Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. 81 (12) 1371–1382 (2016)
JSCS–4934
UDC 633.6+547.543.05:547.458.2:547.455.623
Original scientific paper

Immobilization of maltase from *Saccharomyces cerevisiae* on thiosulfonate supports

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(Received 30 July, revised 14 October, accepted 19 October 2016)

Abstract: In this study, two commercial supports (Eupergit® C and Purolite® A109) were chemically modified in order to introduce thiosulfonate groups, which could subsequently exclusively react with the cysteine residues on the surface of enzymes. Thereafter, the maltase from *Saccharomyces cerevisiae* was immobilized onto the obtained thiosulfonate-activated supports, resulting in high expressed enzymatic activities (around 50 %), while on the other hand, immobilization on unmodified supports yielded expressed activities less than 5 %. Moreover, protein loadings up to 12.3 mg g^{-1} and immobilized activities up to 3580 IU g^{-1} were achieved by employment of these thiosulfonate supports. Desorption experiments, performed on samples taken during immobilization, proved that immobilization on the thiosulfonate supports was the first step of fast adsorption onto the supports and the formation of covalent bonds between the thiosulfonate groups and the thiol groups of cysteine represented a second slower step. More importantly, although enzyme coupling occurred *via* covalent bond formation, the performed immobilization proved to be reversible, since it was shown that 95 % of the immobilized activity could be detached from the support after treatment with a thiol reagent (*β*-mercaptoethanol). Thus, the support could be reused after enzyme inactivation.

Keywords: *α*-glucosidase; covalent disulfide bridges; Eupergit® C; Purolite® A109.

INTRODUCTION

Maltase (*α*-glucosidase, E.C 3.2.1.20) catalyses hydrolysis of *α*-(1→4) glycosydic bonds between two glucose moieties, with a primary biological role to

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doi: 10.2298/JSC160730099M

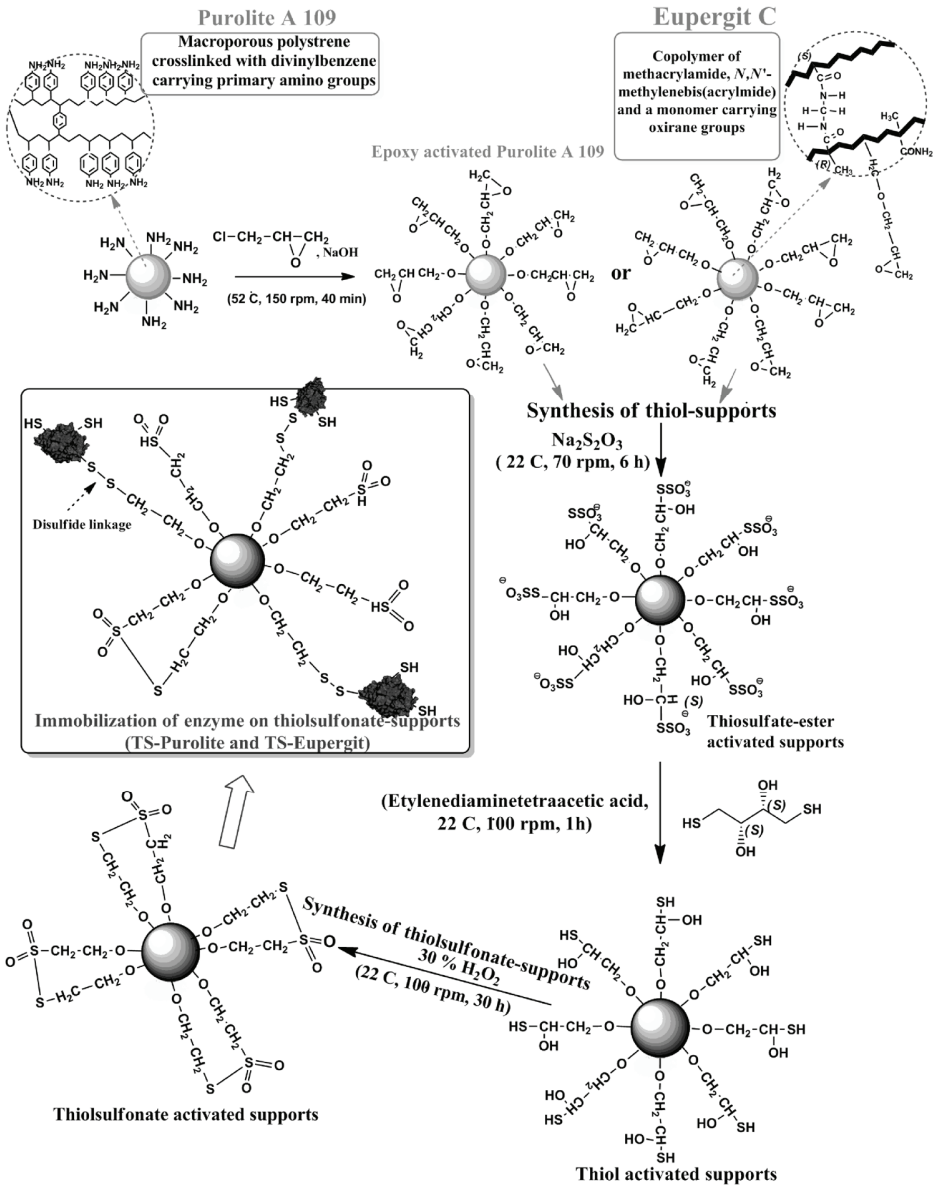
hydrolyse maltose to glucose, thus enabling its prosperous employment in food processing, brewing, distilling and pharmaceutical industries. Nowadays, maltase becomes even more attractive since it can catalyse the reaction of transglucosylation, provided efficient glucosyl acceptor molecules (various hydroxylated compounds) are present in reaction mixture. This corresponds to the contemporary trend of the utilization of the potential of transglycosylation of various glycosidases in the synthesis of bioactive glycosides.[1–4] In previous studies, it was proven that maltase from *Saccharomyces cerevisiae* is a very powerful catalyst since it can be used for the synthesis of glucosides with physiological activity using various aglycons (hydroquinone, *p*- and *m*-hydroxybenzyl alcohol and vanillyl alcohol), and it exhibits high stability in organic solvents.[5–8] Rising interest for the application of this enzyme invokes the development of adequate immobilization protocols and the opportunity for multiple use and application in continuous immobilized enzyme reactors. Hitherto, maltase from *S. cerevisiae* has been immobilized by encapsulation in polygalacturonic beads coated with chitosan,[9] covalent immobilization *via* the oxirane groups on poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate) [poly(GMA-*co*-EDGMA)],[10,11] *via* the carbonyl groups on glutaraldehyde-activated Sepabead® EC-EA support[12] or on cyanogen bromide activated poly(2-hydroxyethylmethacrylate).[13]

The goal of this study was to evaluate supports bearing the thiosulfonate group in the immobilization of maltase from *S. cerevisiae*. Thiosulfonate moieties react only with the cysteine residues on the enzyme surface and form stable covalent disulfide bridges.[14–16] Such a support seems to be a good candidate for immobilization of maltase, since the enzyme activity rapidly diminishes unless a thiol-containing compound is present in solution, plausibly because it prevents unwanted conformational rearrangements.[17] Hence, it seems that unpaired cysteine residues are susceptible to reactions that cause loss of the catalytic conformation of the enzyme and this could be avoided by immobilization on thiosulfonate supports. In addition, it provides the additional advantage of more site-directed immobilization than supports previously applied in maltase immobilization since oxirane or carbonyl groups react with a significantly wider range of amino acid residues.[18] Thiosulfonate supports were obtained by chemical modification of two commercial supports: Eupergit® C, a copolymer of methacrylamide, *N*,*N*′-methylenebis(acrylamide) and a monomer carrying oxirane groups, and Purolite® A109, a styrene–divinylbenzene copolymer with primary amine functional groups (Table I).

TABLE I. The properties of Eupergit® C and Purolite® A109[19,20]

Support	Functional group	Particle size, μm	Pore size, nm	Surface area, $m^2\ g^{-1}$
Eupergit® C	Epoxy	150–300	25–250	180
Purolite® A109	Primary amine	425–1000	4.5	38

Chemistry of the supports and their activation are depicted in Fig. 1. The first step of Purolite® A109 modification was the introduction of the epoxy group with epichlorohydrin, while further steps are mutual for the activation of both supports and result in thiosulfonate groups that react with cysteine residues on the enzyme surface.



Scheme 1. The chemistry of thiosulfonate modification of Eupergit® C and Purolite® A109.

Different supports were applied in order to evaluate the eventual effects of the support surface and particularly two supports (Eupergit® C and Purolite® A109) were chosen bearing in mind their exceptional thermal and chemical stability and favorable mechanical and hydrodynamical features for application in different bioreactor configurations. Moreover, their non-toxicity and biocompatibility ensures their safe application in the food and pharmaceutical industry. High activities and stabilities have already been achieved with these supports in the immobilization of various enzymes,[2,20–25] including maltase.[10]

EXPERIMENTAL

The supports used in this study, Purolite® A109 and Eupergit® C were purchased from Purolite (Philadelphia, PA, USA) and Sigma–Aldrich, respectively. Maltase used throughout the study was isolated from *Saccharomyces cerevisiae* by a previously described procedure.[5] Reagents for activity and protein concentration assays: *p*-nitrophenyl-*α*-D-glucopiranosyde (*p*--NPG), Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) were purchased from Sigma–Aldrich, and all the other chemicals were of analytical grade and obtained from Centrohem (Stara Pazova, Serbia), unless specified otherwise.

All experiments were performed in duplicate and the average values are presented in the figures throughout the paper.

Preparation of epoxy-activated supports (epoxy-Purolite)

In order to introduce epoxy groups on the surface of Purolite® A109, a previously described modification method was applied.[20] The obtained epoxy-activated Purolite® A109 (epoxy-Purolite) was used directly for enzyme immobilization, but it was also further functionalized with purpose of introducing thiosulfonate groups on its surface.

Preparation of thiosulfonate-activated supports (TS-Purolite and TS-Eupergit)

For the introduction of thiosulfonate reactive groups on the surface of the epoxy-Purolite and Eupergit® C, a two-step procedure was performed. The first step comprised the introduction of thiol groups on the surface of the supports, using a slightly modified method previously described by Axen *et al.*[26] Briefly, 1 g of support (epoxy-Purolite or Eupergit® C) was equilibrated with sodium phosphate buffer (0.5 M; pH 6.3), and then filtered through a sintered glass filter. Subsequently, the suction dried support was suspended in 1 mL of the same buffer and 1 mL of 2 M sodium thiosulfate solution was added. The suspension was placed on an orbital shaker at 70 rpm and 22 °C for 6 h, and then the suspension was filtered and washed with an excessive amount of distilled water. Then 1 g of suction dried support was resuspended in 1 mL of sodium bicarbonate buffer (0.2 M; pH 8.5) and dithiothreitol (DTT) solution (prepared by dissolving 200 mg of DTT in 1 mL of 1 mM ethylenediaminetetraacetic acid (EDTA)) was added. The reaction mixture was placed on an orbital shaker at 100 rpm and 22 °C for 1 h. When reaction was completed, the obtained thiol-support (thiol--Purolite or thiol-Eupergit) was filtered through a sintered glass filter, and washed with sodium bicarbonate buffer (0.2 M; pH 8.5), distilled water and 0.1 M acetic acid to eliminate any residual DTT.

In the second step, the thiol groups of the supports (thiol-Purolite or thiol-Eupergit) were converted to thiosulfonate groups.[27,28] Thus, 1 g of suction dried thiol-support was suspended in 3 ml of sodium acetate (0.2 M at pH 5.0) and then 120 μL of 30 % hydrogen peroxide was added. The reaction mixture was placed on an orbital shaker at 100 rpm and 22 °C. During the

course of reaction, aliquots of 150 μL of 30 % hydrogen peroxide were added to the reaction mixture after 0.5 h, 1.5 h and 2.5 h. Total reaction time should be no longer than 30 h. When the reaction was completed, the obtained thiosulfonate-supports (TS-Purolite or TS-Eupergit) were filtered and washed with 0.1 M acetic acid until the absence of peroxide. The TS-supports could be used immediately or stored in sodium acetate (0.2 M; pH 5.0) at 4 °C until use.

Immobilization of maltase

Immobilization of maltase was performed onto several unmodified and modified supports (Purolite® A109, Eupergit® C, epoxy-Purolite, TS-Eupergit and TS-Purolite). The support (0.1 g) was equilibrated with 10 mM phosphate buffer (pH 6.8) and resuspended in 2 mL of maltase solution that was prepared in the same buffer so that 5 mg of proteins per g of support was offered. These suspensions were incubated on an orbital shaker at 25 °C and 100 rpm for 48 h. At different intervals, aliquots of the suspensions were removed and centrifuged for 90 s at 10,000 rpm. The separated immobilized preparations were washed three times with phosphate buffer (10 mM; pH 6.8), centrifuged at 10,000 rpm and then used for the determination of the maltase activity. The supernatants were sequestered for protein and maltase activity determination.

For two selected supports (TS-Purolite and TS-Eupergit), which exhibited the best characteristics during preliminary screening, optimization of immobilization process was performed as follows: 0.05 g of equilibrated support was resuspended in 1 mL of maltase solutions prepared by dissolving different amounts of protein in 10 mM phosphate buffer (pH 6.8) so that 1–100 mg of proteins per g of support was offered. The obtained immobilized preparations were washed, centrifuged and further used as described in the previous paragraph.

Protein assay

Protein concentration was determined according to the Bradford method using bovine serum albumin as the standard protein.[29] The amounts of bound enzyme were determined indirectly from the difference between the amount of enzyme introduced into the immobilization process and the residual amount of the enzyme in the supernatant after the immobilization. Protein immobilization yield (*PIY*) was calculated using the equation:

$$PIY = \frac{\text{Proteins in blank sample} - \text{Proteins in supernatant after immobilization}}{\text{Proteins in blank sample}} \quad (1)$$

Blank sample represents the control sample of enzyme solution without an immobilized support, exposed to the same conditions as the immobilized samples during the immobilization procedure in order to eliminate external factors for eventual activity loss.

Maltase activity assay

The activity of free and immobilized maltase was determined using *p*-nitrophenyl-*α*-D--glucopiranosyde (*p*-NPG) as substrate.[30] The substrate solution was prepared by dissolving 0.3 mg mL^{-1} *p*-NPG in phosphate buffer (50 mM; pH 6.8) and 1 mM *β*-mercaptoethanol (2-sulfanylethanol). For the soluble enzyme, 0.2 mL of substrate solution was mixed with 0.005 mL of free enzyme sample. The enzyme activity was calculated from the slope of the *p*-nitrophenol (*p*-NP) calibration curve. In the assay with immobilized enzyme, *β*-mercaptoethanol was omitted from the substrate solution. The assay mixture for immobilized maltase consisted of 0.005 g of immobilized preparation suspended in 0.4 mL of substrate solution. Liberation of *p*-NP was monitored at 405 nm in 30 s intervals for 2 min. 1 IU of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of *p*-NP at 25 °C in 1 min

from *p*-NPG. The immobilization yield (*IY*), the expressed activity (*EA*) and the specific activity (*SA*) were calculated using the following equations:

$$IY = 100 \times \frac{\text{Activity in blank sample} - \text{Activity in supernatant after immobilization}}{\text{Activity in blank sample}} \quad (2)$$

$$EA = 100 \times \frac{\text{Activity of immobilized preparation}}{(IY/100) \times \text{Activity in blank sample}} \quad (3)$$

$$SA = \frac{\text{Activity of immobilized preparation}}{\text{Bound proteins}} \quad (4)$$

Elution of maltase from TS-Purolite

NaCl treatment. Samples of the immobilized enzyme were taken after 3, 9 and 15 h of immobilization under optimum conditions. The wet immobilized preparations were resuspended in 1 M NaCl and incubated on a thermoshaker at 600 rpm and 25 °C for 2 h. The activities of immobilized maltase and activities in the supernatants prior and after the NaCl treatment were determined by the maltase activity assay. In addition, the protein contents in supernatants before and after treatment were determined by the protein assay.

β-*Mercaptoethanol treatment*. Maltase immobilized on TS-Purolite was treated with 1 M *β*-mercaptoethanol on a thermoshaker at 600 rpm and 25 °C for 10 h. The maltase activity and protein content in the supernatant and the activity of the immobilized preparations were determined by the activity and protein assays prior and after the *β*-mercaptoethanol treatment.

RESULTS AND DISCUSSION

Selection of immobilization support

In a preliminary experiment, maltase from *S. cerevisiae* was immobilized on five supports differing with respect to the polymeric scaffolds and functional groups (primary amine, epoxy or thiosulfonate) in order to provide an adequate evaluation of the efficiency of the immobilization *via* cysteine residues. The obtained results of the protein immobilization yield indicated that the supports with epoxy groups enabled higher protein loadings than their thiosulfonate derivatives (Table II). The differences were more prominent with Purolite as the support, since the second modification step resulted in a decrease of the protein immobilization yield from 88.8 to 62.1 %. Unmodified Purolite, the only support with amino functional groups, showed the lowest *PIY*. With respect to *IY*, the differences between the results obtained with epoxy- and thiosulfonate-supports were insignificant, since yields above 90 % were achieved. As the immobilization yield expresses the decrease of maltase activity in the supernatant, such a trend indicates that both functionalities have high affinity towards maltase immobilization.

Enzyme attachment is not always useful because its orientation and activity is influenced by the type of functional group on the enzyme surface, and hence for future use, immobilized enzymes need to be evaluated through the expressed activity.[31–34] Hugely higher expressed activities (54.6 % for Eupergit and 48.9 % for Purolite) were achieved with thiosulfonate supports than with epoxy supports

(only 4.6 % for Eupergit and 1.5 % for Purolite). Such a trend indicates that maltase retains a higher portion of its initial activity if attached exclusively *via* cysteine residues on thiosulfonate supports, than in case of immobilization on epoxy supports, which can occur *via* both thiol and amino groups on the protein surface.[18]

TABLE II. The efficiency of maltase immobilization on different supports

Support	*PIY* / %	*IY* / %	*EA* / %	*SA* / IU g^{-1} protein
Purolite® A109	55.2	75.1	3.1	0.97
Eupergit® C	96.4	100	4.6	1.10
Purolite-epoxy	88.8	91.2	1.5	0.35
TS-Eupergit	89.3	98.3	52.3	8.16
TS-Purolite	62.1	97.1	44.6	8.83

Since the immobilization yields were similar in all experiments, provided that almost complete activity disappeared from the supernatant, lower activity yields measured on support indicate that attachment on epoxy supports occurred predominantly through basic amino acid residues (mostly lysine residues), which resulted in a less active enzyme conformation.

Furthermore, in terms of evaluating maltase immobilization selectivity towards the examined supports, the specific activities of different immobilized preparations were compared (Table II). The highest specific activities were obtained for the thiosulfonate supports (8.16 IU g^{-1} proteins for Eupergit and 8.83 IU g^{-1} proteins for Purolite). Therefore, it could be concluded that maltase showed particular affinity towards the thiosulfonate supports.

Optimization of the immobilization on the thiosulfonate supports

Therefore, further experiments were focused on optimization of the immobilization on the thiosulfonate supports. The kinetics of the immobilization is depicted in Fig. 1.

Immobilization on the thiosulfonate supports is very slow process, since a constant linear increase in the loaded protein occurred throughout 48 h. However, it is obvious that only initial 24–30 h of immobilization are productive with respect to activity of maltase, since maximum *IY* was reached and it seems that subsequently, only futile attachment of proteins in an inactive conformation occurred. Hence, the optimum immobilization times were 24 h for TS-Purolite and 30 h for TS-Eupergit and all further experiments were performed at these immobilization times.

Subsequent experiments were performed to determine the capacity of the thiosulfonate supports by varying the concentrations of offered protein in the range 10–100 mg per g of dry support. The maximum was reached at offered protein concentration of 60 mg g^{-1} and further increases did not result in any improvements (Fig. 2). Similar protein loadings (12.1 and 12.3 mg g^{-1}) and immo-

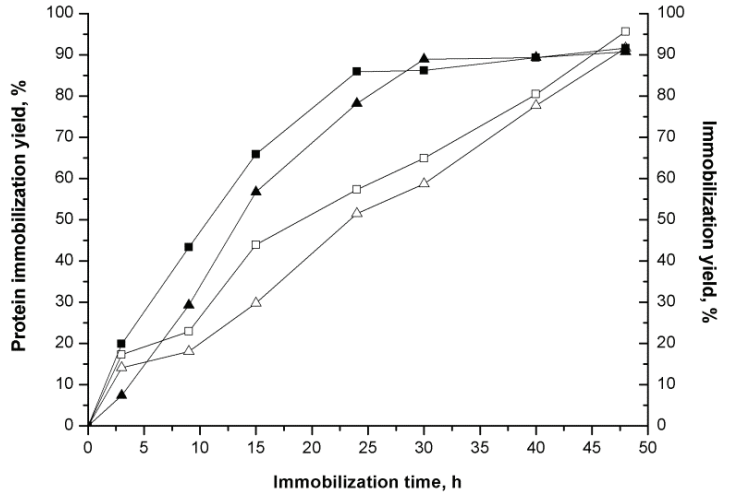


Fig. 1. Immobilization kinetics on TS-Purolite (triangles) and TS-Eupergit (squares). Immobilization yield is presented with filled symbols and protein immobilization yield with hollow symbols.

bilized activities (3520 and 3580 IU g^{-1}) were achieved with TS-Eupergit and TS-Purolite, respectively. When the obtained capacities are compared with those achieved using the other immobilization supports, namely Purolite (35 mg g^{-1} support), Purolite-epoxy (20 mg g^{-1} support) and Eupergit C (90 mg g^{-1} support), it is evident that the immobilizations predominantly occurred *via* thiosulfonate

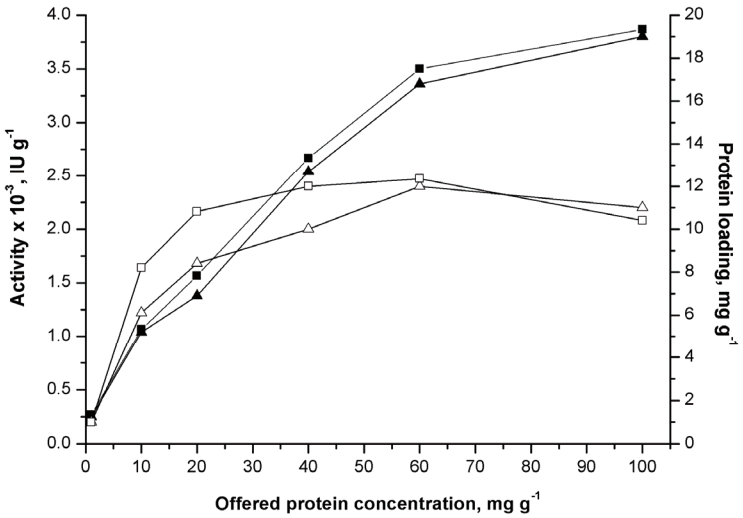


Fig. 2. The effect of the offered protein concentration on maltase immobilization on TS-Purolite (triangles) and TS-Eupergit (squares). Activities are presented with filled symbols and protein loadings with hollow symbols.

moieties, resulting in highly active and favorable enzyme conformations. Moreover, bearing in mind that both thiosulfonate supports exhibited similar results, it could be concluded that the surface of the supports did not influence the immobilization process.

Study of the immobilization mechanism

The results of immobilization onto the unmodified supports (Table II) indicated that significant fractions of the proteins (approximately 50 %) were attached onto the supports, although with low activity yield and hence adsorptive forces between the enzyme and the support scaffolds could not be disregarded. Covalent immobilization *via* thiosulfonate groups is a very slow process and hence fast enzyme adsorption inevitably occurred during the immobilization. However, the adsorption is also a reversible process and the formation of covalent bonds is irreversible under the examined circumstances, and overall immobilization is the sum of these competitive processes. It is plausible that during immobilization, adsorbed molecules gradually form covalent bonds with thiosulfonate moieties when in an appropriate conformation and covalent immobilization becomes predominant in the later stages. In order to test this hypothesis, desorption of immobilized enzymes obtained at different stages of immobilization (3, 9 and 15 h) with 1 M NaCl was performed. Obviously (Fig. 3), in the initial stage of immobilization (3 h), a significant drop in the retained activity (38 %) was obtained by desorption, but during the course of immobilization, it steadily decreased to only 6 % of the desorbed activity. Therefore, it was confirmed that the adsorbed enzyme molecules gradually formed covalent bonds with thiosulfonate groups and a stable covalently immobilized catalyst was obtained.

Finally, in order to confirm that immobilization occurred *via* cysteine residues, the immobilized enzyme was treated with the competitive thiol reagent,

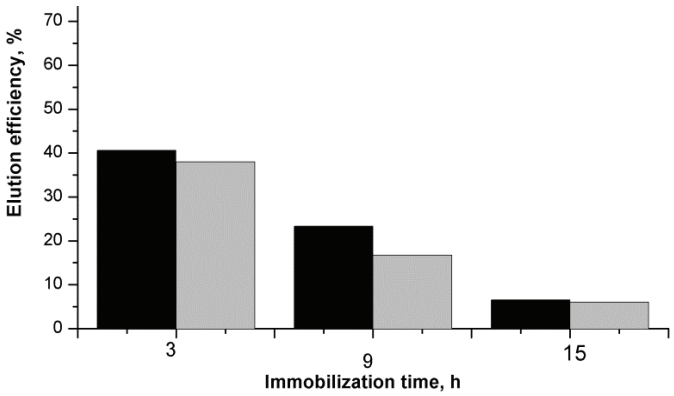


Fig. 3. Enzyme desorption with 1 M NaCl. Desorbed maltase activity is depicted with black bars and desorbed proteins with gray bars.

β-mercaptoethanol. After treatment, 98 % of the proteins and 95 % of the immobilized maltase activity had been released into the supernatant, which is confirmation that immobilization occurred almost exclusively through the formation of covalent bonds between thiol groups of cysteine. More importantly, it also indicates that immobilization is reversible, hence enzyme could be liberated and the support used repeatedly, which significantly reduces the costs of immobilization and enables the use of more expensive immobilization supports.

Thermal stability

Major drawback concerning enzyme employment in industrial bioprocesses is the possibility of thermal inactivation, due to the partial unfolding of the enzyme structure. Therefore, in terms of assessing the potential application of the obtained immobilized preparations (TS-Purolite and TS-Eupergit) on the industrial scale, the thermal stability of free and immobilized maltase was determined at 35 and 45 °C. The results (Fig. 4) clearly indicated that the immobilized enzymes showed moderately higher stability under the examined conditions in comparison to free maltase. As expected, the benefits of immobilization were more pronounced at the higher temperature, confirming that immobilization enables improvement of the rigidity of the tertiary structure of the enzyme and thus prevents its unfolding during heat treatment.

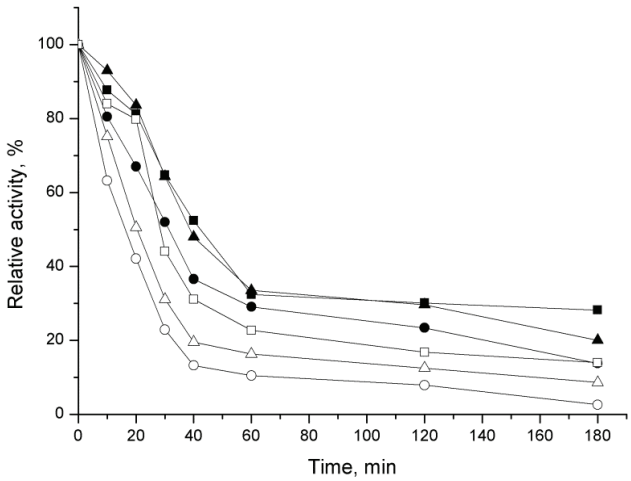


Fig. 4. Thermal stability of free maltase (circles) and maltase immobilized on TS-Purolite (triangles) and TS-Eupergit (squares) at 35 (filled symbols) and 45 °C (hollow symbols).

CONCLUSIONS

In this study, it was undoubtedly shown that thiosulfonate-supports provide favorable chemistry for the immobilization of maltase from *S. cerevisiae*. The fact that similar activities and protein loadings were achieved with two supports

with significantly different polymer scaffolds indicates that this approach could be successfully applied to a wide range of immobilization supports with hydroxyl, amino and epoxy groups. Finally, a simple method for the regeneration of the support using a competitive thiol agent indicates that even expensive immobilization supports could be used for maltase immobilization, since they could be reused after inactivation of the immobilized enzyme.

Acknowledgement. This research is performed within project III 46010 funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

И З В О Д

ИМОБИЛИЗАЦИЈА МАЛТАЗЕ ИЗ *Saccharomyces cerevisiae* НА ТИОСУЛФОНАТНЕ НОСАЧЕ

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У овој студији извршена је хемијска модификација површина два комерцијална носача за имобилизацију ензима (Purolite® A109 и Eupergit® C) са циљем увођења тиосулфонатних група, које би потом искључиво реаговале са остацима цистеина на површини ензима. На модификоване носаче имобилисана је малтаза из *Saccharomyces cerevisiae*. Том приликом је утврђено да је модификацијом површине носача омогућено постизање високих приноса ензимске активности (око 50 %), док су са друге стране, приноси активности у случају немодификованих носача биле мање од 5 %. Концентрација везаних протеина и добијене активности ензима имобилисаног на тиосулфонатне носаче износиле су 12,3 mg g^{-1} и 3580 IU g^{-1}, редом. У експериментима који су подразумевали десорпцију ензима са носача, рађеним на узорцима у различитим фазама имобилизације, доказано је да се имобилизација на тиосулфонатне носаче одиграва у два корака. Први корак подразумева фазу брзе адсорпције ензима на носач са тиосулфонатним групама, док је други корак спорији и подразумева формирање ковалентне везе између тиосулфонатних група носача и тиолних група остатака цистеина на површини ензима. Такође, важно је истаћи да поред формирања стабилних ковалентних веза, ова имобилизација поседује значајне предности, имајући у виду да је експериментално показано да је реверзибилна, односно да се значајан део везане ензимске активности (95%) може десорбовати са носача након третмана са тиолним реагенсом (β-меркаптоетанол) што омогућава поновну употребу носача након инактивације ензима.

(Примљено 30. јула, ревидирано 14. октобра, прихваћено 19. октобра 2016)

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