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USE OF TOYOPEARL AS A SUPPORT FOR THE IMMOBILIZATION OF UREASE

ICHIRO SASAKI, KEISUKE MAKINO* and TAMIO TAKEUCHI
Department of Polymer Science and Engineering, Faculty of Textile Science, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606 (Japan)
and
HIROO SASAKI
Central Research Laboratory, Toyo Soda Manufacturing Co., Tonda, Shinnanyo, Yamaguchi 746 (Japan)
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SUMMARY

A packing material for size-exclusion chromatography, Toyopearl, which was derivatized by introducing amino groups, was found to be a good support for the immobilization of urease. The immobilized enzyme had an optimum pH of 7, the same value as for intact urease. In the use of the enzyme, packed in PTFE tubing (*ca.* 8 cm × 0.7 mm I.D.) in a flow system, the addition of ethylenediaminetetraacetic acid (EDTA) to the eluent was found to be essential. With phosphate buffers containing EDTA, a constant high value of the enzyme activity could be obtained for 21 days at 37°C.

INTRODUCTION

By the application of enzymatic reactions to clinical analysis[1,2], the diagnosis of various diseases has become more accurate and faster. The recent introduction of immobilized enzymes[3–6] has furthered this trend and possible the reuse of expensive enzymes. This unique and powerful method will be needed to overcome the current problem that the number of clinical samples to be examined is increasing more rapidly than that the number of clinical chemists.

Another useful tool recently introduced into clinical medicine is high-performance liquid chromatography (HPLC)[7,8]. These two methods are often combined to produce a more effective method for complex biological samples[9]. In this case, the samples are separated by HPLC and the targeted molecules, which have been isolated from biological contaminants, are subsequently converted into easily detectable compounds by an immobilized enzyme, packed into a column. However, the substrate remaining on the immobilized enzyme column after the measurements can often inhibit the enzyme activity. For example, when controlled-pore glass is used as a support, damage to the enzyme can be caused by substrates remaining on the column through the interaction with free silanols on the support. Also agarose, which is

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preferred as a support for this purpose and is known to be less prone to interaction with components in biological samples, is so soft that the system can hardly be used at a practical, high flow-rate.

In this study, we have examined the applicability of Toyopearl, which has been used as a semirigid gel in size-exclusion chromatography[10,11] and is known to be less adsorptive for components in the samples, as a base matrix for the immobilization of the enzyme. Toyopearl has also been used as a support in affinity columns[12] and has shown excellent properties for biological samples. The enzyme bound to the support in the present investigation was urease (urea amidohydrolase, E.C. 3.5.1.5), and the immobilized enzyme was used for the detection of urea in a flow system.

EXPERIMENTAL

Urease was purchased from E. Merck (Darmstadt, F.R.G.). Toyopearl HW-55S was obtained from Toyo Soda (Tokyo, Japan). The amino derivatization of the gel was performed according to the method reported previously[12]. All other chemicals were purchased from Nakarai Chemicals (Kyoto, Japan) and used without further purification.

Water was purified by a Milli R/Q water purifier (Millipore, Bedford, MA, U.S.A.). The concentration and pH of the phosphate buffer were 50 m*M* and 7, respectively, unless otherwise specified.

An aqueous solution of urease was prepared by dissolving 0.2 g of urease in 5 ml of phosphate buffer and allowing it to stand for 2 h at 5°C. Precipitates were removed by centrifugation at 7880 *g* for 30 min at 4°C. The samples were obtained as supernatants.

The immobilization of urease on the gels was carried out according to the method reported previously for urease bound to alkylaminosilane glass beads[1]. The gel (1 g), which was washed with water, was activated with 25 ml of an aqueous glutaraldehyde solution (2.5%). The suspension was degassed *in vacuo* for 30 min at room temperature and then allowed to stand for 1 h under atmospheric conditions. After the reaction, this activated gel was washed with water. The prepared urease solution (15 ml) was added to the activated gel, followed by degassing for 30 min at room temperature. The immobilization was carried out by shaking the sample under atmospheric conditions for 24 h at 5°C. After the coupling reaction, the suspension was washed with phosphate buffer and then dried *in vacuo* for 1 h at room temperature. The immobilized urease was stored dry in a refrigerator at 5°C.

For the determination of the activity of the immobilized urease in the batch system, the amount of ammonium ion released by the enzyme was determined by the urease–indophenol method, in which urea was used as a substrate. Two reagents were used: solution A containing 12.5 g of phenol and 62.5 mg of sodium nitroprusside per l; solution B containing 1.56 g of sodium hydroxide and 4 ml of 10% sodium hypochlorite solution per l. The determination was carried out as follows. A test sample (5 ml) of substrate was prepared by dissolving 1 g of urea in phosphate buffer. The solution was preincubated for 15 min at 37°C. Subsequently, 20 mg of immobilized urease gel were added. The suspension was incubated for 2 min at 37°C in a shaking incubator. After incubation, the suspension was filtered immediately through a Millex-HA filter (Millipore). To the filtrate (10 μl), 2 ml of solution A and

then 2.5 ml of solution B were added. This mixture was kept for 50 min at 50°C in a shaking incubator. After incubation, the absorbance at 560 nm was measured. A calibration curve used for the calculation of the activity was obtained by use of different concentrations of ammonium chloride and by plotting the concentrations against the observed absorbance.

The column of immobilized urease was prepared as follows. Pre-swollen, immobilized urease gel (200 mg) was suspended in 1.5 ml of phosphate buffer. This slurry was packed in PTFE tubing (50 cm × 0.7 mm I.D.). One end of this column was connected to a pump, Model 5SK25GK-A (Milton Roy Co., Philadelphia, PA, U.S.A.), the other end was plugged with glass wool to retain the gel, and then a stainless-steel tube was attached. Pumping was continued until the gel had settled completely. The column of immobilized urease thus prepared was installed in a flow system in which the column was placed immediately after the sample injector and the column outlet was connected directly to the fraction collector. The determination of the concentration of ammonium ion in the column effluent was performed as follows. A quantity (1 ml) of the effluent was collected immediately after the injection of the sample and then 2.0 ml of solution A and 2.5 ml of solution B were added to the effluent. After the same treatment as that used for the batch system, the absorbance was measured.

RESULTS AND DISCUSSION

First, the effect of the functional groups at the gel surface on the resulting activity of the immobilized urease (IU) was examined. Under the conditions given in the Experimental section, urease was immobilized, by use of glutaraldehyde on two different types of Toyopearl gel: HW-55S, which has hydroxyl groups, and its amino derivative. The resulting activities (U/g IU) were 1.84 and 1520 for Toyopearl HW-55S and its amino derivative, respectively. One unit (U) of enzyme hydrolyzes 1 μmol of urea per minute at 37°C under the conditions described in the Experimental section. The higher activity obtained with the amino derivative suggests that the introduction of amino groups into the gel is essential for immobilization in which glutaraldehyde is used as a spacer. Therefore, the amino derivative of Toyopearl was used as a support for the immobilization in further studies.

The effect of the concentration of the urease solution was then explored. Different concentrations of urease solution were used for the immobilization on 1 g of gel, activated prior to use with glutaraldehyde, and the activity of the immobilized enzyme was determined. The maximum activity was achieved when 15 ml of urease solution (40 g/l) were used with 1 g of gel. A further increase in the amount of enzyme did not lead to an increase in the activity. Although the use of higher concentrations of urease may shorten the reaction time between urease and the gel, activated with glutaraldehyde, our attempts did not lead to better results because of extensive precipitation of urease. Since this precipitation blocks the column, a 40 g/l urease solution was selected for immobilization in the present study.

The effect of the reaction time for the immobilization of urease on the activity of the IU obtained was also explored in order to optimize the immobilizing conditions. A 15-ml volume of urease solution (40 g/l) was used for immobilization on 1 g activated gel, and the activity of the IU thus prepared was determined for each

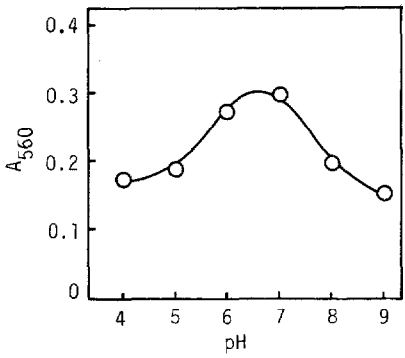


Fig. 1. Relationship between the activity of immobilized urease and pH of the urea solution. The activity is expressed in terms of the absorbance of stained solutions at 560 nm. Conditions: immobilized urease, 20 mg; urea, 1 g; concentration of urea in sample, 200 g/l; temperature, 37°C; reaction time, 2 min.

different reaction time. The maximum activity of IU was obtained with a reaction time of 12 h; longer reaction times did not result in an increased activity of IU. For this study, a reaction time of 24 h was selected. Consequently, in the present work, all reactions were carried out under the following conditions: activated gel, 1 g; volume of urease solution (40 g/l), 15 ml; reaction time between urease and the activated gel, 24 h; temperature, 5°C.

The optimum pH for the enzymatic activity of IU was determined as follows. After enzymatic reaction at different pH values the activity was determined spectroscopically. The change in activity obtained with the pH is depicted in Fig. 1. From the results, the optimum pH for the IU was found to be *ca.* 7. It has been reported that the optimum pH for the enzymatic reaction of intact urease in solution lies between 6.4 and 7.6[13]. Thus, the optimum pH is unchanged by immobilization. This may suggest that slight changes in the enzyme environment, caused by the remaining unreacted amino groups on IU, do not affect the activity.

In practical applications of IU in clinical medicine the enzyme may be stored in slurry form for certain periods before use. Therefore, the change in activity during wet storage was investigated. IU was stored at 5°C in phosphate buffer for 30 days,

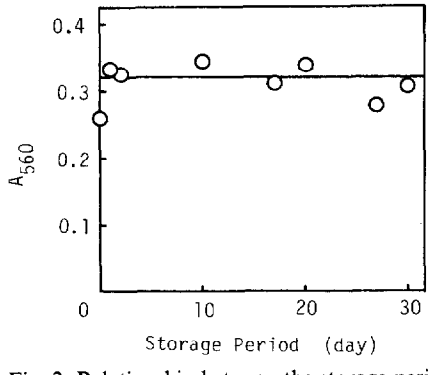
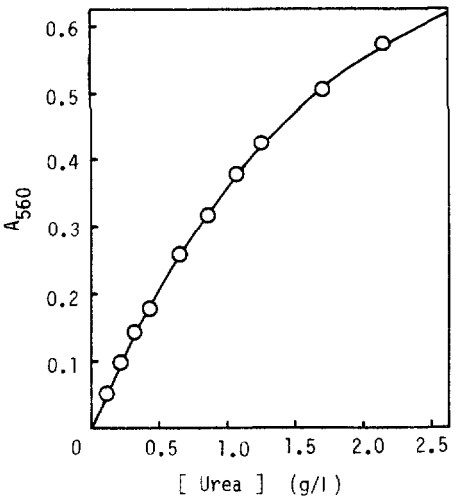


Fig. 2. Relationship between the storage period of wet immobilized urease and its activity. Details as in Fig. 1.

and the residual activity of an aliquot of IU (*ca.* 20 mg) was determined at intervals during that period by the method given in the Experimental section. A new aliquot was used for each measurement. The change in activity is shown in Fig. 2. This result demonstrates that the activity of IU did not decrease in cold phosphate buffer over this period and that the urease immobilized on Toyopearl can be used in clinical practice.

For continuous detection of urea, IU was packed in a column (*ca.* 80 × 0.7 mm) of PTFE tubing and was installed in the flow system. A calibration curve was constructed by introducing samples containing various concentrations of urea into the system and measuring the amount of ammonium ion released in the effluent. The samples volume was 25 μl. The flow-rate of the phosphate buffer, containing ethylenediaminetetraacetic acid (EDTA, 0.2 g/l), used as a carrier, was 0.5 ml/min. The temperature was controlled with a column oven at 37°C. Fractions (1 ml) were collected immediately after the injection and subsequently determined for released ammonium ion. The results in Fig. 3 show a good linear relationship between the concentration of urea (up to *ca.* 0.6 g/l) in the samples and absorbance of the effluent. The highest concentration of urea in normal human sera is *ca.* 0.4 g/l. Therefore, urease immobilized on Toyopearl with glutaraldehyde can be used for the detection of urea in normal human sera in either a flow-injection system or in a post-column detection system for HPLC, because the detection limit of the IU column can be increased by using a longer column.

Since a great number of samples is often analyzed in clinical laboratories, high



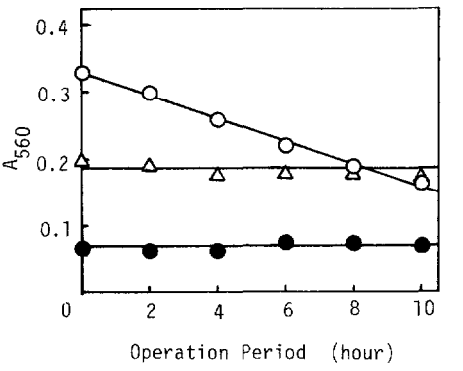


Fig. 3. Relationship between the concentration of urea in the sample loaded on the immobilized urease column and the absorbance at 560 nm of the stained effluents. Conditions: volume injected, 25 μl; column, *ca.* 80 × 0.7 mm; flow-rate, 0.5 ml/min; temperature, 37°C; carrier, phosphate buffer containing EDTA (0.2 g/l); fraction, 1.0 ml. Fractions were stained by reaction with solutions A and B and the absorbance monitored at 560 nm.

Fig. 4. Change in the activity of the immobilized urease column during continuous use for 10 h. Conditions: column, *ca.* 80 × 0.7 mm; concentration of urea in the sample, 0.4 g/l; volume injected, 25 μl; carrier and temperature, phosphate buffer at 37°C (○), phosphate buffer containing EDTA (0.2 g/l) at 25°C (●) and at 37°C (△). Other conditions as in Fig. 3.

stability of the enzyme is required in order to obtain reliable data. Therefore, the operational stability of the IU column (*ca.* 80 × 0.7 mm) was examined over 10 h by assaying samples every 2 h. The IU column was installed in the flow system, 25 μl of a solution containing 0.4 g/l urea were injected and the released ammonium ion was measured. The results are shown in Fig. 4. When phosphate buffer alone was used as a carrier at 37°C the activity of the IU column was found to decrease gradually to almost half of its original value after 10 h. However, the activity of this column could be restored several times, although not completely, by injecting phosphate buffer containing EDTA. Therefore, an EDTA-treated column was subsequently used for the assay with phosphate buffer, containing EDTA, at 37 and 25°C. When phosphate buffer containing 0.2 g/l EDTA was used as a carrier, the activity of the IU column was found to be unchanged after 10 h at 37 and 25°C, as shown in Fig. 4. These results suggest that the decrease in the activity of the IU column with phosphate buffer alone was caused by inhibition due to small amounts of metal in the solution, and that this undesirable contaminant can be removed by adding EDTA to the solution. By comparing the results at 37 and 25°C, it was found that at 37°C a higher sensitivity of detection was obtained, and that more reliable data could be obtained by use of a longer column, with EDTA in the carrier, at this temperature.

Another important factor for a practical column reactor is the reproducibility of over a long period. The column was installed in the flow system, and an assay carried out every day. Between assays, the column was stored with a carrier in a refrigerator at 5°C. The column (*ca.* 80 × 0.7 mm) was used with phosphate buffer containing EDTA (0.2 g/l), 25 μl of urea solution (0.4 g/l) were injected at 37 and 25°C and the amount of ammonium ion released was detected. At both temperatures the activity of each IU column was found to be constant for 25 and 21 days, respectively. During these periods, 62 injections were performed at each temperature.

In conclusion, Toyopearl was found to be highly useful as a support for the immobilization of urease. The immobilized enzyme column could be used in a flow-injection system or as a post-column reactor for HPLC and, therefore, could facilitate speedy and accurate analyses.

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