Conjugation of Penicillin Acylase with the Reactive Copolymer of *N*-Isopropylacrylamide: A Step Toward a Thermosensitive Industrial Biocatalyst

Alexander E. Ivanov, Ewald Edink,† Ashok Kumar, Igor Yu.Galaev, Alexander F. Arendsen,† Alle Bruggink,‡ and Bo Mattiasson*

Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden, DSM Food Specialties, P.O. Box 1, 2600 MA Delft, The Netherlands, and DSM Research, Life Sciences R&D, P.O. Box 18, 6160 MD Geleen, The Netherlands

Conjugation of penicillin acylase (PA) to poly-*N*-isopropylacrylamide (polyNIPAM) was studied as a way to prepare a thermosensitive biocatalyst for industrial applications to antibiotic synthesis. Condensation of PA with the copolymer of NIPAM containing active ester groups resulted in higher coupling yields of the enzyme (37%) compared to its chemical modification and copolymerization with the monomer (9% coupling yield) at the same NIPAM:enzyme weight ratio of ca. 35. A 10-fold increase of the enzyme loading on the copolymer resulted in 24% coupling yield and increased by 4-fold the specific PA activity of the conjugate. Two molecular forms of the conjugate were found by gel filtration on Sepharose CL 4B: the lower molecular weight fraction of ca. 10^6 and, presumably, cross-linked protein-polymer aggregates of MW > 10^7. Michaelis constant for 5-nitro-3-phenylacetamidobenzoic acid hydrolysis by the PA conjugate (20 μM) was found to be slightly higher than that of the free enzyme (12 μM), and evaluation of V_{max} testifies to the high catalytic efficiency of the conjugated enzyme. PolyNIPAM-cross-linked PA retained its capacity to synthesize cephalexin from D-phenylglycin amide and 7-aminodeacetoxycephalosporanic acid. The synthesis-hydrolysis ratios of free and polyNIPAM-cross-linked enzyme in cephalexin synthesis were 7.46 and 7.49, respectively. Thus, diffusional limitation, which is a problem in the industrial production of β-lactam antibiotics, can be successfully eliminated by cross-linking penicillin acylase to a *smart polymer* (i.e., polyNIPAM).

Introduction

Covalent attachment of enzymes and other proteins to poly-*N*-isopropylacrylamide (polyNIPAM) has been widely used as a method for the preparation of thermosensitive protein conjugates during the past 15 years (*1*). Water-soluble polyNIPAM undergoes a phase transition when heated above 32 °C (*2*). Conjugation of polyNIPAM with proteins provides them with thermosensitivity and allows their separation from aqueous solutions stimulated by a small shift of temperature, pH, or ionic strength (*3*). Owing to this unique property polyNIPAM warranted the name *smart polymer* (*1, 3*).

Being employed as reusable biocatalysts, the enzymes conjugated with smart polymers have certain advantages over enzymes immobilized on solid supports. Immobilization of enzymes on or within solid particles often leads to significant loss of the catalytic activity (*4*−*7*), although there are examples of immobilization techniques that result in fully retained activity, such as cross-linked penicillin acylase (PA) aggregates (*8, 9*). Most probable reasons for inactivation are conformational changes in the adsorbed protein globules (*10*), as well as random orientation and blocking of the active sites by the carrier surface (*11*). Mass-transfer of the substrates to the enzyme (and/or the products from it) may be limited as a result of their diffusion in pores of the solid carrier (*12*). Alternatively, the conjugation of enzymes to water-soluble reactive polymers may help to avoid the protein conformation changes (*13*), while the diffusional limitations for substrate are supposed to be strongly reduced (*14*). In fact, trypsin conjugates with end-group activated oligoNIPAM displayed even higher esterase and amidase activities as compared to those of the pure enzyme (*15*).

There are three most common synthetic ways to prepare enzyme−polyNIPAM conjugates:

(1) introduction of polymerizable vinyl groups into the protein followed by their copolymerization with NIPAM (*16, 17*),

(2) acylation of aminogroups of the protein by reactive copolymer of NIPAM carrying the ester functions of *N*-acryloyloxysuccinimide (NASI) comonomer as pendant groups (*14, 18, 19*), and

(3) acylation of aminogroups of the protein by homopolymer of NIPAM carrying reactive *N*-succinimide ester function as the end-group (*15, 18*).

It is worth noting that methods 2 and 3, as well as the properties of the resultant conjugates, were carefully compared using the same system of polyNIPAM conjugated to trypsin (*18*). The enzyme stability in solution was higher in both conjugates compared to that of native trypsin, while the end-group attachment of polyNIPAM yielded the higher stability of the conjugate under

* To whom correspondence should be addressed. Tel: +46-46-2228264. Fax: +46-46-2224713. E.mail: bo.mattiasson@biotek.lu.se.

† DSM Food Specialties.

‡ DSM Research, Life Sciences R&D.

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repeated temperature cycling. Methods 1 and 2 were not compared within the same conjugation system until recently, when studies carried out in our laboratory have shown that method 2 yields higher retained trypsin activity in the conjugate than method 1 does (*20*). On the other hand, chemical modification of enzymes with itaconic anhydride according to method 1 was earlier proven to be an efficient and mild technique: the thus modified β-glucosidase retained 98% enzymatic activity, while 84% of its lysine residues were acylated (*21*).

Although a lot of work has been done on the preparation and characterization of enzymes conjugated with smart polymers, many questions arise when a new enzyme is going to be conjugated and studied as a reusable biocatalyst for large-scale processes. Most important of them are

(a) choice of the conjugation method;

(b) estimation of the proper enzyme loading on the smart polymer with respect to activity, phase transition temperature, and feasibility of preparative isolation of the conjugate;

(c) separation of the conjugate from the nonreacted protein and the polymer;

(d) evaluation of structural features of the conjugate, including protein content, degree of cross-linking, and apparent molecular weight;

(e) estimation of kinetic constants displayed by the conjugated enzyme in the reactions with model substrates and in the target biotranformations; and

(f) overall performance in suitable applications such as simple peptide couplings.

The influence of enzyme loading on its coupling yield and relative specific activity was studied up to now only for low and moderate enzyme loadings (*14*). Cross-linking of enzymes during conjugation with multifunctional reactive copolymers or radical copolymerization was noticed in the literature but not quantified (*22*). Methods for separation of such conjugates, mostly based upon gel filtration or hydroxyapatite chromatography, were briefly described in the literature, although the relevant separation profiles were not reported (*18*, *23*).

The aim of the present study is to make a step toward effective conjugation of polyNIPAM to penicillin acylase (PA), the industrially important enzyme employed for synthesis of penicillin- and cephalosporin-type antibiotics.

The advantage of a smart-polymer-based biocatalyst already mentioned above are particularly true for the semisynthetic penicillins and cephalosporins. Their molecularly inherent instability (β-lactam ring) causes low thermostability and only narrow ranges of pH for reactions, product isolation, and other operations. Moreover, the reaction mixtures obtained after enzymatic coupling of activated side chain (arylglycine esters or amides) with the antibiotic nucleus (i.e., 6-APA or 7-ADCA) contain mixtures of amino acids and coupling products, which can only be separated through subtle pH changes. Recovery of the biocatalyst through a small and simple change of temperature greatly facilitates this downstream processing.

Carrying out the present study we tried to answer the questions formulated in the items a−f above and, therefore, to outline a way to prepare and characterize thermosensitive conjugates of penicillin acylase.

Materials and Methods

Materials. 1.4-Dioxane was dried over potassium hydroxide and distilled (101−102 °C). *N*-Isopropylacrylamide (NIPAM), *N*-acryloyloxysuccinimide (NASI), 5-nitro-3-phenylacetamidobenzoic acid (NIPAB), and 5-nitro-3-phenylglycinamidobenzoic acid (α-NIPAB) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ammonium persulfate and tetramethylethylenediamine (TEMED) were products of Bio-Rad Laboratories (Hercules, USA). 2,2′-Azobis(2-methylpropionitrile) was purchased from ACROS (Malmö, Sweden). Blue dextran (MW 10^6) and Sepharose CL-4B were products of Pharmacia-LKB (Uppsala, Sweden). Penicillin acylase from *E. coli* was supplied by DSM Anti Infectives (Delft, The Netherlands) as an aqueous solution ("Assemblase liquid", 13 U/mL), stabilized with 30% propylene glycol, and was used as received. Filtration membranes (regenerated cellulose, ϕ 25 mm, MWCO 100 kDa) were purchased from Intersep Deutschland (Witten, Germany).

Cephalexin, phenylglycin amide, and 7-aminodeacetoxycephalosporanic acid (7-ADCA) were in-house chemicals of DSM Research (Geleen, The Netherlands).

Methods. ***Copolymerization of NIPAM with N-Acryloyloxysuccinimide (NASI) and Preparation of NIPAM Homopolymers.*** NIPAM (2.15 g, 19 mmol) and NASI (0.169 g, 1 mmol) were dissolved in 20 mL of distilled 1,4-dioxane. AIBN (60 mg) was dissolved in 3 mL of dioxane and added to the above mixture. The solution was filtered through Munktell no. 3 filter paper and kept at 70 °C and nitrogen bubbling for 8 h. Three portions of the reaction mixture (6 mL) were each dropped into 50 mL of diethyl ether at vigorous magnetic stirring. The obtained precipitate was separated from the solvent by filtration through paper filter, washed by ca. 50 mL ether on the filter, and dried under vacuum over anhydrous calcium chloride. The yield of the copolymer was 89%. The activated ester functions in the copolymer were quantified by spectrophotometric estimation of *N*-hydroxysuccinimide ($\epsilon_{260} = 7000\ M^{-1}\ cm^{-1}$) released as result of alkaline hydrolysis in 5 mM sodium hydroxide. The content of monomer units with activated ester functions was ca. 0.5 mol %.

PolyNIPAM-1 was prepared according to the above protocol with no NASI monomer added. PolyNIPAM-2 and polyNIPAM-3 were prepared by polymerization of NIPAM (0.57 g) in water (20 mL) using ammonium persulfate (21 mg) and TEMED (14 μL) as initiators and mercaptoethanol as a chain-transfer reagent (14 and 105 μL of 1%v/v aqueous solution, respectively). The two latter NIPAM homopolymers were precipitated from water at 50 °C, dissolved in 1,4-dioxane, and reprecipitated into diethyl ether.

Viscosity Measurements: Estimation of Molecular Weights of NIPAM Polymers and Copolymers. Molecular weights (MW) of polyNIPAM and NIPAM-NASI copolymers were calculated from their intrinsic viscosity using the formula $[\eta] = 9.59 \times 10^{-5}\ MW^{0.65}$ (*24*). An Ubbelohde viscometer was used to measure intrinsic viscosity of the polymers in tetrahydrofuran at 27 °C.

Conjugation of PA by Copolymerization with NIPAM. Assemblase liquid (1 mL) was diluted with 0.1 M sodium phosphate (9 mL, pH 7.8), containing 5% w/v glucose. Fresh aqueous solution (1 mL) of itaconic anhydride (1 mg/ml) was added to the enzyme solution by portions of 50 μL during 12 min. NIPAM (429 mg, ca. 3.8 mmol) was dissolved in the solution of modified enzyme on shaking. The prepared solution was degassed under vacuum of a filter pump. Aqueous solution of mercaptoethanol (1% v/v, 0.21 mL or 30 μmol), ammonium persulfate (10 mg in 0.3 mL of distilled water) and TEMED (7 μL) were added to the reaction mixture, the latter filtered through Munktell no. 3 filter paper, and allowed to polymerize for overnight at room temperature.

The polymerization product was combined with 2 M NaCl (3 mL), and the mixture was heated to 35−40 °C for precipitation of the conjugate. The obtained precipitate was separated from solution by centrifuging (1 min, 4800 × *g* rcf), and the supernatant was collected and assayed for PA activity. The precipitate was dissolved in 10 mL of distilled water and combined with 3 mL of 2 M NaCl. Thermal precipitation followed by the assay of the supernatant was repeated. Finally, the thus precipitated conjugate was dissolved in 0.02 % (w/v) sodium azide (8 mL) and stored at 8 °C.

Chemical Modification of Penicillin Acylase by NIPAM-NASI Copolymer. *Synthesis of Condensation Conjugate I.* Assemblase liquid (0.25 mL) was diluted with 0.1 M sodium phosphate (4.75 mL, pH 7.8). NIPAM-NASI copolymer (0.107 g) was dissolved in the enzyme solution overnight on a rocking table at 6 °C. The reaction mixture was combined with 2 M NaCl (1.5 mL) and heated to 40−43 °C for precipitation. The obtained precipitate was separated from solution by centrifuging (1 min, 4800 × *g* rcf), and the supernatant was collected and assayed for absorbance at 280 nm and PA activity. The precipitate was dissolved in 5 mL of distilled water and combined with 1.5 mL of 2 M NaCl. Thermal precipitation followed by the assay of the supernatant was repeated. Finally, the thus precipitated conjugate was dissolved in 0.02 % (w/v) sodium azide (5 mL) and stored at 8 °C.

Synthesis of Condensation Conjugate II. Synthesis of condensation conjugate II was performed under conditions of higher enzyme loading (2.5 mL of Assemblase liquid was combined with 2.5 mL of 0.1 M sodium phosphate, pH 7.8) at the same amount of NIPAM-NASI copolymer (0.107 g) as described above. The reaction mixture was diluted with 4 M NaCl (5 mL) prior to thermal precipitation. The precipitated conjugate was dissolved in 0.02 % (w/v) sodium azide (5 mL) and stored at 8 °C.

Gel Filtration Chromatography. *Packing and Calibration of the Column.* A column (1 × 30 cm) packed with 24 mL Sepharose CL-4B gel was washed with 150 mL of degassed working buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.05% NaN_3, pH 7.5) at flow rate of 0.17 mL/min.

The following calibration samples dissolved in 0.5 mL working buffer were applied to the column and eluted at a flow rate of 0.17 mL/min by the working buffer, their elution profiles being registered using the UV detector (Uvicord SII, Amersham Pharmacia Biotech, Uppsala, Sweden) at $\lambda = 280$ nm:

(1) 0.5 mg of Blue Dextran (1.0 mg/mL, MW = 10^6 g/mol)
(2) polyNIPAM-1 (MW = 2.5×10^5)
(3) polyNIPAM-2 (MW = 2.3×10^6)
(4) polyNIPAM-3 (MW = 8.1×10^6)
(5) NIPAM-NASI copolymer (MW = 3.9×10^4)
(6) 10 μL of "Assemblase liquid" (MW = 7.1×10^4)
(7) 10 mg $K_2Cr_2O_7$ (MW = 294)

The calibration was obtained by plotting the logarithm of the MW of samples 2−6 against their intrinsic elution volumes (V_{el}*, see below). Samples 1 and 7 were used to estimate the void volume ($V_0 = 7.9$ mL) and the total volume ($V_t = 24.2$ mL) of the column. The calibration was described by the linear equation

$$\log \mathrm{MW} = A - bV_{el} \qquad (1)$$

where V_{el} is elution volume, $A = 9.9$, and $b = 0.25$. The coefficients A and b were chosen so that the values of number-average molecular weight $M_n = \Sigma H_i/\Sigma H_i/\mathrm{MW}_i$ coincided with the relevant molecular weights of samples 2 and 3 found by viscosity measurements. These samples contained the polymer fractions mostly eluted with $V_{el} > V_0$, the pattern more appropriate for calculation of M_n, while sample 4 contained a large portion of the polymer eluted at V_0, making the calculations less correct. H_i is the height of elution profile above the baseline in arbitrary units at given V_{el}; MW_i is the molecular weight calculated by eq 1 for the given V_{el}. Intrinsic elution volumes V_{el}* were calculated according to eq 1 for M_n of each polymer sample. V_{el} corresponding to the peak maximum was taken for calibration in the case of sample 6 (Assemblase liquid).

Gel Filtration Chromatography of Polymers and Conjugates. Aqueous solution of NIPAM-PA conjugate or NIPAM homopolymer (0.5 mL) was applied to the Sepharose CL-4B column equilibrated with the working buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.05% NaN_3, pH 7.5). Fractions of 1 mL were collected at a flow rate of 0.17 mL/min and assayed for PA activity, absorbance at 280 nm, and turbidity. Turbidity (470 nm) was measured after 5-fold dilution of the chromatographic fractions with the working buffer followed by their heating for 10 min in a water bath at 50 °C.

Estimation of Enzymatic Activity of Penicillin Acylase. A sample of penicillin acylase or its conjugate (5−100 μL) was added to a 250 μM solution of α-NIPAB in 50 mM sodium phosphate, pH 7.0 to the final volume of 2.5 mL. Absorbance of 3-amino-5-nitrophenylbenzoic acid (ANPBA), a product of α-NIPAB hydrolysis, was recorded at 405 nm each 30 s for 5 min. The rate of α-NIPAB hydrolysis was calculated using the molar extinction coefficient of ANPBA equal to 9090 M^{-1} cm^{-1}. One unit of enzymatic activity was defined as the amount of enzyme that hydrolyses 1 μmol of α-NIPAB in 1 min. This method for estimation of PA activity was used in all experiments carried out in the present study besides the estimation of kinetic constants of NIPAB hydrolysis catalyzed by the conjugated and free PA.

Membrane Filtration of Condensation Conjugate II. A solution of condensation conjugate II prepared as described earlier was dialyzed and diluted 10-fold with distilled water to the final volume of 5.0 mL. The sample was filtered through an Intersep membrane (100 kDa cutoff) with a flow rate of 0.5 mL/min under pressure of nitrogen, until 4.0 mL of filtrate was collected. Distilled water (4.0 mL) was added to the filtration unit, and the process was repeated. The filtered conjugate and the filtrate were assayed for absorbance at 280 nm and PA activity.

Estimation of Kinetic Constants of NIPAB Hydrolysis Catalyzed by Conjugated and Free Penicillin Acylase. Initial rate of enzymatic hydrolysis of NIPAB was determined from the increase in ANPBA concentration detected by its absorbance at 405 nm (see above). The concentration of NIPAB was varied from 3.5 to 350 μM. Assemblase liquid and condensation conjugate II were diluted 50-fold and 10-fold, respectively, by 0.1 M potassium phosphate (pH 7.0). Aliquots of the diluted enzyme preparation (50 μL) were added to a NIPAB solution (2.45 mL) in the same buffer, and the kinetics of hydrolysis was followed as described above. Kinetic constants were calculated from Lineweaver−Burk plots of the initial rates of enzymatic hydrolysis.

Cephalexin Synthesis Activity Measurements. Cephalexin synthesis experiments were done essentially as described elsewhere (*25*).

Results and Discussion

Comparison of PA Conjugates Prepared by Copolymerization of PA with NIPAM and by Covalent Attachment to NIPAM-NASI Copolymer. To choose the most preferential method for PA conjugation from the viewpoint of the enzyme coupling yield and retained activity, a comparison of two different techniques has been carried out. According to the first one, amino groups of the enzyme were acylated with itaconic anhydride for introduction of polymerizable double bonds to the protein molecule, which was subsequently copolymerized with NIPAM. According to the second technique, amino groups of the enzyme were acylated by the active esters of NIPAM-NASI copolymer.

It should be noted that *N*-hydroxysuccinimide ester groups simultaneously undergo two chemical reactions: amidation with amino groups of the protein and hydrolysis. The input of each of these processes depends on the conditions of the conjugation, in particular, on the concentration of amino groups carried by the protein (*19*). In the course of conjugation, the nonamidated ester groups of NIPAM-NASI copolymer transform into carboxylic acids so that the nonconjugated copolymer is a copolymer of NIPAM and a salt of acrylic acid (NIPAM-AA copolymer).

For making a correct comparison, equal weight ratios of NIPAM:enzyme and NIPAM-NASI copolymer:enzyme (ca. 35) were used at the same concentration of penicillin acylase in the reaction mixtures. Radical polymerization of NIPAM carried out in the absence of PA in the reaction mixture resulted in almost quantitative conversion of the monomer into polyNIPAM, so that the amount of the polymer accessible for conjugation was equal in both coupling techniques. Figure 1 illustrates the decrease in the enzyme activity resulting from its acylation with itaconic anhydride (a) and NIPAM-NASI copolymer (b), as well as distribution of the activity between the thermally precipitated conjugates and the supernatants. Obviously, acylation of penicillin acylase with NIPAM-NASI copolymer has led to a smaller decrease in the activity of the enzyme: 82% activity was retained against 60% retained by the enzyme acylated with itaconic anhydride. Further, the coupling yield of penicillin acylase obtained by its conjugation with NIPAM-NASI copolymer was also much higher: 37% of the initial enzyme activity was found in the reprecipitated conjugate against 9% found in the conjugate prepared by copolymerization technique. Attempts to increase the amounts of itaconic anhydride used at the enzyme modification step did not improve coupling yield but resulted in even stronger inactivation of PA (data not shown). Thus the conjugation of PA with NIPAM-NASI copolymer is clearly preferential among the compared techniques.

To make sure that the conjugation of PA to NIPAM-NASI copolymer proceeded as a result of covalent binding and did not involve a physical uptake of the enzyme by the precipitating polymer, a control experiment has been undertaken. A sample of polyNIPAM prepared similarly to NIPAM-NASI copolymer was added to the solution of PA in conditions identical to those of the above conjugation, and repeated thermal precipitation of the polymer has been carried out. The precipitate entrapped less than 1% of the initial PA activity (against 37% in the covalent conjugate), so that noncovalent binding of PA was proven to be negligibly low.

Gel Filtration Chromatography of Penicillin Acylase Conjugates. Condensation of proteins with multifunctional reactive copolymers may result in the conjugates containing several protein and/or copolymer macromolecules covalently attached to one another as a result of intermolecular cross-linking. The same holds true for the conjugates prepared by radical copolymerization of low molecular weight monomers such as NIPAM with the proteins carrying double bonds. Provided the molecular weight characteristics of the conjugates differ strongly from the parent enzyme and the reactive copolymer, the composition of the obtained conjugates might be assayed and their apparent molecular weights may be estimated by gel filtration chromatography.

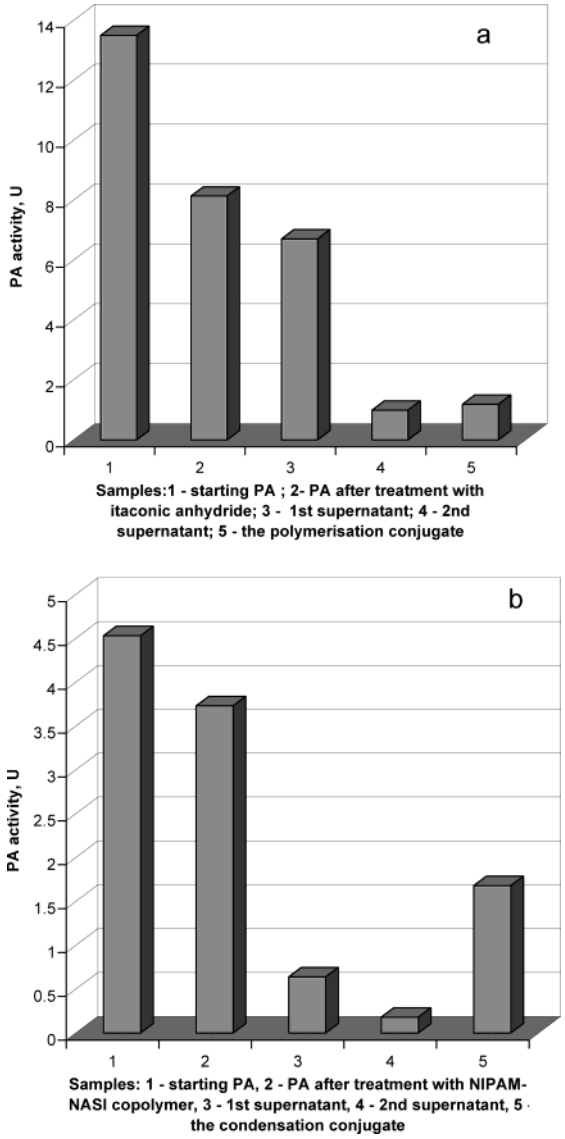

Figure 1. Enzymatic activity of penicillin acylase in the samples of the enzyme chemically modified with (a) itaconic anhydride and (b) NIPAM-NASI copolymer; distribution of the activity among the precipitated conjugates and supernatants.

Calibration of the Sepharose CL-4B chromatography column is illustrated in Figure 2. The solid line relates to gel chromatography of polyNIPAM of intermediate molecular weight (points 2 and 3, see Methods). Although NIPAM-NASI copolymer (5) and proteins of Assemblase liquid (4) do not exactly obey the given calibration, it still can be used for evaluation of apparent molecular weights of the prepared conjugates. Figure 3 illustrates the separation profiles of the copolymerization and condensation conjugates obtained by gel filtration chromatography. The main chromatographic peaks of conjugates registered by the turbidity of chromatographic fractions

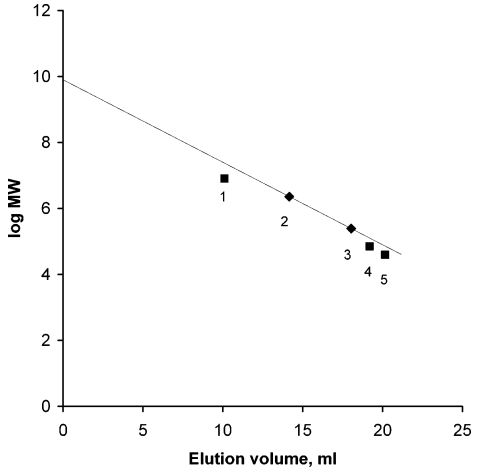

Figure 2. Calibration of the Sepharose CL-4B gel filtration column (1 cm × 30 cm): (1−3) polyNIPAM, MW = 8.1×10^6, 2.3×10^6, 2.5×10^5, respectively; (4) Assemblase liquid (penicillin acylase, MW = 7.1×10^4); (5) NIPAM-NASI copolymer, MW = 3.9×10^4. The solid line is given by the equation log MW = $9.9 - 0.25V_{el}$ developed from the chromatographic profiles of polyNIPAM samples 2 and 3 (see Methods for calibration details).

(λ = 470 nm, 50 °C) exhibited elution volumes (V_{el}) of ca. 19 and 20 mL, respectively, which indicates their apparent molecular weight of $\leq 10^5$). Obtained under the conditions of large mass and molar excess of either NIPAM-NASI copolymer or NIPAM comonomer (107 mg copolymer per 3 mg PA), the samples of conjugated PA contain a lot of nonconjugated NIPAM-AA copolymer or polyNIPAM exhibiting the above elution volumes.

The most remarkable feature of the obtained separations are elution volumes corresponding to the maximal PA activity in the fractions: V_{el} = 16−17 mL for the copolymerization conjugate and V_{el} = 14 mL for the condensation conjugate, both being markedly smaller than V_{el} = 19.2 mL for free PA. Obviously, the activity elution profiles testify to the higher hydrodynamic volumes and higher apparent molecular weights of the conjugates compared to the starting copolymer and the enzyme. It is worth noting that almost no free PA was found in the conjugates. The separation of conjugates from the nonconjugated enzyme by means of repeated thermal precipitation was, therefore, quite effective.

The amount of protein in the conjugates of PA can be evaluated on the ground of their UV spectra (see Table 1). Provided the absorbencies of the conjugate solution at 220 and 280 nm are known, one can evaluate the contents of both the polymer and the protein in the conjugate. Although none of the conjugates could be completely separated from the excess of nonconjugated polymer (see Figure 3), the separation of condensation conjugate proceeded to a higher extent and allowed its isolation and characterization (fractions 8−18, Figure 3b, were combined and assayed by UV spectrometry). The content of PA in the thus isolated condensation conjugate was evaluated as 7 % (w/w). One may conceive the following structures contributing to the conjugate:

(1) single PA globule chemically modified with many chains of NIPAM-NASI-copolymer (V_{el} = 14 mL, see Figure 3b, apparent molecular weight of ca. 10^6) and

(2) multiple PA globules chemically cross-linked and modified with many chains of NIPAM-NASI-copolymer (V_{el} = 8−10 mL, see Figure 3b, apparent molecular weight of $>10^7$)

Apparently, the first type of structure is mainly presented in the sample.

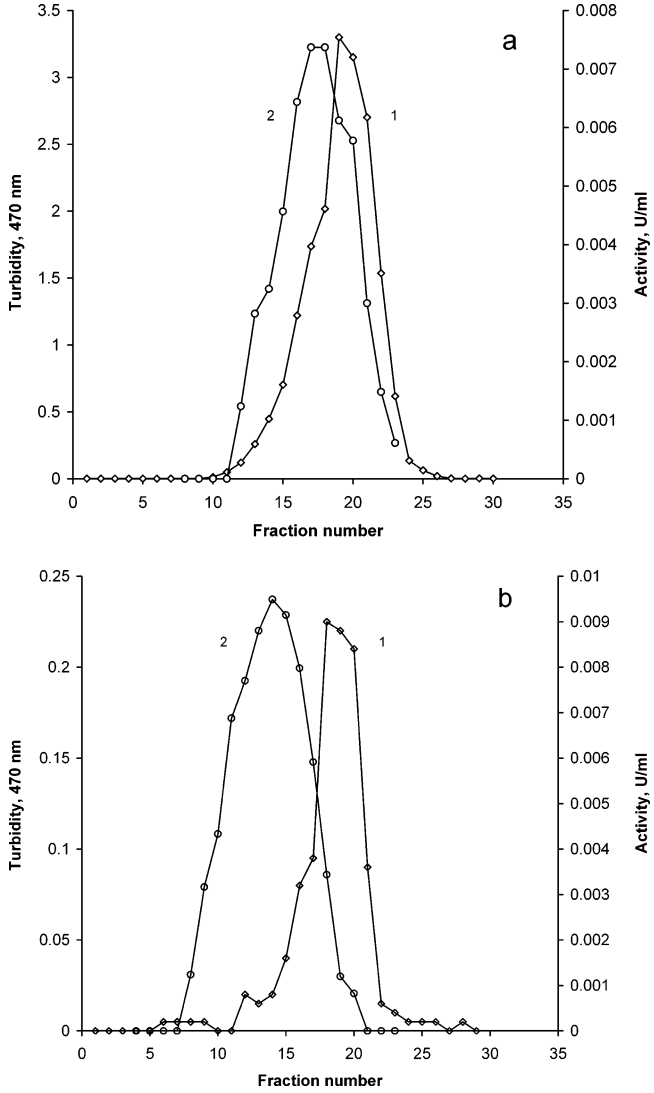

Figure 3. Gel filtration of penicillin acylase conjugates with polyNIPAM prepared by (a) polymerization and (b) condensation techniques: (1) turbidity (measured in the fractions at 50 °C) and (2) penicillin acylase activity profiles.

Table 1. Extinction Coefficients of PolyNIPAM and Penicillin Acylase in Aqueous Solution (mL/mg·cm)

sample	ϵ_{220}	ϵ_{280}
polyNIPAM	1.13	7×10^{-3}
penicillin acylase	10.5	1.7

Conjugation of NIPAM-NASI-Copolymer with Penicillin Acylase at Increased Enzyme Loading (Condensation Conjugate II). Conjugation of NIPAM-NASI copolymers with proteins is most often performed under conditions of a large (8- to 100-fold) weight excess of the copolymer (*14*, *19*, *22*). From a practical viewpoint, preparation of thermosensitive PA conjugates with maximal protein content and activity is of high importance. Therefore we attempted to prepare the condensation conjugate of PA at 3.5-fold weight excess of the copolymer, i.e., to increase the amount of the enzyme taken for conjugation by 10 times.

Figure 4 illustrates the distribution of PA activity between the thus prepared thermally precipitated condensation conjugate II and the relevant supernatants. The coupling yield of penicillin acylase was still rather high: 24% of the initial enzymatic activity contained in the reprecipitated conjugate; its specific activity was 4-fold higher as compared to that of the previous prepa-

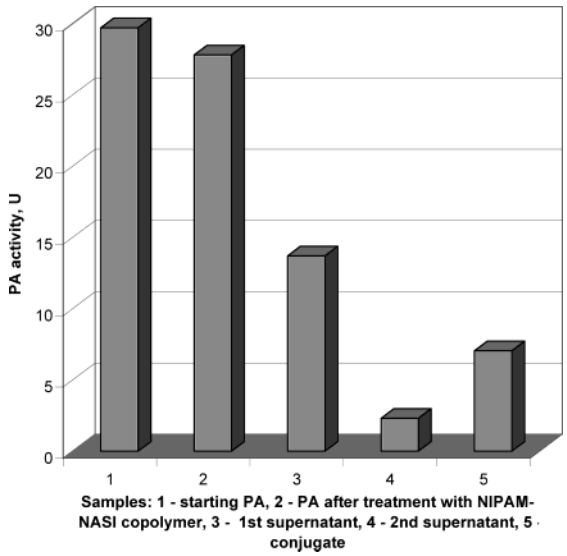

Figure 4. Enzymatic activity of penicillin acylase in the sample of condensation conjugate II and distribution of the activity among the precipitated conjugate and supernatants.

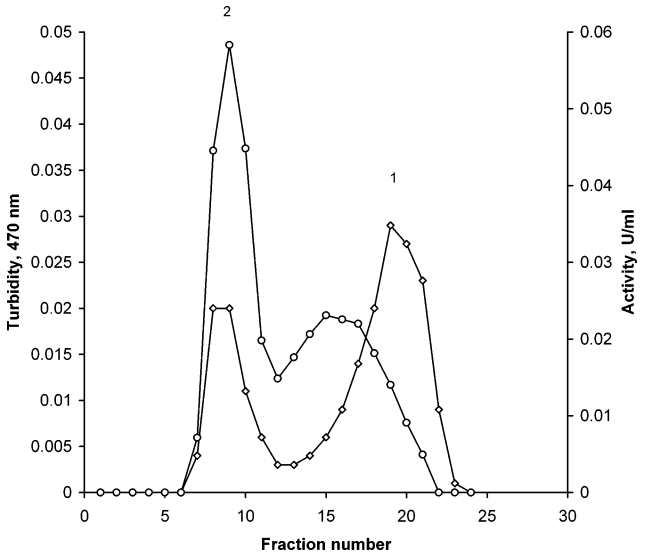

Figure 5. Gel filtration of condensation conjugate II: (1) turbidity (measured in the 5-fold diluted fractions at 50 °C) and (2) penicillin acylase activity profiles.

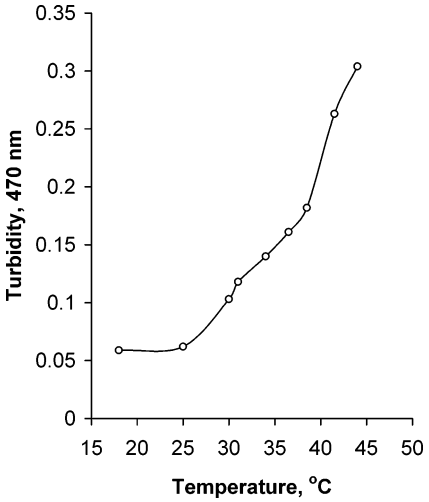

Figure 6. Turbidity of condensation conjugate II purified by gel filtration, as a function of temperature.

ration (see Figures 1b and 4). In the case of conjugate II, 94% activity was retained after treatment of the enzyme by the reactive copolymer, and the activity summed up in the fractions of bound and nonbound enzyme was 74% from the starting PA (see Figure 4). The coupling technique, therefore, does not strongly deteriorate the enzyme activity. Important technical feature of the new conjugate was its weaker response to a temperature shift: although its solution became turbid at 40 °C in the presence of 0.5 M NaCl, a collectable precipitate did not form, i.e., it could not be settled down by centrifugation at 8000 × *g* rcf in a reasonable time (up to 30 min). Preparative sedimentation of the conjugate became possible in 2 M NaCl at 40 °C.

Figure 5 illustrates the gel filtration chromatogram of the prepared conjugate. Free enzyme (V_{el} = 19.2 mL) almost does not contribute to the activity separation profile: it was effectively separated during the thermal precipitation of the conjugate. The separation profile is clearly bimodal with two peaks of the enzymatic activity at V_{el} = 9 mL and V_{el} = 15 mL. The first peak most probably corresponds to the high molecular weight (>10^7) cross-linked conjugates eluted nearby the void volume of the column (V_{el} = 8 mL). The second peak is similar to that previously observed in the separation profile of the condensation conjugate I (V_{el} = 14 mL, see Figure 3b). Apparently, the 10 times increased loading of PA resulted in higher degree of intermolecular cross-linking between the enzyme and NIPAM-NASI copolymer, and yielded a larger share of high molecular weight products. Judging by the turbidity profile (Figure 5), some portion of NIPAM-NASI copolymer still remained nonconjugated and transformed into NIPAM-AA copolymer (V_{el} = 19−21 mL) even under conditions of the increased PA loading. On the other hand, a relatively high turbidity was displayed by the high molecular weight fractions of the conjugate (0.15 M NaCl, 50 °C), indicating the increased concentration of the enzyme-bound copolymer. The conjugate fractions (8−16, see Figure 5) were combined and assayed for their UV absorbance at 220 and 280 nm. The content of PA in the thus isolated conjugate was evaluated as 13 % (w/w), i.e., the increased amount of protein was found in the conjugate prepared under conditions of increased enzyme loading. Thermal sensitivity of the conjugate is illustrated in Figure 6; formation of large, visible particles was observed at 44 °C, which were easily separated from the supernatant by low-speed centrifugation.

Further increase of enzyme loading was also tested in the present study: 1.25-fold weight excess of the copolymer was used. The thus obtained conjugate could hardly be precipitated at 40 °C even in the presence of 2 M NaCl; its solution was rather viscous, most probably because of abundant intermolecular cross-linking and formation of high molecular weight products. Although the NIPAM-NASI-copolymer can be highly loaded by PA, thermal precipitation of the conjugates becomes difficult, most probably because of the high amount of hydrophilic protein contained therein.

As indicated earlier, effective separation of the conjugate from NIPAM-AA copolymer has been achieved by gel filtration. However, this method does not seem to be technically feasible because of its low speed and, therefore, a low productivity. For preparative separation of the conjugates a membrane filtration seems to be more promising.

Separation of Condensation Conjugate II by Membrane Filtration. To evaluate the technical feasibility of membrane filtration as a method for purifica-

Table 2. Characteristics of Condensation Conjugate II Exposed to Membrane Filtration

sample	volume (mL)	A_{280nm}	PA activity (mU/mL)
condensation conjugate II before filtration	5 + 4 mL distilled water[a]	0.167	25
after filtration	0.85	0.032[b]	135
filtrate	8.1	0.031	3.4

[a] See Methods. [b] Determined after 20-fold dilution.

tion of PA conjugates, the condensation conjugate II was exposed to filtration through cellulose membrane with 100 kDa cutoff (see Methods for experimental details). Parameters of the starting conjugate solution, the purified conjugate and the filtrate are given in Table 2. Most of the enzyme (>90% activity) was retained in the membrane filtration (MF) unit whereas the filtrate contained a small amount of protein. The condensation conjugate II was analyzed by gel filtration chromatography before and after membrane filtration as illustrated in Figure 7. A small decrease in the PA enzymatic activity and protein content observed in the filtered sample was very likely due to a partial breakthrough of its lower molecular weight fraction ($<10^6$, V_{el} = 14−20 mL, see Figures 5 and 7). On the other hand, the nonconjugated NIPAM-AA copolymer turned out to be efficiently removed from the sample of the conjugate: very low turbidity was detected in the chromatographic fractions 18−21 mL. This elution volume is characteristic of the copolymer. One may conclude that as a result of prevailing formation of high molecular weight conjugates the suggested MF technique quite effectively separates them from the excess of nonconjugated copolymer.

Catalytic Properties of the Conjugated Penicillin Acylase. ***Hydrolysis.*** Kinetic constants (k_{cat} and K_m) of the reactions catalyzed by immobilized enzymes are usually different from those obtained with the free enzymes. The constants are sensitive to even slight changes in accessibility of the active sites, conformational changes in the protein globules, and diffusional limitations for substrates (*5*, *10*). It seemed, therefore, promising to characterize the prepared PA-poly(NIPAM) conjugates by comparison of kinetic parameters of NIPAB hydrolysis to those found with free PA. The obtained Michaelis constants (K_m) and the maximal rates of enzymatic hydrolysis (V_{max}) are summarized in Table 3. As follows from the table, a slight increase in K_m resulted from the conjugation of PA to NIPAM-NASI copolymer. It can be attributed to a lower local concentration of the substrate near to the enzyme, i.e., within relatively large cross-linked PA-polyNIPAM conjugate. Similar effect was earlier registered for poly(NIPAM)-conjugated α-chymotrypsin (*14*). The value of K_m obtained in the present study for free PA agrees well with that reported earlier, 11.3 μM (*26*).

As the exact concentration of PA in the Assemblase liquid is unknown, the calculation of k_{cat} from the obtained values of V_{max} is not possible. One can correlate, however, the concentrations of protein in the 50-fold diluted Assemblase liquid and in the 10-fold diluted condensation conjugate II stock solution used in the above experiments. Surprisingly, it turned out that the lower concentration of protein in the condensation conjugate II displayed the higher values of V_{max} compared to that of the sample of free enzyme (see Table 3). This may be explained either by preferential coupling of PA to NIPAM-NASI copolymers during conjugation, compared to the other proteins of Assemblase liquid, or by higher turnover number (k_{cat}) of the conjugated enzyme.

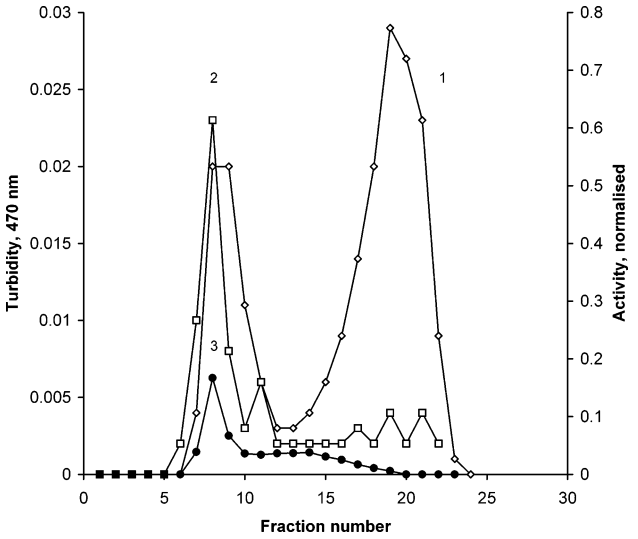

Figure 7. Gel filtration of condensation conjugate II purified by membrane filtration. Turbidity measured in the 5-fold diluted fractions at 50 °C (1, ◇) before and (2, □) after membrane filtration; (3, ●) penicillin acylase activity in the fractions of the filtered conjugate.

Table 3. Kinetic Parameters of NIPAB Hydrolysis Catalyzed by Penicillin Acylase

sample	K_m (μM)	V_{max} (μM/s)	protein concn in PA-containing samples (mg/mL)
Assemblase liquid, 50-fold diluted (free PA)	12	0.235	0.24
condensation conjugate II, 10-fold diluted	20	0.47	0.16

A similar effect was reported earlier for trypsin conjugated with the end-group activated oligoNIPAM: k_{cat} versus *N*,α-benzoyl-D,L-arginine-*p*-nitroanilide increased with increasing the molar ratio oligoNIPAM:trypsin (*15*). So far, one may conclude, at least, that catalytic efficiency of PA active site was not much affected during the enzyme conjugation to NIPAM-NASI copolymers.

Cephalexin Synthesis. Enzymatic production of cephalexin involves coupling of D-phenylglycin amide (D-PGA) or ester derivative of D-phenylglycine (D-PG) to 7-aminodeacetoxycephalosporanic acid (7-ADCA) using immobilized penicillin acylase. This coupling is thermodynamically unfavorable; the reaction equilibrium is shifted toward hydrolysis of the product and formation of D-PG and 7-ADCA. This is a reason the enzymatic cephalexin production curve is bell-shaped in time (a thermodynamic equilibrium cannot be changed by enzymes) and the reaction, which is kinetically controlled, must be stopped prior to net hydrolysis of cephalexin. Selectivity of cephalexin synthesis can be characterized by synthesis/hydrolysis ratio (S/H), i.e., the amount of produced antibiotic divided by the amount of D-PG formed by hydrolysis of both D-PGA and the target cephalexin. The value of S/H is of great importance; a low S/H implies low production capacity and a low utilization of substrates.

Immobilization of enzymes often results in mass-transfer limitations, which are especially critical in enzymatic coupling of activated side chains with β-lactam nuclei (*27*) and have a negative effect on S/H. The loss in S/H increases with increasing enzyme loading, although in terms of economics, stability of products, and downstream processing, high enzyme loading is preferred. This poses serious problems in developing better-

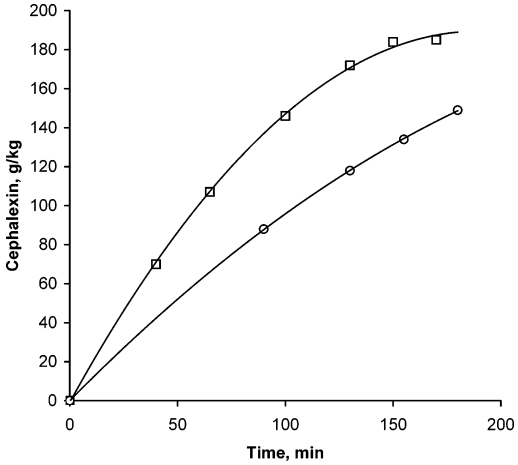

Figure 8. Cephalexin synthesis catalyzed by (□) Assemblase liquid (0.28 mg PA/mL) and (○) polyNIPAM condensation conjugate II (4.6 mg conjugate/mL).

immobilized PA for cephalexin production. We believed that PA immobilized to *smart polymers* may not suffer from a decrease in S/H because of the putative absence of diffusion limitation.

It was already shown that penicillin acylase retained its α-NIPAB and NIPAB hydrolyzing activity after immobilization. To test whether poly-NIPAM conjugated penicillin acylase was still capable of coupling 7-ADCA to PGA, we performed enzymatic synthesis of cephalexin using PA-polyNIPAM condensation conjugate II, as well as Assemblase liquid. The final reaction mixture (approximately 70 mL) contained 0.28 mg/mL free penicillin acylase or 4.6 mg/mL penicillin-polyNIPAM condensation conjugate II. Figure 8 shows formation of cephalexin as a function of time. From this graph it is obvious that after cross-linking to polyNIPAM penicillin acylase retains its ability to synthesize cephalexin from phenylglycin amide and 7-ADCA.

To find out if the conjugation of penicillin acylase to poly-NIPAM had any effect on the selectivity of reaction, we calculated the S/H of cephalexin synthesis catalyzed with Assemblase liquid and PA-polyNIPAM condensation conjugate II before maximum cephalexin formation was attained. Thus, the apparent S/H were 7.46 and 7.49 for for Assemblase liquid and penicillin-polyNIPAM condensation conjugate II, respectively. These nearly identical ratios demonstrate that conjugation of *E. coli* penicillin acylase with the copolymer of *N*-isopropylacrylamide (NIPAM-NASI) results in a conjugate, which not only retains its capacity to couple 7-ADCA to D-PGA but also behaves as a free enzyme with respect to the synthesis/hydrolysis ratio. These results demonstrate that synthesis of cephalexin using reusable penicillin acylase is feasible without loss of selectivity, which is usually associated with immobilized penicillin acylases. This opens the way to a reusable, thermosensitive industrial biocatalyst for the production of β-lactam antibiotics.

Such a biocatalyst may be feasible not only in reaction mixtures where substrate and product are soluble, such as, for example, the synthesis of cephalexin, but also for "solid-to-solid" biosynthesis or reactions carried out in suspension ("slurry") (*28*). In true solid-to-solid synthesis the biocatalyst is soluble, whereas reactants and products are in the solid state. Although a soluble PA biocatalyst would in principle be applicable in such reactions, an inevitable consequence of solid-to-solid application is limitation of mass transport. The other type of reaction, "slurry reaction", provides a more practical option for soluble PA biocatalysts. In slurry reactions, actual catalysis is preceded by dissolution of the substrate and/or followed by precipitation of the product, whereby high product yield can be achieved in the case of product precipitation because of the concomitant shift of the reaction equilibrium toward product formation. With a conventional biocatalyst (enzyme bound to a porous solid carrier) precipitation of the product onto the carrier can result in severe activity loss. Clearly, a soluble PA biocatalyst will not suffer inactivation through product precipitation.

Conclusions

We have prepared and isolated several thermosensitive biocatalysts based on PA conjugated with polyNIPAM. It was found that condensation of the enzyme with reactive NIPAM-NASI copolymer is advantageous over copolymerization of NIPAM with chemically modified PA, in regard to the coupling yield and retained enzymatic activity. The condensation conjugates are PA molecules chemically modified and/or cross-linked by polyNIPAM chains. Owing to thermosensitivity of the polymer, the conjugates can be precipitated and collected on heating to 44 °C in the presence of 2 M NaCl. Kinetic constants (K_m and V_{max}) of NIPAB enzymatic hydrolysis indicate that catalytic efficiency of PA active site was not much affected by the conjugation, and moreover, the conjugated enzyme was also able to catalyze formation of cephalexin from D-PGA and 7-ADCA. Rates of the cephalexin synthesis and hydrolysis relate similarly to those observed with free PA and suggest no diffusional limitations for the substrates.

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References and Notes

(1) Hoffman, A. S. Bioconjugates of intelligent polymers and recognition proteins for use in diagnostics and affinity separations. *Clin. Chem.* **2000**, *46*, 1478−1486.

(2) Heskins, M.; Guillet, J. E. Solution properties of poly(*N*-isopropylacrylamide). *J. Macromol. Sci. Chem.* **1968**, *A2*, 1441−1455

(3) Galaev, I. Yu.; Mattiasson, B. Affinity precipitation of proteins using smart polymers. In *Smart Polymers for Bioseparation and Bioprocessing*; Galaev, I., Mattiasson, B., Eds.; Taylor and Francis: London and New York, 2002; pp 55−77.

(4) Messing, R. A. Immobilized RNAse by adsorption on porous glass. *Enzymologia* **1970**, *38*, 370−372.

(5) Sears, P. S.; Clark, D. S. Comparison of soluble and immobilised trypsin kinetics: implications for peptide synthesis. *Biotechnol. Bioeng.* **1993**, *42*, 118−124.

(6) Kennedy, J. F.; White, C. A. Principles of immobilization of enzymes. In *Handbook of Enzyme Biotechnology* ; Wiseman, A., Ed.; Ellis Horwood: Chichester, 1985; pp 147−207.

(7) Mohy Eldin, M. S.; Schroen, C. G. P. H.; Janssen, A. E. M.; Mita, D. G.; Tramper, J. Immobilisation of penicillin acylase onto chemically grafted nylon particles. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 445−451.

(8) Cao, L.; van Rantwijk, F.; Sheldon, R. A. Cross-linked enzyme aggregates: a simple and effective method for the immobilisation of penicillin acylase. *Org. Lett.* **2000**, *2(10)*, 1361−1364.

(9) Cao, L.; van Langen, L. M.; van Rantwijk, F.; Sheldon, R. A. Cross-linked aggregates of penicilin acylase: robust ca-

talysis for the synthesis of β-lactam antibiotics. *J. Mol. Catal. B: Enzym*. **2001**, *11*, 665−670.
(10) Kondo, A.; Oku, S.; Murakami, F.; Higashitani, K. Conformational changes in protein molecules upon adsorption on ultrafine particles. *Colloids Surf., B* **1993**, *1*(3), 197−201.
(11) Fusek, M.; Turkova, J.; Stovickova, J.; Franek, F. Polyclonal anti-chymotrypsin antibodies suitable for oriented immobilisation of chymotrypsin. *Biotechnol. Lett.* **1988**, *10*, (2), 85−90.
(12) Barros, R. J.; Wehtje , E.; Adlercreutz, P. Mass transfer studies on immobilized α-chymotrypsin biocatalysts prepared by deposition for use in organic medium. *Biotechnol. Bioeng.* **1998**, *59*(3), 364−373.
(13) Caliceti, P.; Schiavon, O.; Veronese, F. M. Immunological properties of uricase conjugated to neutral soluble polymers. *Bioconjugate Chem*. **2001**, *12*(4), 515−522.
(14) Chen, J.-P.; Hsu, M.-S. Preparation and properties of temperature-sensitive poly(*N*-isopropylacrylamide)-chymotrypsin conjugates. *J. Mol. Catal. B: Enzym*. **1997**, *2*, 233−241.
(15) Ding, Z.; Chen, G.; Hoffman, A. S. Unusual properties of thermally sensitive oligomer-enzyme conjugates of poly(*N*-isopropylacrylamide)-trypsin. *J. Biomed. Mater. Res*. **1998**, *39*, 498−505.
(16) Shoemaker, S. G.; Hoffman, A. S.; Priest, J. H. Synthesis and properties of vinyl monomer/enzyme conjugates: conjugation of L-asparaginase with *N*-succinimidyl acrylate. *Appl. Biochem. Biotechnol.* **1987**, *15*, 11−24.
(17) Auditore-Hargreaves, K.; Houghton, R. L.; Monji, N.; Priest, J. H.; Hoffman, A. S.; Nowinski, R. C. Phase-separation immunoassays. *Clin. Chem*. **1987**, *33*(9), 1509−1516.
(18) Matsukada, M.; Aoki, T.; Sanni, K.; Ogata, N.; Kikuchi, A.; Sakurai, Y.; Okano, T. Effect of molecular architecture of poly(*N*-isopropylacrylamide)-trypsin conjugates on their solution and enzymatic properties. *Bioconjugate Chem*. **1996**, *7*, 96−101.
(19) Uludag, H.; Norrie, B.; Kousinioris, N.; Gao, T. Engineering temperature-sensitive poly(*N*-isopropylacrylamide) polymers as carriers of therapeutic properties. *Biotechnnol. Bioeng.* **2001**, *73*, 510−521.
(20) Hao, Y.; Andersson, M.; Virto, C.; Galaev, I. Yu.; Mattiasson, B.; Hatti-Kaul, R. Stability properties of thermosensitive poly(*N*-isopropylacrylamide)-trypsin conjugates. *Biocatal. Biotransform*. **2001**, *19*, 341−359.
(21) Fischer, L.; Peissker, F. A covalent two-step immobilisation technique using itaconic anhydride. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 129−135.
(22) Caliceti, P.; Schiavon, O.; Hirano, T.; Ohashi, S.; Veronese, F. M. Modification of physicochemical and biopharmaceutical properties of superoxide dismutase by conjugation to the copolymer of divinyl ether and maleic anhydride. *J. Controlled Release* **1996**, *39*, 27−34.
(23) Monji, N.; Cole, C.-A.; Hoffman, A. S. Activated, N-substituted acrylamide polymers for antibody coupling: application to a novel membrane-based immunoassay. *J. Biomater. Sci., Polymer Ed.*, **1994**, *5*(5), 407−420.
(24) Fujishige, S. Intrinsic viscosity−molecular weight relationships for poly(*N*-isopropylacrylamide) solutions. *Polymer J*. **1987**, *19*, 297−300.
(25) Schroën, C. G. P. H.; Nierstrasz, V. A.; Moody, H. M.; Hoogschagen, M. J.; Kroon, P. J.; Bosma, R.; Beeftink, H. H.; Janssen, A. E. M.; Tramper, J. Modeling of the enzymatic kinetic synthesis of cephalexin−influence of substrate concentration and temperature. *Biotechnnol. Bioeng.* **2001**, *73*, 171−178.
(26) Alkema, W. B. L.; Floris, R.; Janssen, D. B. The use of chromogenic reference substrates for the kinetic analysis of penicillin acylases. *Anal. Biochem*. **1999**, *275*, 47−52.
(27) Bruggink, A.; Roos, E. C.; de Vroom, E. Penicillin acylase in the industrial production of β-lactam antibiotics, *Org. Proc. Res. Dev*. **1998**, *2*(2), 128−133.
(28) Straathof, A. J. J.; Litjens, M. J. J.; Heijnen, J. J. Enzymatic transformations in suspensions. In *Enzymes in Nonaqueous Solvents: Methods and Protocols*; Vulfson, E. N., Halling, P. J., Holland, H. L., Eds.; Humana Press Inc.: Totowa, NJ, 2001; pp 603−610.

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