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Synthesis of Thermal Phase Separating Reactive Polymers and Their Applications in Immobilized Enzymes*

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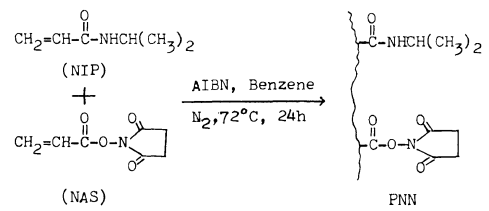
ABSTRACT: This paper describes the synthesis of active ester copolymers from *N*-isopropylacrylamide (NIP) with *N*-acryloxysuccinimide (NAS), aminolysis of active esters, and determination of their average molecular weights. As a model, thermolysin was attached to the polymer to form soluble–insoluble immobilized enzymes capable of being centrifuged above their T_c, because poly(*N*-isopropylacylamide) (poly(NIP)) has a lower critical solution temperature (T_c) of about 31—33°C. The effects of immobilization of enzymes have been studied including the amount of active ester, time, pH, temperature, and ratio of polymer to enzyme. The tests of thermal stability and repeated precipitation and separation showed that the use of T_c as the technique of separation is possible.

KEY WORDS Polymerization / Phase Separating Reactive Polymers / Poly(*N*-isopropylacrylamide) / Immobilization of Enzyme / Thermolysin /

The immobilization of enzymes using insoluble carriers has been widely studied.[1,2] However, because of the solid substrate and/or product in carriers, effective enzyme reaction does not occur in its insoluble form. In order to overcome the problems of insoluble enzyme reaction systems, the preparation of soluble–insoluble enzymes has been attempted. This type of immobilized enzyme shows a soluble state during catalytic reaction but an insoluble form after the reaction. Several papers have reported such immobilized enzymes by changing the pH or salt concentration of reaction medium after the reaction to bring about this reversible procedure.[3-8]

It is well-known that aqueous solutions of poly(*N*-isopropylacrylamide) (poly(NIP)) exhibit a lower critical solution temperature (T_c), at which demixing occurs, of about 31—33°C throughout a wide concentration range (up to *ca.* 5%).[9,10] This thermally reversible characteristic has been successfully exploited in an immunoassay and protein separation, in which many of the advantages of both homogeneous and heterogeneous methods are obtained.[11,12] So we are encouraged to extend this technique to immobilize enzymes.

This paper describes the synthesis of thermally-reversible reactive water–soluble copolymers obtained by copolymerizing *N*-isopropylacrylamide (NIP) with *N*-acryloxy-



Synthesis of PNN

Scheme 1.

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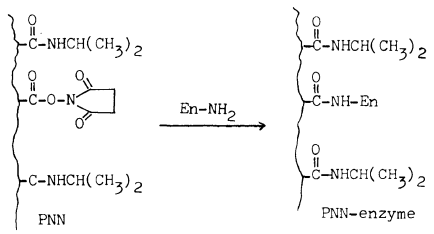

Immobilization of enzyme

Scheme 2.

succinimide (NAS) (Scheme 1), assays of active ester and determination of number average molecular weights. As a model thermolysin was attached to the polymers to form water-soluble immobilized enzymes (Scheme 2). The immobilization conditions were studied and the stability of the immobilized thermolysin was also investigated.

EXPERIMENTAL

Materials

Thermolysin and casein were purchased from Sigma (St. Louis, Mo.). *N*-Acryloxysuccinimide (NAS)[9] and acryloylchloride[13] were synthesized according to reported methods. 2,2′-Azobisisobutyronitrile (AIBN) (mp 103°C), trichloro acetic acid (TCA), mercaptoethanol (bp 157°C), isopropylamine (bp 34°C), ethylene diamine tetraacetic acid (EDTA) (mp 250°C (dec.)), and tris(hydroxymethyl)aminoethane (Tris) were all purchased and used without further purification.

Instruments

IR spectra were recorded on a Nicolet 170SX spectrophotometer. UV spectra were run with a Shimadzu UV-240 spectrophotometer. Viscosity was determined by a Ubbelodhe viscosimeter.

Methods

Synthesis of N-Isopropylacrylamide (NIP). NIP was synthesized with acryloylchloride (27.3 g, 0.3 mol) and isopropylamide (35.4 g, 0.6 mol) in ethylacetate at 0°C with stirring for 4 h. After the reaction the precipitate was filtered, the filtrate was concentrated and then was applied to a Vacuum liquid Chromatography (VLC).[14] All collected fractions were put together and the solvent was removed. The residue was dried and then recrystallized twice with a mixed solvent of benzene and hexane. The colorless needle crystals were separated by filtration. Yield: 24.4 g (72%) mp 62—63°C (lit.[9] mp 62—63°C); IR (KBr) cm^{-1}: 3300, 3090, 2990, 2890, 1660, 1620, 1550, 1250, and 920. NMR: 7.70—8.30 (s, 1H), 6.20—6.40 (d, 2H), 5.30—5.70 (t, 1H), 3.70 −4.40 (s, 1H). *Elemental analysis*, Calcd: C, 63.68%; H, 9.80%; N, 12.38%. Found: C, 63.66%; H, 9.85%; N, 12.30%.

Synthesis of Poly(N-isopropylacrylamide-co-N-acryloxysuccinimide) (PNN). The copolymerization of *N*-acryloxysuccinimide (9.9 g, 87.5 mmol), *N*-acryloxysuccinimide (0.93 g, 5.5 mmol), was carried out in the presence of AIBN (30 mg) in 200 ml of benzene at 72°C for 24 h. Ethyl ether (400 ml) was added at the end of reaction to precipitate the polymr. After removal of the supernatant, the polymer was dissolved in 200 ml of acetone, and then the acetone solution was pured into vigorously stirred ethyl ether (800 ml). The precipitated fluffy polymer was washed on the funnel four times with 100 ml aliquots of ethyl ether. This procedure was repeated twice. Then the polymer was dried in a vaccum desiccator in 1.0 Torr for 48 h at ambient temperature to constant weight: 10.2 g (94%). IR (KBr) cm^{-1}; 3080, 2990, 1750, 1660, 1375, 1215, and 1075.

Determination of the Molecular Weight of PNN

The number average molecular weights of aminolyzed PNN samples were calculated from their intrinsic viscosities,[10] determined as described by Flory.[15] Aminolysis of PNN was accomplished by treatment of the polymer (50 mg) with 2 ml of isopropylamine for 24 h

at room temperature. Then the solvent was distilled and the residue was dissolved in acetone and precipitated by ethyl ether. This procedure was repeated three times.

Assay for the Active Ester Content of PNN

PNN (50 mg, about 53 μmol of active ester groups, dried under vacuum at 1 Torr and 25°C for 48 h) was dissolved and made up to volume in distilled water in a 5 ml volumetric flask. A 50 μl of this solution was added into a 5 ml quartz cuvette containing 3000 μl of Tris–HCl buffer (0.1 M, pH 7.5), 50 μl of 1 M isopropylamine aqueous solution and 10 μl of 1 M solution of mercaptoethanol. The rate of the appearance of *N*-hydroxysuccinimide was followed spectrophotometrically at 259 nm at 25°C.[16] After the reaction was completed and increase of absorbance level off, the active ester concentration was calculated.

Immobilization of Thermolysin

PNN (500 mg) was placed in a beaker containing a small stirring bar, and 10 ml of 0.01 M $Ca(OAc)_2$ buffer pH 7.3 and 10 ml of 0.2 mg ml^{-1} thermolysin in 0.01 M $Ca(OAc)_2$ solution were added. The polymer was dissolved within 0.5 h at about 10°C by stirring. The polymer solution was stirred magnetically for 24 h at 17°C to ensure complete immobilization of the enzyme. After the reaction, the solution was removed to a centrifuge tube and centrifuged for 30 min at 10000 rpm in a 35°C air bath. The supernatant was removed. The precipitate was then dissolved in 20 ml Tris–HCl buffer pH 7.5 and stored at 4°C.

Assay for Enzyme Activity

1) Assay for Activity of Free Enzyme.[17] 0.1 ml enzyme solution (1 mg/10 ml) and 1.9 ml of 0.01 M Tris–HCl buffer pH 8.0 were pipetted into a test tube. 1 ml casein (2% pH 8.0) was also pipetted into the tube. The solutions were mixed well and the reaction was carried out for 10 min at 35°C. At the end of the reaction, 2 ml of TCA were added. After being shaken, the tube was kept in a 35°C water bath for 30 min. The precipitate was filtered off through filter paper, and absorbance of the filtrate was measured at 280 nm. The activity unit was corrected by a standard curve and represented by $[PU]^{ca.\ 35°C,280\,nm}$.[18] The specific activity of the thermolysin is calculated to be 157.

2) Assay for Activity of Immobilized Enzyme. 0.2 ml of immobilized enzyme solution and 1.8 ml of 0.01 M Tris–HCl buffer pH 8.0 were pipetted into a test tube, and 1 ml casein (2%, pH 8.0) was pipetted as well. The other steps were repeated as described above.

Measurement for the Activity of Immobilized Thermolysin after Repeated Use

0.2 ml immobilized thermolysin solution was added to 2.8 ml Tri–HCl buffer solution (pH 8.0). After being stirred at 35°C for about 10 min, the mixture was centrifuged at 35°C for 30 min. The supernatant was removed, and the precipitate dissolved again with 2.0 ml Tris–HCl buffer. When the immobilized thermolysin was completely dissolved, its activity was assayed according to the method above, and the cycles of this process was counted as the number of repeated use. In this way we measured the activity of immobilized thermolysin for repeated use from one to five times.

RESULTS AND DISCUSSION

Preparation of Copolymers and Their Characteristics

PNN was prepared by the free-radical polymerization of *N*-isopropylacrylamide (NIP) and *N*-acryloxysuccinimide (NAS) in benzene solution, using thermal initiation with AIBN (Scheme 1). This system was used in order to provide suitable molecular weight polymers, which dissolved quickly and generated an aqueous solution of suitable viscosities and could also be precipitated effectively above T_c. A buffer solution containing the

Table I. Preparation of NIP/NAS copolymers and their characteristics[a,b]

Polymer	Reaction conditions			Yield	$\bar{M}_n \times 10$	Content of active ester ($\times 10^2$)
	$R \times 10^2$	T/°C	t/h	%		
PNN-1	1.54	72	24	95	1.85	1.43
PNN-2	3.42	72	24	96	1.75	3.15
PNN-3	6.82	72	24	94	1.78	5.78
PNN-4	12.3	72	24	94	1.72	11.1
PNN-5	22.4	72	24	93	1.60	19.7

[a] Ratio of moles of active ester to moles of NIP.
[b] Ratio of moles of measured active ester to moles of NIP.

polymer up to 5% by weight formed steadily. Conversion of monomers to copolymers was consistently high. They appeared to be indefinitely stable at room temperature in a desiccator under dry air; the active ester groups, however, hydrolysed on exposure of PNN to undried air, but this hydrolysis was slow. The polymer was assayed for active ester by allowing it to react with an isopropylamine solution and measuring the absorbance due to the anion of *N*-hydroxysuccinimide (E_{259} 8600 $M^{-1} cm^{-1}$). Usually, the content of active ester groups in a sample of polymer was 85—95% as that expected on the basis of the composition of monomers. Several polymers differing in active ester content were used in this experiment.

The number average molecular weights of the polymers were estimated by converting them to poly(*N*-isopropylacrylamide by reacting with excess of isopropylamine. The viscosities of their aqueous solutions were measured by a Ubbelodhe Viscosimeter. The difference between *N*-hydroxysuccinimide and isopropylamine was also considered in the calculation of M_n by viscosity. All experimental data are listed in Table I.

Precipitation and Separation

It has been known that poly(*N*-isopropylacrylamide) and PNN-protein have a T_c of about 31—33°C throughout a wide concentration range. We utilized this demixing behavior of poly(NIP) at or above the T_c as the separation technique in our experiment above 33°C, phase separation occurred; however, precipitates were difficult to filter and collect; because their viscous flocculate hindered the performance of ultrafiltration. But we found that polymers could be centrifuged almost completely from solution for 30 min at 10000 rpm in a 35°C air bath.

Immobilization of Enzyme

Thermolysin was coupled to the active ester copolymers *via* formation of amide bond by nucleophilic substitution of succinimidyl groups in the polymers (Scheme 2). After coupling, we did not separate the enzyme-immobilized polymer from the unreacted polymer, because when compared to that in unseparated system, the activity of the immobilized enzyme dropped after being separated from the unreacted polymer by the method of Hoffman *et al.*[19] So the unreacted polymer has no influence on the activity of immobilized enzyme.

Table II shows that PNN-3 is a suitable carrier to immobilize an enzyme, because the relative activity of the immobilized enzyme with PNN-3 as carrier is highest, and the remaining activity is also perfectly good. In order to find optimum immobilization conditions, we immobilized thermolysin at different pH, temperatures, times, and ratios of polymer to enzyme. From Table III we can see

Table II. Characterization of immobilized thermolysin

Polymer	Remaining activity[a]	Relative activity[b]
	%	%
PNN-1	15	85
PNN-2	21	89
PNN-3	30	92
PNN-4	32	90
PNN-5	35	87

Note: Immobilization was carried out under the same conditions: pH, 7.2; T, 17°C; t, 30 h; ratio of PNN to enzyme (w/w), 250.

[a] Remaining activity (%) = activity of immobilized enzyme/activity of free enzyme in solution × 100%.

[b] Relative activity (%) = activity of immobilized enzyme/(activity of free enzyme in solution − activity of free enzyme in filtration solution) × 100%.

Table III. Immobilization conditions and remaining activity of PNN-3–thermolysin

pH[a]	RA[b]	T/°C[c]	RA[b]	R[d]	RA[d]	IT[e]	RA[b]
6.2	15.6	0	25.0	500	22.0	1.0	8.0
7.2	29.9	17	29.7	250	19.8	8.0	17.8
7.8	29.7	23	28.5	125	18.2	20.0	24.3
8.1	22.4	30	7.9	42	16.7	30.0	29.7

[a] T, 17°C; t, 30 h.

[b] Remaining activity (%).

[c] pH 7.2; t, 30 h.

[d] Ratio of PNN-3 to thermolysin (w/w): pH 7.2; T, 17°C; t, 30 h.

[e] Immobilization time (h): pH 7.2; T, 17°C; ratio of PNN-3 to thermolysin (w/w) was 250:1 in a, c, and e.

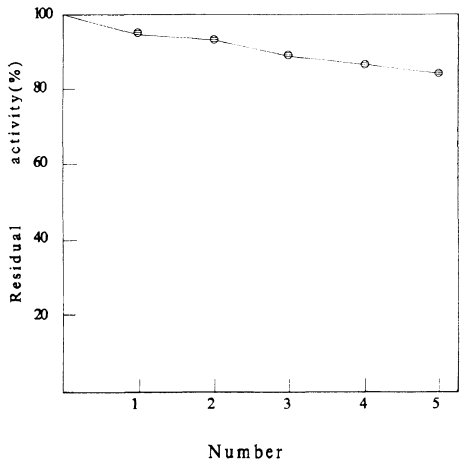

Figure 1. Effects of the repeated use on the residual activity of casein hydrolysis at pH 8.0.

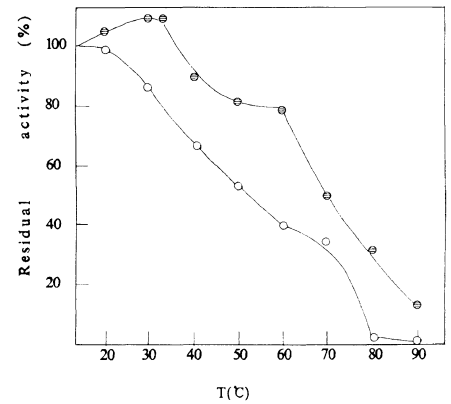

Figure 2. Effects of heat treatment at given temperatures and pH 8.0 for 1 h on the residual activity of casein hydrolysis at pH 8.0 and 35°C. (○), native enzyme; (⊜), PNN-enzyme.

that the optimum conditions are: pH 7.2, temperature 17°C, ratio 500, time 30 h.

Effect of Repeated Usage

The durability of immobilized enzymes is very important to application, because they are subject to repeated use on residual activity (highest activity among tested immobilized enzymes was 100%) of casein hydrolysis by immobilized thermolysin. From Figure 1 it can be seen that the activity of immobilized thermolysin does not decrease very much with increase of cycles and remains over 80% after five times.

Effect of Heat Treatment

We know that the thermal stability of immobilized enzymes is a very important characteristic in usage. This is also the basis for the use of T_c as the separation technique in our lab. From Figure 2 it can be seen that the immobilized thermolysin is more stable than free native thermolysin at all tempera-

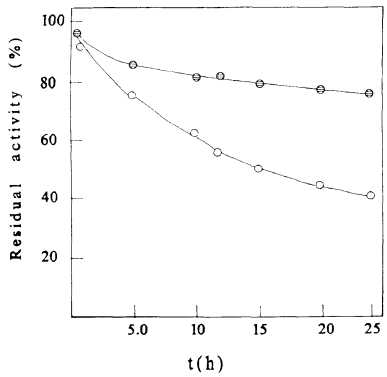

Figure 3. Effects of storage in buffer at pH 8.0 and 50°C on the residual activity of casein hydrolysis at pH 8.0 and 35°C. (○), native enzyme; (⊜), PNN-enzyme.

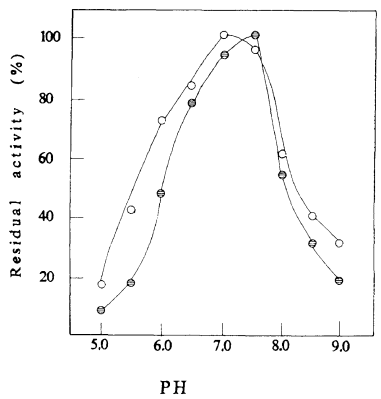

Figure 4. Effects of pH on the residual activity of casein hydrolysis at pH 8.0 and 35°C, treatment at given pH and 25°C for 30 min. (○), native enzyme; (⊜), PNN-enzyme.

tures. When kept for 1 h within 40°C, the activity of thermolysin did not decrease. So we believe that the technique used in our lab is available.

Storage Stability

The immobilized thermolysin and free native thermolysin were stored at 50°C in 0.01 M Tris–HCl buffer (pH 7.2), and after a certain interval, the activities of immobilized thermolysin and free native enzyme were assayed. From Figure 3 we can see that the immobilized thermolysin was more stable than the free native enzyme.

Effect of Temperature

In Figure 4 the immobilized and free native thermolysin hydrolysed the casein at different temperatures. It can be seen that the optimum temperature of immobilized thermolysin shifted to 5°C higher compared with that of the free native enzyme.

Optimum pH

The effects of pH on the rate of hydrolysis of casein by native thermolysin and immobilized thermolysin were compared. As shown in Figure 5, the optimum pH and pH–activity profile of immobilized thermolysin shifted in parallel *ca.* 0.7 pH units to the alkaline side

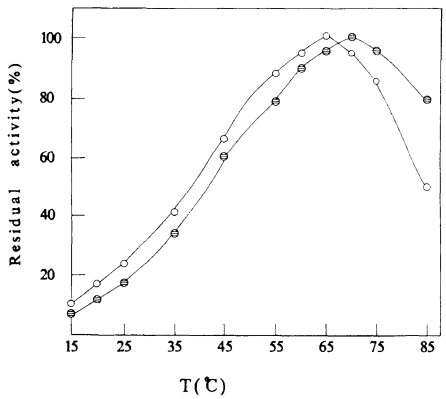

Figure 5. Activity was assayed at different temperatures and pH 8.0 on the hydrolysis of casein. (○), native enzyme; (⊜), PNN-enzyme.

compared with those of native thermolysin.

Effect of EDTA

EDTA is a strong inhibitor of thermolysin.[20] So we assayed the activity of thermolysin and native enzyme at different concentrations of EDTA. From Figure 6 we can see that immobilized thermolysin is more stable than native enzyme at all concentrations of EDTA.

CONCLUSIONS

The results obtained in our study show that

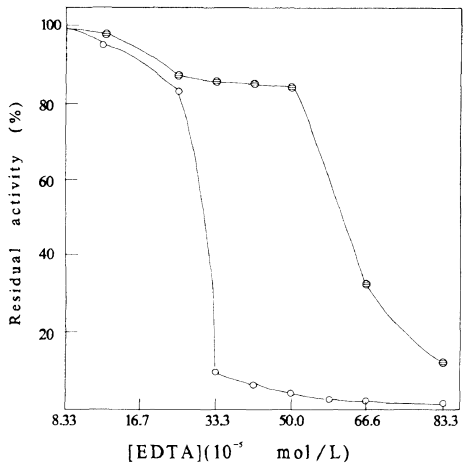

Figure 6. Effects of different concentration of EDTA on the residual activity of casein hydrolysis at pH 8.0 and 35°C, EDTA = 8.33×10^{-5} M. (○), native enzyme; (⊖), PNN-enzyme.

the polymer we synthesized in our lab can be used for the immobilization of an enzyme, and because of its characteristics of T_c, it can also be precipitated by raising the temperature and separated. In this procedure, the activity of the immobilized enzyme does not decrease. The major advantage of this method is that it avoids using strong acid or a solution with a high concentration of salt as the means of precipitating the polymer, or else, the enzyme would be destroyed. For instance, a pH-sensitive enzyme such as thermolysin would be damaged by strong acid. The thermal stability of most enzymes after being immobilized, can be greatly increased so that most of them withstand heat treatment around 35°C without loss of activity. The method used in our lab can bring about enzyme reaction in its soluble form, and separation in its insoluble form. We are now studying possible applications of this technique to enzyme-catalytic reaction.

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