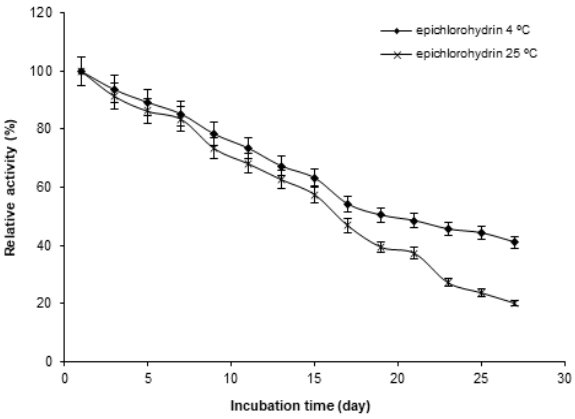

Fig. 8: The relative activity of GSTs, which is covalently immobilized to the MNPs via the ECH intermediate arm, depending on the storage time at 25 ºC and 4 ºC.

The re-use stability of GSTs, which was covalently immobilized to the MNPs via the epichlorohydrin intermediate arm, was determined. After taking 70 mg and 100 mg samples, it was determined that GSTs activities were about 38.6% and 47.6%, of respectively.

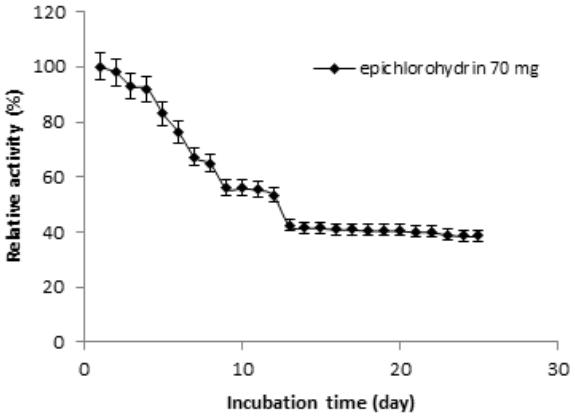

Fig. 9: Changes of the relative activity of GSTs, covalently immobilized to the MNPs via the ECH (70 mg) intermediate arm, depending on the number of re-uses.

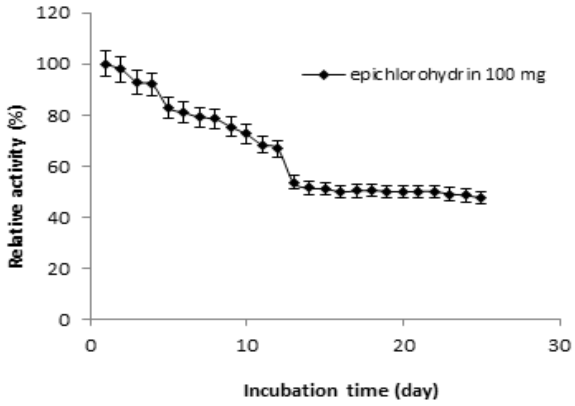

Fig. 10: Changes of the relative activity of GSTs, covalently immobilized to the MNPs via the ECH (100 mg) intermediate arm, depending on the number of re-uses.

The activity of the free enzyme and GSTs covalently immobilized to MNPs through the intermediate arm of ECH was measured at different substrate concentrations under the determined optimum conditions and a Lineweaver-Burk plot was plotted using the Sigma Plot Enzyme Kinetic Module program and the kinetic parameters are shown in Table 1.

Table-1: K_m, V_{max} values of free enzyme and immobilized enzymes.

Name	K_m (mM)	V_{max} (U/mg prot.$^{-1}$)
Free enzyme (1 mM GSH) (0.3-2 mM CDNB)	0.2010	194.30
Free enzyme (1 mM CDNB) (0.3-2 mM GSH)	0.1873	20.80
Immobilized enzyme ECH (70 mg Intermediate Spacer Arm)	0.2523	33.29
Immobilized enzyme ECH (100 mg Intermediate Spacer Arm)	0.3042	33.08

In this study, GSTs enzyme was covalently immobilized on to MNPs via an epichlorohydrin intermediate arm. The average size of the magnetic nanoparticles synthesized using the co-precipitation method was determined to be 158.2 nm. The magnetic support was activated by ECH and the enzyme preparation was bound. Immobilized enzymes are more advantageous than free enzymes. The immobilization studies with GSTs are limited. For this reason, GSTs preparation obtained from sheep liver was immobilized to magnetic nanoparticles using the covalent binding method for the first time.

The covalent binding technique was used for the immobilization of the GSTs rich preparation. ECH is more stable and keeps enzymes active better than other bifunctional linkers like glutaraldehyde because its chain is flexible and it doesn't break down enzymes as much during immobilization. MNPs were tested as carriers. Empty (no enzyme) MNPs prepared as carriers were used in the covalent binding technique. ECH intermediate arm was attached to the MNP for activation of the covalent binding. Immobilization efficiencies were evaluated in terms of relative enzymatic activity and total protein amount, while the results are presented, respectively. The pH-related changes of the GSTs activities for free and immobilized enzymes are shown together in Fig. 1.

The most important factor governing the activity of enzymes is the pH effect. It is a variable dependent on a series of experimental parameters such as optimum pH of an enzyme, reaction time, temperature, substrate structure and concentration, ionic strength of the medium, and purity of the enzyme. Since biochemical reactions take place in an aqueous medium, pH greatly affects the overall charge and activity of enzymes. The effect of pH on immobilized and free GST activity is given in Fig. 1. As can be seen in the figure, while the optimum pH value of the free enzyme is 7.0, the optimum pH of the immobilized enzyme is 6.0. In a different study on sheep liver GSTs, the optimum pH value of the free enzyme was reported as 7.0, whereas the optimum pH value of the immobilized enzyme was 7.5 [15].

In the literature, the optimum pH values for GSTs range between 7.0 and 8.5 [16, 17]. pH change after enzyme immobilization can be explained by three factors; anionic or cationic character of the support materials, modification of the enzyme molecule during immobilization, the products being acidic or basic after enzymatic reaction [18]. It may be considered that factors are important in this study due to both the modification of GSTs molecules during

immobilization and the formation of HCl acid after the enzymatic reaction. Therefore, it has been observed that pH shifts towards the acidic region after immobilization. The pKa values of the enzyme's functional residues can also be used to understand this. Immobilization can modify the microenvironment of ionizable groups, changing optimal pH through alterations in ionization equilibria.

Temperature-related changes in the activities of free and immobilized glutathione-S-transferases are shown together in Fig. 2. The effect of temperature on free and immobilized GSTs activity was analysed from the graphs depicting the change of relative activity with temperature. In general, as the incubation time increases, the optimum temperature decreases due to thermal denaturation. The optimum temperature of the enzyme usually changes after immobilization [19]. As seen in Fig. 2, the optimum temperature for the free and immobilized enzyme was determined as 37 °C and 40 °C, respectively. This shows that immobilization process does not significantly change the optimum temperature of GSTs. In a study for sheep liver GSTs, the optimum temperature for both free and immobilized enzyme was reported as 37 °C [15]. Enzymes are large and highly complicated proteinaceous molecules. The three-dimensional structure of an enzyme must be preserved to maintain its catalytic activity. The reaction rate increases as the temperature increases. However, after a certain temperature, activity decreases due to the denaturation of the enzyme. The temperature at which the enzyme shows the maximum activity (optimum temperature) is especially important as it is an operational parameter. The difference in the optimum temperature for the free and the immobilized enzymes can often be caused by the modification in the structure of the immobilized enzyme molecules, especially when covalent binding is employed for enzyme immobilization. The effect of substrate concentration on the activity of free and immobilized GSTs and their kinetic parameters were determined using substrate (CDNB) and co-substrate (GSH). The effect of CDNB concentration on the enzymatic activity is given in Fig. 3 and Fig. 4. When the dependence of GSTs activity on CDNB concentration was evaluated from the graph, it was found to be 1 mM for both free and immobilized enzymes. No significant increase was observed in the substrate concentration of CDNB. In a study on sheep liver GSTs, the substrate concentration of CDNB was also reported as 1 mM for both free and immobilized enzymes [15].

Lineweaver-Burk diagrams of CDNB (1/V versus 1/S) are presented in Fig. 3 and Fig. 4. Kinetic parameters were calculated using the Sigma Plot software. Figures 5 and 6 show how the concentration of GSH affects the activity of the enzyme.

When the dependence of GSTs activity on GSH concentration was graphically evaluated, it was found to be 1 mM for both free and immobilized enzymes. No significant increase was observed on the substrate concentration of GSH. In a study on sheep liver GSTs, it was reported as 1 mM for both free and immobilized enzyme [15].

Lineweaver-Burk diagrams of GSH (1/V versus 1/S) are given in Fig. 5 and Fig. 6. Kinetic parameters were calculated on the computer using the Sigma Plot program. Michaelis-Menten constant (K_m), refers to the substrate concentration in an enzymatic reaction when the reaction rate reaches half the maximal rate and is a measure of the affinity of the enzyme to the substrate. A high Michaelis-Menten constant shows that a high substrate concentration is required to achieve half-saturation, and the enzyme has little interest in the particular substrate.

As seen in Table I, there is no significant change between the K_m values of free and immobilized enzymes. However, K_m value is slightly higher and V_{max} values are lower for immobilized GSTs preparation. This is due to restricted diffusion as seen in other enzymes immobilized by the covalent binding method. In other words, in the covalent immobilization method, not only the enzyme was modified and its structure was impaired but also the mobility of the immobilized enzymes was decreased. Therefore, K_m values were higher and V_{max} values were lower for the immobilized GSTs. Considering the reaction mechanism of GSTs, the enzyme needs GSH as a cofactor, which acts as a secondary substrate. The effect of substrate concentration on the enzymatic activity of GSTs have been extensively studied in plants and mammals. In a study conducted in the human liver, K_m value was found as 0.37 mM for GSH and 1 mM for CDNB [20]. In the same study, V_{max} value was expressed as 3.3 μmol/min mg protein^{-1}. In another study conducted with beef liver, K_m value was 3.33 mM for GSH, and V_{max} value was 2.5 μmol/min mg prot.$^{-1}$ [21]. In a study conducted with fire and, the K_m values were reported as 0.84 mM for GSH and 0.13 mM for CDNB, while the V_{max} value was noted as 87.4 μmol/min mg prot.$^{-1}$ for GSH [17]. In a study on GSTs from *Atactodea striata*, it has been reported that K_m values were 0.19 mM for GSH and 0.43 mM for CDNB, whereas V_{max} value was 0.00017 μmol/min mg prot.$^{-1}$ for GSH and 0.00024 μmol/min mg prot.$^{-1}$ for CDNB [16].

One of the factors influencing long-term stability is the thermal stability of both free and immobilized GSTs. Thermal denaturation happens quickly at higher temperatures, even though enzymes are typically more stable at lower temperatures.

In order to investigate the thermal stability of GSTs, both free and immobilized enzymes were first incubated at different temperatures between 40 °C and 60 °C for 24 hours, and their activities were measured under optimum reaction conditions. At 40 °C, maintained enzymatic activities for free and immobilized enzymes were 63% and 55%, respectively, whereas, the enzymatic activities reduced to 49% and 40% respectively for free and immobilized enzymes. At this temperature, it has been observed that the immobilized enzyme maintains its activity by 40%.

In a study on sheep liver GSTs, it was reported that immobilized enzyme activity was preserved by 93% at 30 °C, and 61% of the free enzyme was retained during the immobilization process [15]. After immobilization, the thermal stability of the enzyme may increase, decrease, or remain the same. However, immobilization generally increases the thermal stability of the enzyme. The thermal stability of the immobilized enzymes is one of the most important criteria in terms of applications. Enzymes remaining stable at high temperatures can be used in many industrial processes that require high operational temperatures. After the initial activities of the free and immobilized GSTs were determined, they were stored in enclosed containers either at 4 °C or room temperature (25 °C). The remaining activities of both sets of enzymes were measured at certain time intervals over the course of 30 days. The remaining activities for the free GSTs were determined to be 32% for enzyme stored at 4 °C and 19% for the enzyme stored at RT, respectively, whereas the remaining activities for the immobilized GSTs were 41% for enzyme stored at 4 °C and 20% for the enzyme stored at RT, respectively. In a study on sheep liver GSTs, it was reported that 18% of the original activity of the free enzyme and 68% of the immobilized enzyme were retained after 6 months of storage [15].

Storage stability is an important factor, especially in the industrial applications of immobilized enzymes. Storage stability is a parameter that depends on the storage conditions of the enzyme. Generally, water-insoluble enzyme preparations can be stored at 4 and 5 °C either in lyophilized or suspension form (Fig. 7 and Fig. 8.).

The re-use stability of GSTs, which were covalently immobilized to the MNPs via the ECH intermediate arm, was determined as 38.6% and 47.6% for 70 mg and 100 mg sample sizes, respectively. Another parameter that affects the industrial value of immobilized enzymes is that these preparations can be used many times and for a long time. The reusability of the immobilized GSTs has been tested through trials for this purpose. As seen in Fig. 9 and Fig.10, after 4 times of use, approximately 92% of its activity was maintained, while the activity started to decline gradually after the 5th use. When the immobilized GSTs were used 10 times, it still maintained 72% of its original activity. For such an impure GSTs preparation, these results indicate that the enzyme may be suitable for the detoxification of some xenobiotics.

Conclusion

In this study, GSTs were successfully immobilized onto MNPs via epichlorohydrin spacer arms. When glutathione-S-transferases were immobilized, their thermal and storage stability increased. The enzyme retained as much as 72% activity after 10 cycles, which is a considerable contribution to cost-effectiveness for biotechnological processes. Long-term storage data favors a potential industrial application. After 20 cycles, the GSTs@MNPs-ECH maintained 50% of its initial value, and after 30 days of storage at 25 °C and 4 °C, the immobilized enzyme's preserved enzyme activity was 20% and 41%, respectively. Since MNPs are non-toxic, cheap, and biocompatible, GSTs@MNPs-ECH may find potential uses to remove and catalyze the detoxification of carcinogens, therapeutic chemicals, and environmental toxin applications.

Acknowledgements

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