Thermosensitive Poly(*N*-isopropylacrylamide) Hydrogel for Refolding of Recombinant Bovine Prethrombin-2 from *E. coli* Inclusion Bodies

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ABSTRACT: The poly(*N*-isopropylacrylamide) (PNIPA) hydrogel, which is a kind of temperature-sensitive polymer, was synthesized by inverse suspension polymerization. The microscopy and scan electron microscopy (SEM) of PNIPA hydrogel were studied. The microscope photograph showed that the particles were in the range of 0.2–0.5mm in diameter, with numerous conjoint pores about 1–2μm spreading all over the surface of the beads. The swelling properties of PNIPA gel beads indicated that the lower critical solution temperature (LCST) of the gel was 33°C. The PNIPA prepared was applied to the renaturation of bovine prethrombin-2 (pThr-2) from inclusion bodies produced in *E. coli*. It was observed that PNIPA was quite efficient in assisting protein renaturation at high protein concentration. When mixing with 105mg/mL PNIPA hydrogel during the refolding, the total activity of the thrombin was about 6222U/mL, compared with only 2800U/mL by simple dilution refolding. The kinetics of pThr-2 refolding with the absence or the presence of PNIPA was also studied respectively. The time required for the refolding with PNIPA gel was a little bit longer than that by the dilution method owing to the diffusion resistance of the protein into the network of the gel and the hydrophobic interaction between the protein and the polymer. The mechanism of the enhancement for the PNIPA gel to the refolding was further discussed. The porosity of the PNIPA hydrogel allows penetration of the unfolded protein into the inside of the polymer with a hydrophobic side chain, which can facilitate the formation of intermediate via hydrophobic interaction with the unfolded protein and the folding intermediate that are liable to re-aggregation. About 1.2mg of purified active thrombin could be recovered from 1 L of cells, which greatly facilitated the scale-up to the quantities of protein necessary for further functional and structural studies. A novel protein renaturation method mediated by PNIPA hydrogel beads, which highly increases the refolding efficiency with easy handling, recycling, and low cost, was proposed. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 96: 1734–1740, 2005

Key words: stimuli-sensitive polymers; poly(*N*-isopropylacrylamide); hydrogels; swelling; bovine prethrombin-2; protein refolding

INTRODUCTION

In producing recombinant proteins, an *E. coli* expression system is often first employed because it is simple and provides extremely high yields.[1] However, the overproduction of proteins in *E. coli* usually leads to the formation of insoluble protein aggregates (inclusion bodies), which don't assume to possess the correct three-dimensional structure.[2] To obtain protein molecules with biological activity, an additional step must be used to convert the inactive and misfolded inclusion body proteins into soluble bioactive products, what is called *in vitro* protein refolding.[3] The insoluble protein pellets must be separated from other cellular components, usually by homogenization or sonication, washing, and centrifugation. Inactive pellets are then solubilized in a mixture composed of denaturants (such as 7mol/L guanidine hydrochloride or 8mol/L urea) and reducing reagents, which could unfold the protein molecules and break disulfide bonds to yield monomeric peptide chains. With the removal of denaturant and excess reducing reagent, the unfolded reduced polypeptide chain can gradually fold into its native structure, and the native disulfide binds form. In recent years, many researchers[4] have attempted to improve the efficiency of refolding by minimizing the formation of aggregation at high protein concentration. These approaches involve refolding in reversed micelles utilizing solid–liquid extraction,[5] refolding with aqueous two-phase systems,[6] refolding with hollow-fiber membrane,[7] and refolding with high performance liquid chromatography (SEC, HIC, IEC, AFC, etc.).[8,9] On the other hand, some additives such as poly(ethylene glycol)[10] that can reduce the possibility of undesired protein–protein interaction are also employed in some processes.

Poly(*N*-isopropylacrylamide) (PNIPA) hydrogel is a typical temperature-sensitive hydrogel and exhibits a lower critical solution temperature (LCST), which is a nonionic polymer with many hydrophobic side

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chains. The network of PNIPA hydrogel will collapse and precipitate from the water solution as the temperature is increased above the LCST. When the temperature is below the LCST, PNIPA hydrogel can absorb water and swell again. In recent years, a great deal of attention has been paid to this temperature-sensitive hydrogel, and the property of phase separation has been used for application in many fields, such as controlled drug delivery,[11] recyclable absorbents,[12] immobilization of enzyme, and reactivity control[13,14].

Enlightened by the report that adding of water-soluble uncrosslinked PEG or PNIPA[15] can inhibit aggregation and facilitate the protein refolding, in this work, we will prepare PNIPA gel particles by inverse suspension polymerization to use them to assist the refolding of prethrombin-2 (pThr-2) inclusion bodies in *E. coli*, because refolding of pThr-2 from inclusion bodies is a difficult problem for the four intramolecular disulfide-bonds it contains, which are prone to forming intermolecular non-native disulfide bonds and causing aggregation during refolding.[16] Based on this application, we try to explore the application field for the PNIPA hydrogel and to establish a more efficient protein refolding method.

METHODS

Materials

N-isopropyl acrylamide (NIPA) was purchased from ACROS. N,N′-Methylene-bisacrylamide (Bis) was from Fluka. Penicillin G, Isopropylthiogalactoside (IPTG), Dithiothreitol (DTT), reduced and oxidized glutathione (GSH and GSSG), HEPES, *N*-benzoyl-Phe-Val-Arg-P-Nitroamide Hydrochloride (S2160), and Snake Venom from *Eshis Carinatus* were purchased from Sigma Co. All other chemicals were of analytical grade.

Microorganism and culture

E. coli BL21(DE3) harboring bovine prethrombin-2 (pThr-2) (preserved by the Institute of Bioengineering, Zhejiang University) was grown in 2L 2XTY medium in a 3.7L fermenter (KLF2000, Bioengineering, Switzerland). The expression of pThr-2 was induced by IPTG to a final concentration of 0.1 m*M* when OD_{600} of cells was 0.8. After a further 6 h growth, the culture was subsequently harvested by centrifugation at 10,000g for 10 min.

Isolation of inclusion bodies

The cell pellets harvested were resuspended in 10mL 50 m*M* Tris-HCl buffer at pH 8.0 and sonicated. The precipitates containing cell debris and protein inclusion bodies were collected by centrifugation and were subsequently resuspended in a 10mL 50 m*M* Tris-HCl washing buffer containing 3mol/L Urea and 0.6%Triton X-100, and stirred at 37°C for 2 h to remove the membrane components and other contaminants.

Preparation of PNIPA hydrogel

The reaction was performed in a 500mL cylindrical round-bottom glass flask fitted with mechanical stirrer, nitrogen inlet, and an oil–water separator. A thermostatic water bath was used for isothermal control. Paraffin oil (as a continuous phase) and Tween80 (as the dispersant) were first added into the flask and stirred. The monomer NIPA, crosslinker Bis (here, W_T represents the mass percent of monomer and crosslinker in the reaction solution, while W_C represents the crosslinker mass percent relative to the total monomer plus crosslinker), and 1.7 wt % initiator APS (with respect to W_T) were dissolved in deionized water. From the results of our previous work,[17] the gel with higher W_T could greatly facilitate the refolding of lysozyme. Thus, here the gels with W_T = 14%, W_C = 5 and 10% were synthesized, respectively. When the solution was added into the flask for about 30 min, following the adding of activator TEMED, the polymerization took place in 3 h. The whole process was carried out with purging of the nitrogen. The water was thus separated through a water–oil separator.

Characterization of PNIPA hydrogel

A microscope equipped with a Nikon camera, TCI System, and the Software for image analysis were employed to determine the particle size of the hydrogel beads. A Scanning Electron Microscope (SEM) (JEOL, JSM5510LV) was used to study the surface characteristics of the microspheres.

Measurement of swelling ratio

The dried particles (with dry weight W_d) were ground into powder and packed into a measuring cylinder with deionized water. The volumes of the wet gels were recorded. The density of the dried particles was about 1.25g/mL, the volume of dried particles was calculated, and the swelling ratio (S_R) was then obtained by dividing the swollen gel volume by the dried gel volume.

Renaturation of pThr-2 inclusion bodies

Purified pThr-2 inclusion bodies were solubilized by incubating with 50 m*M*, pH 8.0 Tris-HCl buffer containing 8mol/L Urea and 0.3mol/L DTT at 37°C for 2 h. The total protein concentration was about 4mg/mL; more than 60% of the solubilizate, as estimated by SDS-PAGE, were pThr-2. The refolding buffer con-

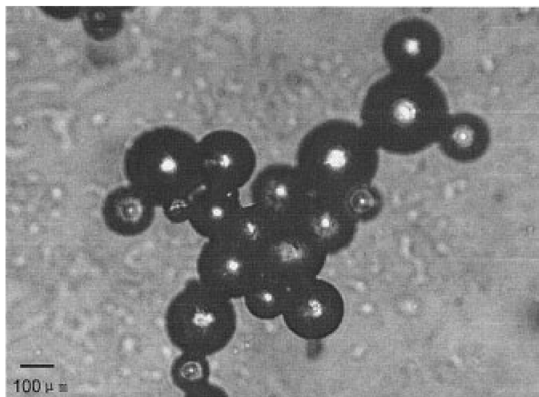

Figure 1 Micrograph of PNIPA gel particles at dry state (W_T = 14%, Wc = 5%).

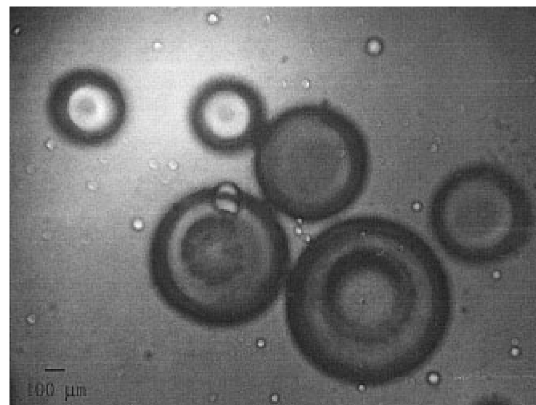

Figure 3 Micrograph of PNIPA gel particles after swelling (W_T = 14%, Wc = 5%).

tains 2.7mol/L Urea, 0.6mol/L GSSG, GSSG/GSH 0.9, 0.1% PEG6000, and 0.5mol/L L-Arg in 50 m*M*, pH7.4 PBS buffer. First the pThr-2 was renatured by diluting the solubilized pThr-2 into different fractions of the refolding buffer according to the dilution factor. The mixture was vortexed and shaken in the incubator at room temperature for 24 h. Then PNIPA gel particles were added into the mixture. With the swelling of the PNIPA gel, the denatured pThr-2 penetrated into the porous gel and refolded step by step with the assistance of the PNIPA gel. The supernatant was collected after refolding. Finally, by heating the swelling gel particles to 37°C for 10 min, the pThr-2 entrapped in the gel network could be retrieved, which also refolded correctly. After refolding, the solution of pThr-2 was dialyzed at 4°C against 25 m*M*, pH7.4 PBS buffer, 0.1% PEG 6000, 0.15mol/L NaCl overnight.

Assay of thrombin

The refolded pThr-2 was activated to thrombin by Snake Venom for the activity assay as reported.[18] The activity of thrombin[19] was measured by the hydrolysis to the chromogenic substrate S2160. One unit is defined as the amount of enzyme required to increase OD_{405} for 0.001 in 1 min. The results of the refolding process were expressed by the activity of thrombin we got from 1mL denatured protein.

Purification of active thrombin

The activated thrombin was purified by loading onto a heparin affinity column (Amersham Biosciences, Sweden) equilibrated with 50 m*M* sodium phosphate, pH 6.5. The unbounded impurities were washed by this buffer. The thrombin with active sites was bounded onto the heparin affinity resin and eluted with 1mol/L NaCl for 5 CV in the same buffer.

RESULTS AND DISCUSSION

Characterization of PNIPA hydrogel

The micrographs of the PNIPA hydrogels in dry state are shown in Figures 1 and 2, and the gels in swelling

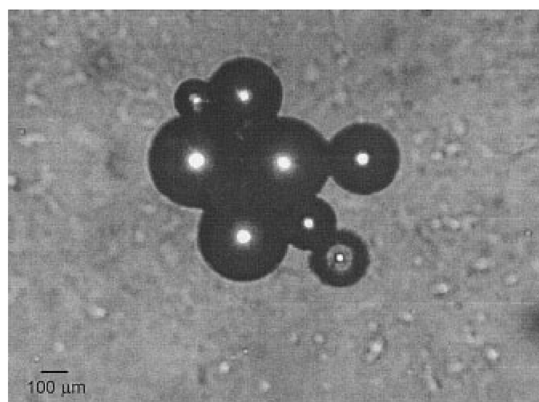

Figure 2 Micrograph of PNIPA gel particles at dry state (W_T = 14%, Wc = 10%).

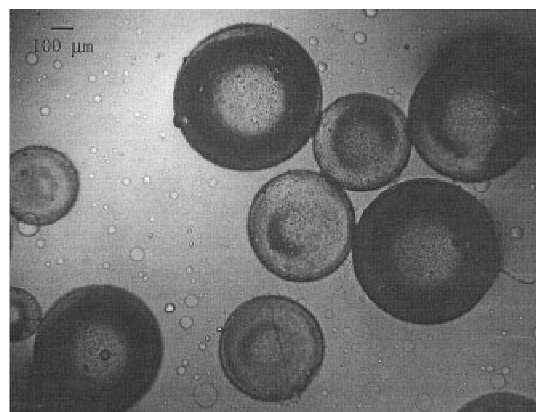

Figure 4 Micrograph of PNIPA gel particles after swelling (W_T = 14%, Wc = 10%).

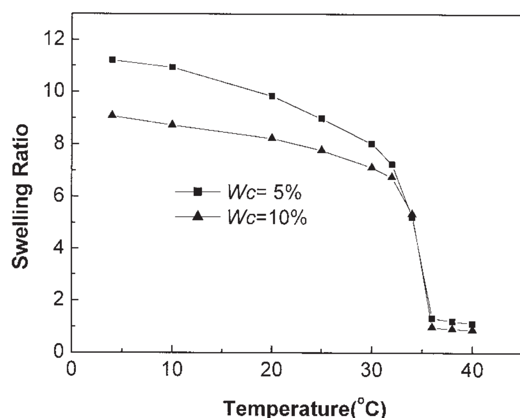

Figure 5 Swelling ratio of PNIPA gels at different temperatures (W_T = 14%).

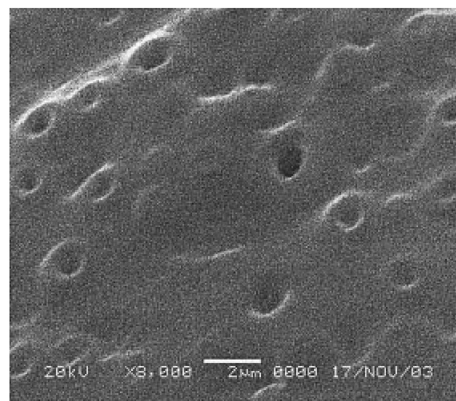

Figure 7 SEM image of PNIPA network (W_T = 14%, Wc = 5%).

state are shown in Figures 3 and 4 (at 4°C), respectively. The average diameter of the dried particles was 0.31mm with a relatively narrow size distribution ranging from 0.2 to 0.5mm. After having swollen at 4°C, the particles were enlarged to 1mm in diameter. Thus, the gel volume is increased about 10 times upon swelling.

For the study of temperature sensitivity of PNIPA gel particles, the swelling ratio of the particles at different temperature was investigated. The results were shown in Figure 5. It can be seen that the lower critical solution temperature (LCST) of the PNIPA hydrogel was at about 33°C. At a certain temperature, it was notable that the gels with lower crosslinker concentration exhibited higher swelling ratio. To understand the temperature-responsive rate of the gel particles, the dried gels were immersed in deionized water at room temperature. The S_R was reported at a definite time interval. The swelling rates of two gels with Wc = 5% and Wc = 10% are shown in Figure 6. The PNIPA network with Wc = 10% can reach the swelling equilibrium in about 3 min, which swells faster than the gel with Wc = 5%.

From the SEM images shown in Figures 7 and 8, we can identify the pores and the connectivity of these pores in the gel particles. The pores of both gels were about 1–2μm in diameter. When W_C = 5%, the pores didn't crosslink with each other, and were sporadically distributed at the surface of the particles. As to the gels with W_C = 10%, numerous conjoint pores spread all over the surface of the particles. It has been demonstrated[20] that the crosslinker mass percent Wc could greatly influence the swelling rate and the texture of the crosslinked hydrogel. Although the polymer with W_C = 10% had more pores than the network with W_C = 5%, the holes of the gel with W_C = 5% looked deeper than W_C = 10%, thus holding larger total pore volume, which could explain the higher swelling ratio of the gel with W_C = 5%. The connectivity of pores played a crucial role in the fast swelling kinetics of the gels. Water and the solute can enter or exit to the gel through the interconnected pores by convection. Moreover, increasing the internal surface area of the networks also increased the contact area between water and the polymer, and led to their accelerating volume swelling rate.

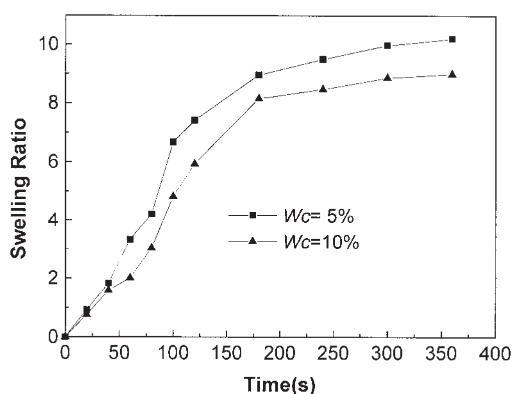

Figure 6 The swelling rate of PNIPA hydrogel (W_T = 14%, 25°C).

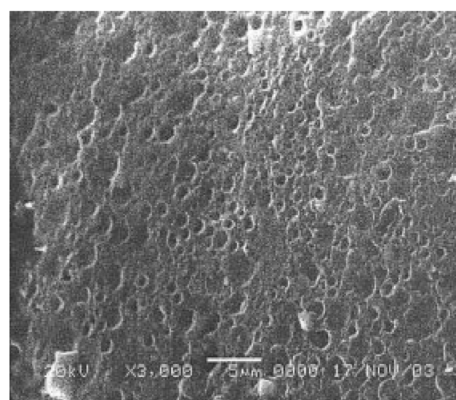

Figure 8 SEM image of PNIPA network (W_T = 14%, Wc = 10%).

TABLE I
Refolding of pThr-2 at Different Dilution Factors (Initial Denatured pThr-2 Concentration: 12.5mg/mL)

Refolding Mode	Thrombin activity (U/mL) at Different Dilution Factors			
	5	20	50	100
Refolding by dilution	1537	24200	82000	206100
Refolding with gel (W_T = 14%, W_C = 5%)	1912	24600	90000	206500
Refolding with gel (W_T = 14%, W_C = 10%)	3162	25900	96200	212500

Effect of PNIPA gel beads on the refolding of pThr-2

In designed experiments, 80mg/mL gels were applied to assist the pThr-2 refolding process. To illustrate explicitly the effect of PNIPA on the protein refolding, the initial denatured pThr-2 concentration here was 12.5mg/mL, which was much higher than that in simple dilution refolding and difficult to perform by regular refolding techniques. The results are shown in Table I.

The higher dilution factor (lower final protein concentration) could get higher thrombin activity. Compared with dilution refolding, the effect of PNIPA on the refolding of pThr-2 was much more notable at lower dilution factors. For example, when the dilution factor was 5 and after refolding with gel (W_C = 10%), 1mL denatured pThr-2 could recover 3162U/mL while refolding by simple dilution only got 1537U/mL, which is an increase of about 106%. When the dilution factor was 50, it increased only 17%. This indicated that the adding of PNPIA could greatly facilitate the protein refolding at high concentration. That is the key point in the refolding process because lower protein concentration means large reactor volume and high capital cost. For the dilution factors used in our experiments, both gels could increase the activity recovery of pThr-2 compared with the dilution process; meanwhile, the gel with W_T = 14% and Wc = 10% worked more efficiently than the gel with W_T = 14%, Wc = 5%. For example, when the dilution factor was 5, after refolding, the thrombin activity was 1537U/mL by dilution, while it was 1912 U/mL and 3162U/mL with PNIPA gel Wc = 5% and Wc = 10%, respectively. This result may be attributed to the more porous structure of the gel with higher crosslinker density, which can be confirmed by the SEM images of these gels. During the protein refolding, the denatured protein can freely penetrate into the gel through the pores, and thus can interact with the isopropyl side chains of the PNIPA polymer through hydrophobic interaction as soon as possible. The inclination to the aggregation between unfolding proteins and the refolding intermediates due to the hydrophobic interaction for the abundant exposed hydrophobic groups contained in them was inhibited. So the nonionic PNIPA hydrogel can assist the refolding of pThr-2, which can be seen in Table I. The interconnected pores of the gel with W_C = 10% facilitate the faster interaction between the denatured protein and the polymer chains, thus giving higher total activity than the gel with W_C = 5%.

Refolding of pThr-2 mediated by PNIPA

From the results of Table I, the gel with W_T = 14% and W_C = 10% was subsequently used to study the effect of PNIPA concentration on the refolding of pThr-2 and its refolding process. The results are represented in Figures 9 and 10. Figure 9 indicates that the total activity of thrombin first increased with the concentration of PNIPA, while further increase of PNIPA concentration led to lower protein recovery due to more protein adsorption into the network of PNIPA, which resulted in the decrease of thrombin activity. PNIPA content of 105mg/mL was optimal for the refolding of pThr-2. When mixing with the 105mg/mL PNIPA hydrogel during the refolding, the total activity of the thrombin was about 6222U/mL, compared with only 2800U/mL by simple dilution refolding, an increase of about 122%. When the linear PNIPA was applied into the refolding of β-lactamase,[15] it could increase β-lactamase activity only about 41%. Despite the target protein being different, the results could still show us that the PNIPA hydrogel particles had great potential in the field of protein refolding due to its reutilization.

Figure 10 shows the refolding process of pThr-2 in the presence or the absence of PNIPA (W_T = 14%, W_C

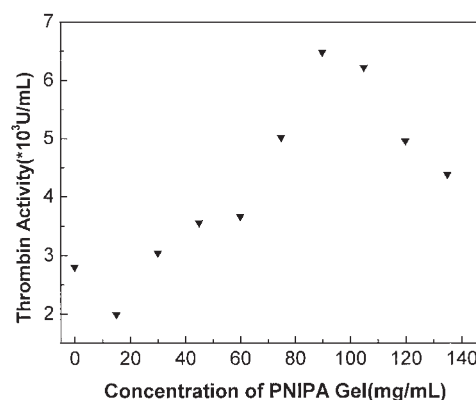

Figure 9 Effect of PNIPA concentration on the refolding of pThr-2 (initial denatured pThr-2 5.8mg/mL, dilution factor 20).

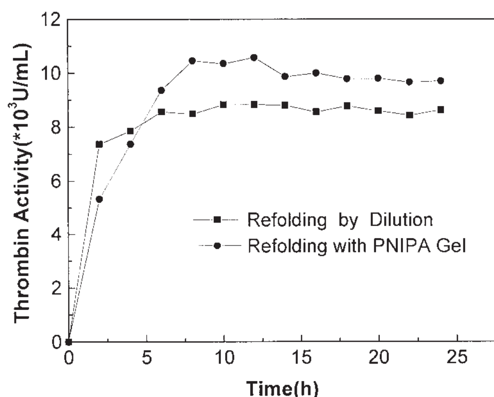

Figure 10 Refolding kinetics of pThr-2 with or without PNIPA particles (initial denatured pThr-2 8.4mg/mL, dilution factor 20).

= 10%, 105mg/mL). During pThr-2 refolding by simple dilution, the process can reach equilibrium within about 6 h. As to the refolding in the presence of the PNIPA gel, the equilibrium needs 8 to 10 h, which is a bit longer than the dilution refolding owing to the diffusion of the protein into the network of the gel and the hydrophobic interaction between the protein and the polymer. But with the assistance of PNIPA to the protein renaturation, the refolding of pThr-2 can be finally achieved in 10 h and the gel can increase the activity by 20% at this condition.

Heparin affinity purification of thrombin

After the recombinant pThr-2 refolded, it was activated into thrombin with an active site. Purification of thrombin on a heparin affinity column was designed due to the heparin binding site on thrombin. As shown in Figure 11, the thrombin eluted from the heparin column when NaCl concentration was 1mol/L. The actual protein concentration was estimated using $E^{1\%} = 19.5$ at 280nm[18]. Based on absorbance at 280nm, about 1.2mg of purified active thrombin could be recovered from 1 L of cells (which could get about 110mg pThr-2 inclusion bodies), so the refolding yield was about 1.1%. Compared with the result gotten by Dibella,[16] who reported that the refolding yield was about 0.7% and about 0.5–0.7mg of thrombin could be isolated from 1 L of *E. coli* cell culture, the PNIPA gel was shown to be an efficient additive in protein renaturation.

CONCLUSIONS

The temperature-sensitive poly(*N*-isopropylacrylamide) (PNIPA) hydrogel particles were prepared by inverse suspension polymerization, and the characterization of the PNIPA gel was described. The external and texture properties of PNIPA networks were studied by microscope and scan electron microscope (SEM). The average diameter of the particles was 0.31mm with a narrow size distribution from 0.2 to 0.5mm. There are numerous conjoint pores about 1–2μm spreading all over the surface of the particles. The swelling properties of PNIPA indicated that the LCST of the gel was about 33°C. The application of PNIPA for the renaturation of pThr-2 showed that PNIPA was effective in enhancing protein renaturation at high protein concentration. The optimal concentration of PNIPA during pThr-2 refolding was 105mg/mL, and at this condition refolding with the PNIPA hydrogel could greatly increase the thrombin activity by about 122%. However, further increase of PNIPA concentration led to lower protein recovery due to more protein adsorption into the network of PNIPA. The time need for the refolding with the PNIPA gel was longer than the refolding by dilution owing to the diffusion of the protein into the network

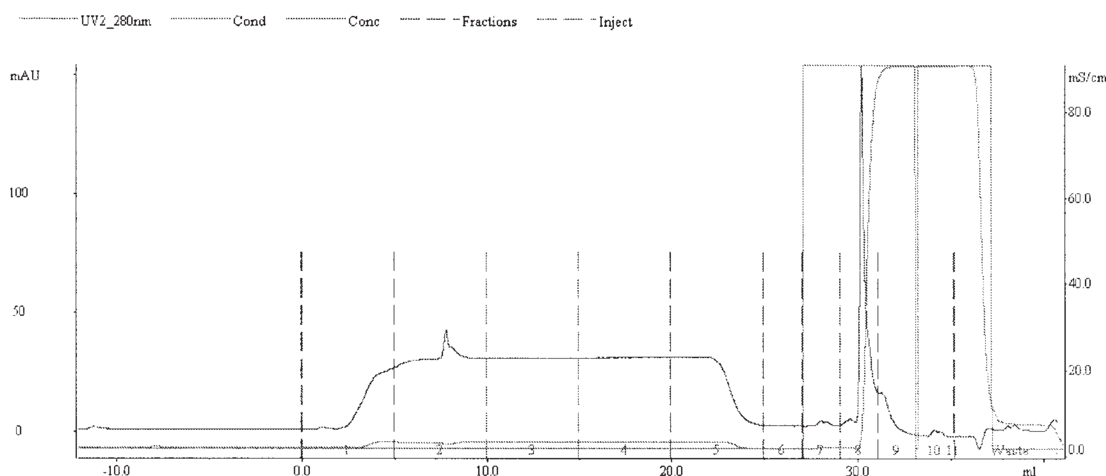

Figure 11 Chromatogram of thrombin by heparin affinity.

of the gel and the hydrophobic interaction between the protein and the polymer. The porosity of the PNIPA hydrogel allows the penetration of the unfolded protein into the inside of the polymer with hydrophobic pendant chain. On the other hand, just as revealed by Lin,[15] the extensive hydrophobic pendants of the linear PNIPA could facilitate the formation of intermediate via hydrophobic interaction with the unfolded protein and the first folding intermediate that are prone to re-aggregation. In a word, the porous interconnected microstructure of the gel and the pendant hydrophobic isopropyl groups play an important role in the protein refolding. Furthermore, after refolding, heated above its LCST, the PNIPA gel could be easily recycled and reused into the refolding process repeatedly. The activated thrombin was purified by the HiTrap heparin affinity column and about 1.2mg of purified active thrombin could be recovered from 1 L of cells, which greatly facilitated the scale-up to the quantities of protein necessary for further functional and structural studies. A novel protein renaturation method by the adding of PNIPA hydrogel, which highly increases the refolding efficiency with easy handling, recycling, and low cost, was expected to be established, thus benefiting us to greatly enlarge the application field for the PNIPA hydrogel.

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