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Testing of the Effect of Reaction Parameters on the Enzyme Immobilization by Adsorption and Cross-Linking Processes with Kinetic Desorption Method

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Summary

The activity of enzymes after the immobilization by weak interactions such as adsorption or adsorption followed by a cross-linking reaction can change easily not only during further application but during the activity measured *via* desorption and/or inactivation of enzymes. The changes in activity could be a consequence of the interactions between the enzyme and support. In this work a quick and efficient method is developed that permits studying of the strength and properties of the forces between the enzymes and support by examining the kinetics of desorption and/or inactivation. Applying this new cyclic kinetic desorption method, the effect of reaction parameters on immobilization could also be studied. The efficiency of this method was tested for optimizing the parameters for immobilization of β-glucosidase on Amberlite IRA 900 anion exchange resin by an adsorption followed by a cross-linking with glutaraldehyde to form potential preparation for food industry. The following parameters were chosen: concentration (0.10 M) of the buffer (sodium acetate, pH=5.5) and the ratio of carrier to enzyme (10:1) for the adsorption step, then the time of treatment (1 min) and concentration (0.25, by mass per volume) of glutaraldehyde for the cross-linking by using the kinetic desorption method. The activity of this preparation was 57 μmol/(min·g) in respect of dry resin. It was established that the effect of different parameters on this enzyme immobilization could be characterized by the new cyclic kinetic desorption method in a quick and efficient way; furthermore, it permitted separate testing of the effect of parameters on the adsorption and cross-linking processes.

Key words: enzyme immobilization, adsorption and cross-linking, β-glucosidase, Amberlite IRA-900, kinetic desorption method

Introduction

Enzyme immobilization provides many important advantages over the use of native enzymes, namely reusability, continuous operation, controlled product formation, easy separation, use in nonconventional media and simple processing (*1*). It can also increase the stability of enzymes. However, in the case of multimeric enzymes, only a multisubunit immobilization could be advantageous for stability (*2*,*3*). Various techniques have been developed for enzyme immobilization, including adsorption, covalent linking to insoluble supports, entrapment in polymeric gels, encapsulation in membranes and cross-linking with bifunctional reagents. Different combinations of immobilization methods are also known (*4*).

One of these combined methods can be a two-step procedure where the first step is the adsorption of enzymes to the surface of an insoluble carrier as an ion exchange resin followed by a cross-linking with glutaraldehyde as a bifunctional reagent (*5–7*). The number and

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the strength of adsorptive bonds can be influenced mostly by parameters like pH and ionic strength. The pH of the immobilization medium has to be chosen on the basis of the isoelectric point of enzyme and the nature of carrier to create the appropriate charges for ionic bonds. As the adsorptive bonds are weak, by using *e.g.* glutaraldehyde as a cross-linking agent, further linkages can be generated to stabilize the interactions. As glutaraldehyde is a versatile reagent, these bonds can be formed between subunits of enzymes, enzyme molecules, or enzyme and carrier, depending on the conditions used in immobilization reaction (concentration of glutaraldehyde, properties of carrier, time of reaction) (*8*).

It is widely known that after enzyme immobilization and before activity measurement, a preparation is only washed several times with water and/or buffer at appropriate pH until there is no detectable enzyme in the last filtrate (*9*). In the case of weak interactions, this procedure may not be enough because the activity of immobilized enzyme preparation can be changed not only in further applications but during the measurement of enzyme activity as well. In the latter case, the desorption and/or inactivation could be a consequence of the interactions between the enzyme and the support.

Earlier a cyclic method called operational stability method (*10*) was used for the study of reusability of different immobilized enzyme preparations for industrial use (*11*). In this method the immobilized enzyme was used in the reaction solution for its application several times in cycles, and the activity of the enzyme was tested after each cycle. In this way the cycles take too long (hours, days) to detect the kinetics of desorption and/or inactivation. Therefore, a special method is required for the study of enzyme immobilization by an adsorption or an adsorption followed by a cross-linking to characterize the strength and properties of forces between the enzyme and the carrier. With the modification of operational stability method, another cyclic method called kinetic desorption method was created. In this technique the measuring of enzyme activity is repeated several times using defined short terms for activity measurement. After the first activity measurement the preparation is separated (filtered) from the reaction mixture, washed and its activity is remeasured in a fresh reaction mixture. The activity of the filtrate of previous reaction mixture is also measured. This cycle is then repeated. The usability of our method is presented on the example of β-glucosidase extracted from almond immobilized on the macroporous anion exchange resin Amberlite IRA-900 (styrene--DVB with quaternary amine active group) by adsorption followed by cross-linking with glutaraldehyde.

Enzyme β-glucosidase (EC 3.2.1.21) is an exohydrolase cleaving β-glucosidic linkages of conjugated glucosides, disaccharides and some oligosaccharides (*12*). β-glucosidase from almond is a glycoprotein composed of two active subunits and has a pH optimum and isoeletric point at pH=5.5 (*13*). In the food industry β-glucosidase can be used for the improvement of organoleptic properties in the production of wine and fruit juices (*14*). It can also be used for the synthesis of glycoconjugates by reversing the normal hydrolytic reaction in nonconventional media (*15*).

In the carbohydrate chemistry Amberlite IRA-900, which is cheap, easily available and has good mechanical properties, was earlier used only in transglycosylation reactions of other glycosidases with immobilization by adsorption (*16*), and with immobilization by a combination of adsorption and cross-linking with glutaraldehyde (*17*). β-Glucosidase was immobilized only on Amberlite IRA 67 by adsorption and it was used for transglycosylation and reverse hydrolytic reactions (*9*).

Earlier we used β-glucosidase immobilized on a modified polyacrylamide-type bead support (Acrylex C-100) for reverse hydrolysis of environmentally friendly surfactant *O*-alkyl glucosides used in the food industry (*18*). β-Glucosidase was immobilized on S-layer followed by entrapment in calcium alginate gel. This preparation was used for flavour enrichment of wines (*19*).

Here the results of the study of the usability of new kinetic desorption method for testing the effect of reaction parameters on the enzyme immobilization by adsorption and cross-linking processes are presented.

Materials and Methods

Chemicals

Amberlite IRA-900 chloride form (mean particle size 0.65–0.82 mm and pore size 40–75 nm) was obtained from Rohm and Haas Company (Philadelphia, PA, USA). β--Glucosidase from almonds (EC 3.2.1.21), *p*-nitrophenyl--β-glucopyranoside (*p*NPG) and other chemicals were from Sigma and Fluka/Riedel-de Haën (Budapest, Hungary).

Preparation of Amberlite IRA-900 hydroxide form

Amberlite IRA-900 hydroxide form was prepared by washing the chloride form with sodium hydroxide (4 %) and then with water according to the literature (*20*). The resin hydroxide form was dried in the presence of sodium hydroxide pellets at 25 °C.

β-Glucosidase activity assay

The activity of native or immobilized β-glucosidase was determined by a modified version of the method described in the literature (*21*) using *p*-nitrophenyl-β-glucopyranoside (*p*NPG) as substrate (0.01 M) in 0.10 M sodium acetate buffer, pH=5.5 (3.0 mL) containing native (0.1 mg) or immobilized (20 mg) enzyme. The reaction mixture was agitated on a rotary shaker (150 rpm) at 25 °C. The concentration of the released *p*-nitrophenol was measured at 400 nm in 0.20 M sodium carbonate buffer (1.0 mL), pH=10.2, containing a sample removed from the reaction mixture every minute (0.02 mL). One unit (U) of enzyme activity corresponded to the mass of enzyme (mg) or dry resin (g) required for hydrolysis of one μmol of substrate ((*p*NPG)/min). The activity of native β-glucosidase from almonds was 3.64 μmol/(min·mg) for Sigma and 10.54 μmol/(min·mg) for Fluka products by this method.

Immobilization method for the adsorption

Dried Amberlite IRA-900 hydroxide form (20 mg) and β-glucosidase (0.5–10.0 mg) in 0.10 or 0.01 M sodium

acetate buffer (pH=5.5) or in deionized water (1.0 mL) were agitated on a rotary shaker (150 rpm) at 25 °C for 24 h. The precipitate was filtered off (the activity of the filtrates was also measured) and washed with 0.10 M sodium acetate buffer, pH=5.5, until no enzyme was detected in the filtrate, then it was examined in wet form by the kinetic desorption method.

Immobilization method for the combination of adsorption and cross-linking

Dried Amberlite IRA-900 hydroxide form (20 mg) and β-glucosidase (2.0 mg) in 0.10 M sodium acetate buffer, pH=5.5 (1 mL) were agitated on a rotary shaker (150 rpm) at 25 °C for 24 h. After decantation the precipitate was treated with glutaraldehyde solution of 0.025–2.0 (mass per volume) in 0.10 M sodium acetate buffer (2.0 mL), pH=5.5, for 0.5–2.0 min on a rotary shaker (150 rpm) at 25 °C. The immobilized enzyme was filtered off (the activity of the filtrates was also measured), washed with 0.10 M sodium acetate buffer, pH=5.5, until no enzyme was detected in the filtrate, then it was examined in wet form by the kinetic desorption method.

Kinetic desorption method

The activity of wet immobilized enzyme preparation was measured in the reaction mixture described above for 10 min and the concentration of *p*-nitrophenol released was measured every minute. Then the enzyme preparation was filtered off (the activity of the filtrates was also measured), washed twice with 0.10 M sodium acetate buffer (2.50 mL), pH=5.5, and this two-step cycle was repeated several times.

Statistical analysis

All the experiments were repeated at least three times and the results were reproducible. The data points represent the mean values within ±5 % of the individual values.

Results and Discussion

The kinetic desorption method was used for the examination of some parameters of the immobilization of β-glucosidase on Amberlite IRA-900 by an adsorption followed by a cross-linking with glutaraldehyde. Since this technique applied two different kinds of chemical interactions for immobilization, different parameters are necessary for each step. Therefore, both steps of the combined method had to be examined separately. Our method permitted the testing of interactions after each step, depending on the applied parameters. In these preliminary tests the concentration of the buffer and the ratio of carrier to enzyme during the adsorption, then the time of treatment and the concentration of glutaraldehyde during cross-linking were studied.

The effect of buffer concentration and the ratio of carrier to enzyme during the adsorption

Buffer concentration

Adsorption of an enzyme on ionic exchangers requires a multipoint enzyme-support interaction. The enzyme interacts with the support only *via* the areas where a higher degree of ionic exchanges is possible (*22*). It is known that high buffer concentration can decrease the efficiency of enzyme immobilization by adsorption (*4*) because the ions in the buffer can reduce the possibility of connection between the enzyme β-glucosidase and the carrier Amberlite IRA-900 hydroxide form. The effect of pH and buffer concentration could not be examined separately since the concentration of buffer solution had an influence on the pH. Therefore, deionized water and 1.0 mL of sodium acetate buffer (pH=5.5) at two concentrations (0.01 and 0.10 M) were used in the reaction mixtures containing the carrier and the enzyme (10:1). It was found that higher buffer concentration resulted in higher pH value of the reaction mixture at the end of immobilization process, which can be attributed to a partial hydroxide-acetate anion change between the buffer solution and the carrier. At the end of immobilization process, the initial pH=5.5 increased to pH=8.2 and 7.0 when sodium acetate buffer was used at concentrations of 0.10 and 0.01 M, respectively, while no change was detected when deionized water (pH=7.0) was used. As β-glucosidase has an isoelectric point at pH=5.5, these high pH values (pH=8.2 and 7.5) could generate negative charge for the enzyme. On the basis of the enzyme activity of filtrates, there was no detectable enzyme desorption (or only inactivated enzymes were desorbed) during the first washing after the immobilization process. Using kinetic desorption method in all of the cases, a decrease in the immobilized activity was found. Contrary to expectation, the best results were detected for the highest buffer concentration (0.10 M sodium acetate buffer, pH=5.5) (Fig. 1). As the increase of buffer concentration can increase the pH of the reaction mixture, it can generate higher negative charge for the enzyme β-glucosidase. It is supposed that higher negative charge of the enzyme causes better connection to Amberlite IRA-900 anion exchange resin despite the fact that high buffer concentration usually decreases the efficiency of enzyme immobilization by adsorption.

The cycles were repeated until the value of the enzyme activity stopped or almost stopped. By using kinetic desorption method, these decreases in activities were attributed to desorption, but not to inactivation of enzyme molecules.

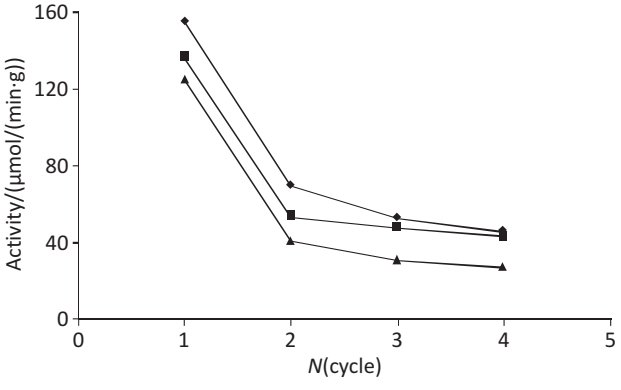

Fig. 1. The effect of buffer concentration on the activity of β-glucosidase immobilized by adsorption to Amberlite IRA-900 resin; media: ◆ 0.10 M sodium acetate buffer, pH=5.5, ■ 0.01 M sodium acetate buffer, pH=5.5, ▲ deionized water

Ratio of carrier to enzyme

In the mixtures, dried Amberlite IRA-900 hydroxide form (20 mg) and β-glucosidase (0.5–10.0 mg) reacted, and after separation the immobilized enzyme samples were tested using the kinetic desorption method. The activity of the samples was compared after the first cycle because these activities were involved in further cross-linking reactions. After a period of approximately linear increase up to the carrier-enzyme ratio of 10:1, the activities of the preparations reached a maximum value and at the ratio of 10:6 and higher, the activities decreased (Fig. 2). This decrease can be attributed to the accumulation of too many enzyme molecules on the surface of the support, which could reduce the possibility of connection of substrate molecules to their active site (*23*), or the amount of enzyme could generate diffusion problems due to a higher rate of hydrolysis than that of substrate entry. On the basis of the activities of β-glucosidase preparations immobilized by adsorption, besides economical considerations, the ratio of Amberlite IRA-900 hydroxide form to β-glucosidase of 10:1 was chosen for further investigations.

It was found that the presence of substrate (cellobiose as a natural substrate of β-glucosidase at 0.10 M concentration) and temperature (between 10 and 30 °C) had no considerable effect on the adsorption process when using the chosen parameters (0.10 M sodium acetate buffer, pH=5.5, and support-enzyme ratio of 10:1).

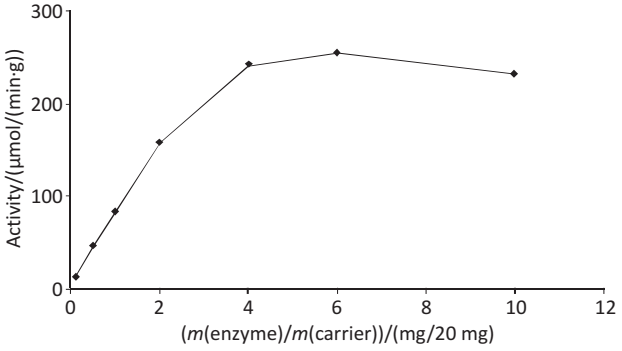

Fig. 2. The effect of the carrier to enzyme ratio on the activity of β-glucosidase immobilized by adsorption to Amberlite IRA-900 resin after the first cycle of the kinetic desorption method

The effect of the time of treatment and concentration of glutaraldehyde on the cross-linking of β-glucosidase adsorbed to resin

As the chemical structure of Amberlite IRA 900 does not permit the generation of covalent or other chemical bonds with glutaraldehyde, the possibility of interaction between the enzyme and support with glutaraldehyde was not studied. Accordingly, it was presumed that enzymes located close to each other were cross-linked by glutaraldehyde on the surface of the support. It is known that in most cases glutaraldehyde cross-linking can cause notable decrease in the catalytic activity of enzymes, which is mostly influenced by the time of treatment and the concentration of glutaraldehyde (*24*). At the same time, it can also stabilize the structure of enzymes and decrease the possibility of desorption by cross-linking the enzymes previously adsorbed on the surface of the support. In this manner this dual effect of cross-linking with glutaraldehyde *via* the change of concentration and time of treatment of glutaraldehyde was studied.

Time of treatment

The effect of time of glutaraldehyde treatment was studied in reaction mixtures containing 0.25 (mass per volume) glutaraldehyde in 0.10 M sodium acetate buffer, pH=5.5. In agreement with literature data (*24*), a notable decrease was observed in catalytic activity of immobilized preparation after glutaraldehyde treatment compared to the activity measured in the first cycle after the adsorption step. Similarly to the earlier experience (*7*), in this experiment reaction time longer than 15 min caused a too strong decrease in the immobilized activity, therefore short treatments (0.5, 1.0 and 2.0 min) were also studied. By using the kinetic desorption method, at first an increase in the immobilized activity was found (Fig. 3). This enhancement is attributed to an advantageous change in the structure of the enzyme layer after the cross-linking reaction. The cycles were repeated until the enzyme activity stopped changing. On the basis of the measurements of enzyme activity of the reaction mixture filtrates, there was no detectable enzyme desorption (or only inactivated enzymes were desorbed), so the preparation can be considered stable. However, a really short reaction time (0.5 min) gave the highest immobilized activity, but because of technological reasons a slightly longer (1 min) reaction time was chosen for our further investigations. Despite the fact that the cross-linking caused a notable decrease in catalytic activity, in the fourth cycle of the kinetic desorption method the cross-linked preparation had higher catalytic activity (57 μmol/(min·g) in respect of dry resin) than the preparation without cross-linking (44 μmol/(min·g) in respect of dry resin). Furthermore, catalytic activity and the interactions between the enzyme and the support were stable in the case of cross-linked preparation, while in the case of preparation without cross-linking the decrease in catalytic activity and the desorption of enzymes were not completely stopped in the fourth cycle.

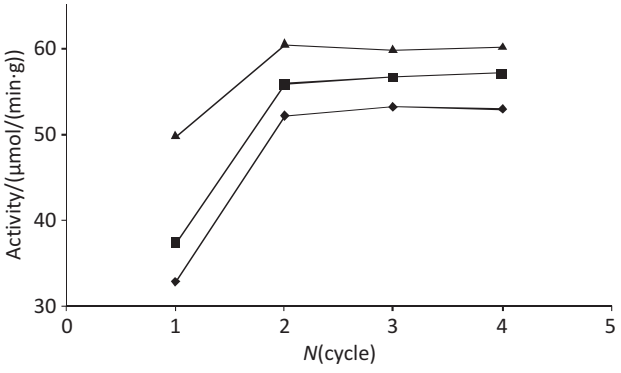

Fig. 3. The effect of the time of treatment with glutaraldehyde on the activity of β-glucosidase preparations immobilized by cross-linking of β-glucosidase adsorbed to Amberlite IRA-900 resin; time of treatment: ◆ 2 min, ■ 1 min, ▲ 0.5 min

Concentration of glutaraldehyde

As the concentration of glutaraldehyde can affect the formation of intra- and intermolecular interactions between enzymes (*25*), various quantities of glutaraldehyde were used in the literature for immobilization reactions by cross-linking. The concentration of glutaraldehyde in the reaction mixtures changed from 0.125 to 10 (mass per volume) (*7,18*) to form mostly enzyme-enzyme interactions. In this study, the decrease in the concentration from 2.0 to 0.50 (mass per volume) caused an increase in the immobilized activity (Fig. 4).

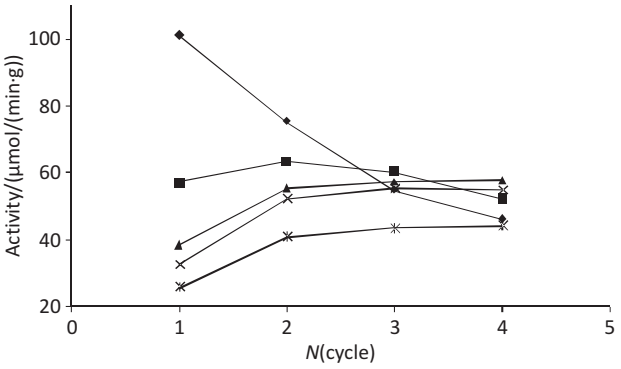

Fig. 4. The effect of glutaraldehyde concentration on the activity of β-glucosidase preparations immobilized by crosslinking of β-glucosidase adsorbed to Amberlite IRA-900 resin; glutaraldehyde concentration (mass per volume): ◆ 0.025, ■ 0.10, ▲ 0.25, × 0.50, ∗ 2.0

At the concentration of glutaraldehyde of 0.25 and 0.50 (mass per volume) almost the same immobilized activities were measured. At very low glutaraldehyde concentration (0.025, by mass per volume), a minimal effect of cross-linking reaction was found. At a slightly higher glutaraldehyde concentration (0.10, by mass per volume), the cross-linking resulted in a stronger interaction but this was not enough to form a stable immobilized preparation. By using the kinetic desorption method, the decrease of activity of these preparations (prepared with 0.025 and 0.10 (mass per volume) glutaraldehyde) was attributed to desorption but not to inactivation of enzyme molecules as in the case of preparations immobilized by adsorption. Since reduced enzyme activity was found at the highest glutaraldehyde concentration examined (2, by mass per volume), the lowest effective glutaraldehyde concentration (0.25, by mass per volume) was chosen for further detailed investigations.

It was found that the presence of substrate (cellobiose at 0.10 M concentration) and pH (between pH=5.5 and 7.0; 0.10 M sodium acetate buffer) had no considerable effect on the cross-linking when using the chosen parameters (1 min glutaraldehyde treatment and 0.25 (mass per volume) glutaraldehyde). Furthermore, it was observed that the highest catalytic activity in the fourth cycle was 57 μmol/(min·g) in respect of dry resin when using the above chosen parameters.

Conclusion

It was established that the effect of different parameters on the enzyme immobilization reactions can be characterized by our cyclic kinetic desorption method in a quick and efficient way. This method can be used primarily for the examination of immobilization processes that apply weak interactions as adsorptive bonds between enzyme and support or apply combined immobilization techniques in which weak interactions are also present, *e.g.* adsorption followed by a cross-linking with a bifunctional reagent. The effect of parameters on the immobilization of β-glucosidase on Amberlite IRA-900 by an adsorption followed by a cross-linking with glutaraldehyde was studied using our kinetic desorption method. Further advantage of this method was that it permitted separate testing of the effect of parameters on the adsorption and cross-linking processes. In this study, the parameters of immobilization were optimized to determine the effect of the preparation on the reaction mixture. The kinetic desorption method can be highly effective for the optimization of parameters of enzyme immobilization to produce stable and efficient preparations for given food industrial utilizations.

Detailed studies of the stability of the preparation for its utilization in food industrial processes (cellobiose degradation, reverse hydrolysis to produce *O*-alkyl glucosides and barley β-glucan oligosaccharides degradation) are in progress in our laboratory.

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