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METAL-COPOLYMER COMPLEXES OF N-ISOPROPYLACRYLAMIDE FOR AFFINITY PRECIPITATION OF PROTEINS

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ABSTRACT

Copolymers of N-isopropylacrylamide (NIPAM) with styrene derivative of iminodiacetic acid (st-IDA) and 1-vinylimidazole (VI) were synthesized by radical copolymerization and metal complexation characteristics of the copolymers were investigated. The theromoprecipitation property of the copolymers indicate the application of Cu(II) loaded copolymer of poly(VI/NIPAM) as a potential carrier for the metal chelate affinity precipitation of proteins. The studies carried out on the purification of protein inhibitors from different cereals, suggest the specific interaction of metal ions bound on the copolymer and the histidine residues on the surface of the target protein. The recovered copolymers could be reloaded with metal ions and can be reused number of times with high efficiency.

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INTRODUCTION

The metal complexation behavior of polymeric ligands has attracted much attention in areas of metal ion separation, catalysis and bioinorganic chemistry [1]. In recent years, much interest has been developed in utilizing metal-polymer complexes for the affinity separation of proteins e.g., immobilized metal affinity chromatography (IMAC) [2]. Another area which is growing constantly is the application of stimuli-responsive polymers [3]. These polymers exhibit reversible phase changes in response to changes in environmental factors such as temperature, pH, ionic strength, electric or magnetic fields [3, 4] and such properties have found their applications ranging from drug delivery to diagnostics, separations and robotics. In temperature responsive polymers, poly(N-isopropylacrylamide) (poly-NIPAM) is well-known as a thermally-reversible water-soluble polymer. The aqueous solutions of the polymer exhibit a sharp phase transition at around 32°C (called the lower critical solution temperature, LCST, or cloud point). This transition occurs because of the reversible shift from hydrophilic below this temperature to hydrophobic above it [5, 6]. The application of this polymer in drug delivery research is well recognized and has been extensively studied for use in drug delivery systems, where time-dependent pulses of drug release are achievable [7]. Our goal has been to utilize the phase separation property of this polymer in the separation of proteins by affinity precipitation.

In affinity precipitation, the bioconjugate is synthesized by coupling a ligand to a phase separating water soluble polymer. The ligand-polymer conjugate selectively binds the target protein from the crude extract and the protein-polymer complex is precipitated from the solution by a simple change in the environment (pH, temperature, ionic strength or addition of some reagents). Finally, the desired protein is dissociated from the polymer and the later can be recovered and reused for another cycle. A variety of ligands like protease inhibitors, antibodies, nucleotides, carbohydrates and triazine dyes have been succesfully used in affinity precipitation [8]. However, the application of metal ion affinity ligands have not been fully exploited in affinity precipitation, which otherwise makes an attractive approach in the chromatographic mode [2]. Metal chelate affinity precipitation makes it more feasible and cost effective when the intended applications are for large scale process. Metal ligands, generally Cu(II) or Ni(II) are covalently coupled to the water soluble-insoluble polymers. The metal ion specifically binds the target protein via the histidine residues on the surface of the protein. The protein-polymer complex is precipitated and later the

protein is dissociated from the polymer. The strategy of precipitation of the target protein and finally recovery of the protein from the metal polymer complex is depicted in Figure 1. The metal affinity offers a number of important advantages over other "biospecific" affinity techniques for protein purification, particularly with respect to ligand stability and protein loading. More important has been the easy protein recovery by removing the metal ion via quenching the chelating agent.

An ideal metal chelate affinity precipitation system must have a reversibly precipitating polymer backbone with reactive groups for the chelating ligand. Poly-NIPAM as such does not provide the reactive groups to be used directly for coupling of affinity ligand. Thus, monomers containing reactive groups are copolymerized with NIPAM. More recently, a new type of polymer, namely copolymer of vinylimidazole (VI) and N-isopropylacrylamide,

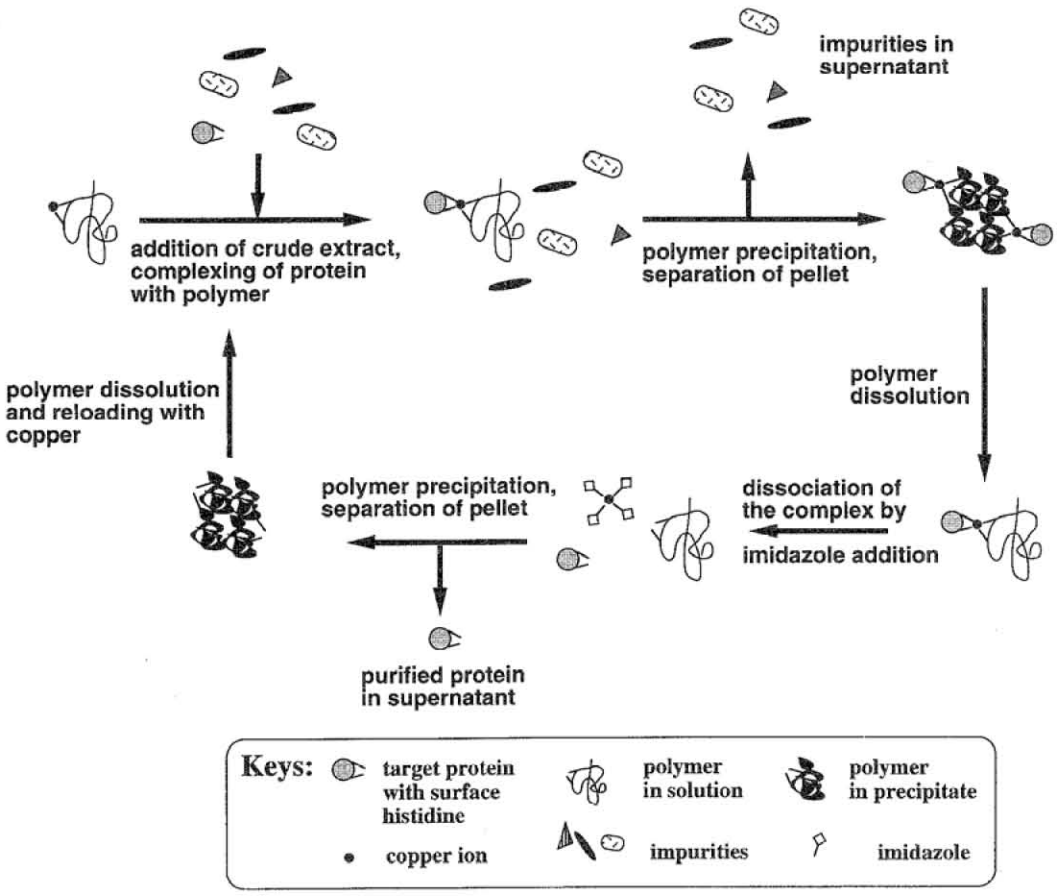

Figure 1. Scheme of metal chelate affinity precipitation of proteins.

poly(VI/NIPAM), was introduced at this laboratory [9]. Several imidazole ligands on a flexible polymer in solution can come close enough to interact with the same metal ion and thus provide sufficient strength of polymer-metal interaction. The metal ion later binds to the target protein via imidazole group in the histidine residues [10]. This can provide an efficient system for the metal chelate affinity precipitation of proteins.

Synthesis and Properties of NIPAM Copolymers

Copolymers of NIPAM with VI or styrene derivative of iminodiacetic acid (st-IDA) were synthesized by radical polymerization [10, 11]. Polydentate carboxy containing ligands like IDA or nitrilotriacetic acid (NTA) have been successfully used for metal chelate mediated purification of proteins [2, 12]. Ligand coordination of IDA or NTA to the metal ion is stronger and there are still coordinating sites on the metal ion available for binding the target protein. When IDA-styrene derivative, synthesized via reacting IDA with benzyl chloride, was copolymerized with NIPAM, the copolymer had a drastically decreased efficiency of precipitation with temperature compared to homopolymer. Charged moieties render the poly-NIPAM macromolecule more hydrophilic and hinders the aggregation and precipitation of the polymer. With increasing amount of IDA in copolymer, the precipitation temperature or cloud point increased significantly. Even with 1 M NaCl, which promotes hydrophobic interactions, the cloud point of the copolymer with 3 mol% IDA was as high as 65°C (Figure 2). It was observed that when the copolymer was loaded with metal ions, which neutralized some of the charges, the copolymer can be precipitated at temperatures slightly above those for poly-NIPAM when moderate salt concentrations were used (upto 0.5 M NaCl). In the presence of 1 M NaCl, the precipitation temperature decreased slightly by increasing the amount of IDA in the copolymer (Figure 2). This possibly indicates that higher IDA moieties will form more inter- and intramolecular crosslinking with the metal ions which help in aggregation and finally precipitation of the copolymer. Similar effect was also reported earlier for VI and NIPAM copolymers [13]. The thermoprecipitation of such polymers around 30-35°C allows their application only for purification of thermostable proteins. Although, we have also observed that the copolymer could be precipitated quantitatively around 15-20°C using 10% acetonitrile and 20 mM $CaCl_2$ (unpublished data). Incorporation of solvent promotes aggregation of the polymer. A similar effect was also reported earlier on a pH sensitive copolymer of methyl methacrylate and methacrylic acid (Eudragit S-100) [14]. Still the appli-

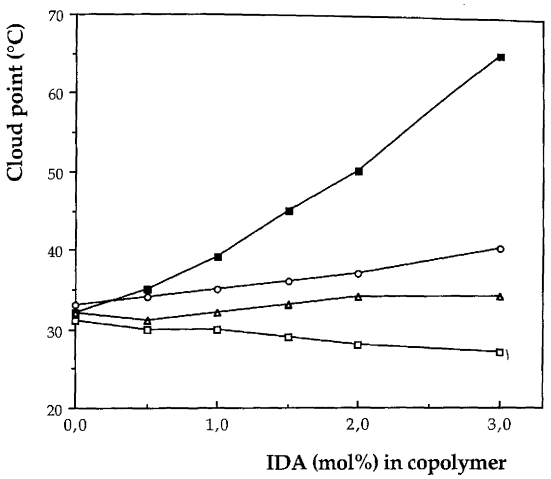

Figure 2. Plot of cloud point of 0.1% (w/v) copolymer solution of poly(st-IDA/NIPAM) against content of iminodiacetic acid (IDA) in the copolymer. Poly (st-IDA/NIPAM) at pH 6.0, precipitated with 2 M NaCl (■); Cu(II)-poly(st-IDA/NIPAM), precipitated with (O) 0.2 M NaCl; (Δ) 0. 5 M NaCl and (□) 1 M NaCl at pH 6.0. Cloud point was taken as the temperature where 0.1% (w/v) copolymer solution shows the half maximum absorbance at 470 nm.

cation of organic solvents may be a limiting factor. Another option is to use three-component copolymers with the third component containing hydrophobic side chains and hence decreasing LCST of the polymer.

The greater success was achieved when using imidazole ligand [9]. Copolymers of vinylimidazole (VI) with NIPAM, poly(VI/NIPAM), were synthesized by radical polymerization in aqueous solution. Started with 5, 10, 17 and 33 mol% VI in the reaction mixture, 5, 10, 15.6 and 26.8 mol% of VI were copolymerized with NIPAM respectively as calculated using ^{1}H-NMR spectra [10, 11]. A clear separation of the peaks from imidazole protons and from N-isoporopyl amide protons was observed (Figure 3). In this copolymer also, the incorporation of relatively hydrophilic imidazole moieties hindered the hydrophobic interactions of the native poly-NIPAM and resulted in an increase in the precipitation temperature (Figure 4). The effect was more evident at lower pH values were imidazole moieties where protonated and hence render the polymer more hydrophilic. Poly(VI/NIPAM) (15.6 mol% VI), for instance, did not precipitate at all on heating up to 70°C at pH 4 and 6, whereas precipitation of homopolymer, poly-NIPAM was independent of pH (Figure 4).

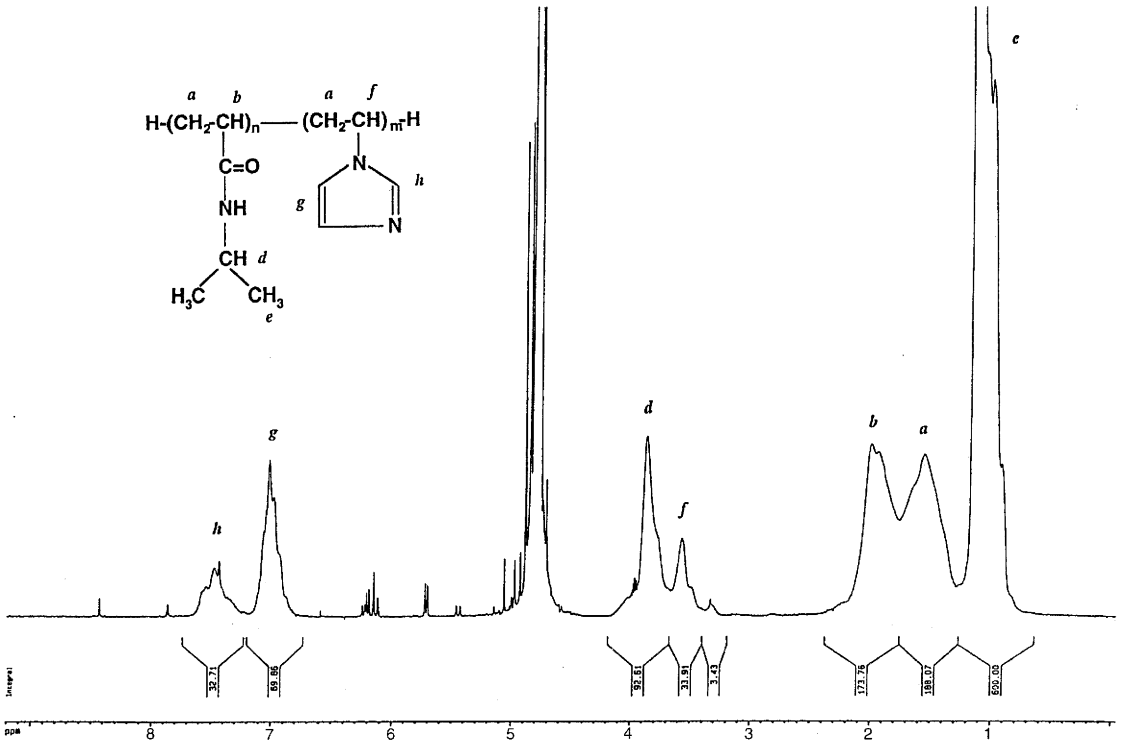

Figure 3. 500 MHz ^{1}H-NMR spectrum of poly(VI/NIPAM) (D_2O, 25°C).

Metal Complexation of Poly(VI/NIPAM) Copolymers

When loaded with metal ions e.g., Cu(II), Zn(II), Ni(II) or Co(II) the precipitation efficiency of the poly(VI/NIPAM) copolymers still decreased and even at pH 8.0, the polymer could not achieve any significant precipitation by heating up to 70°C. This was because the metal ions induce more positive charges to the copolymer and renders it more hydrophilic. It was shown earlier that different metal ions did not show any major differences in this effect [13]. To achieve complete precipitation of such copolymers, one should incorporate conditions promoting hydrophobic interactions and reducing mutual repulsion caused by similar charges. The increase in ionic strength and hence decrease in charge repulsion by adding NaCl facilitated precipitation of the Cu(II) bound poly(VI/NIPAM) copolymer (Figure 5). Other metal bound copolymers also precipitated efficiently in the temperature range of 15-25°C.

Using a different spectroscopic technique, we studied the interaction of Cu(II)-ion with poly(VI/NIPAM). Cu(II) interaction with vinylimidazole result-

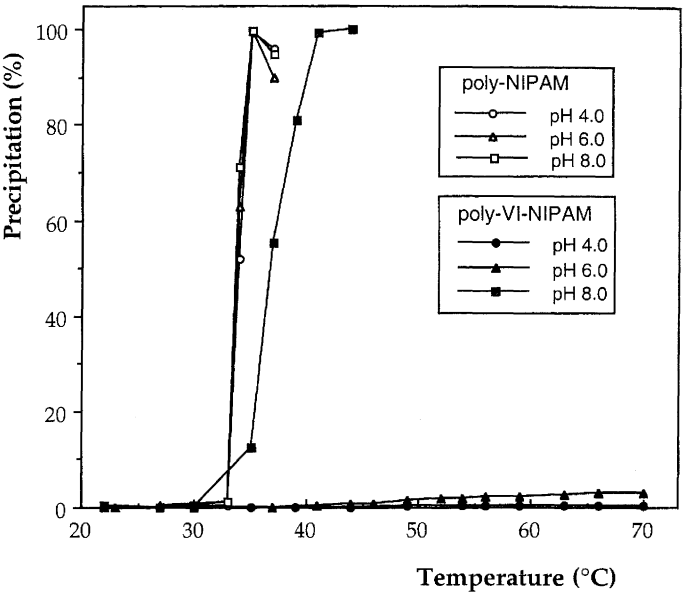

Figure 4. Thermoprecipitation of poly-NIPAM and poly(VI/NIPAM) (15.6 mol% VI) from aqueous solution at various pH values monitored as turbidity at 470 nm. Polymer concentration 1.0 mg/ml.

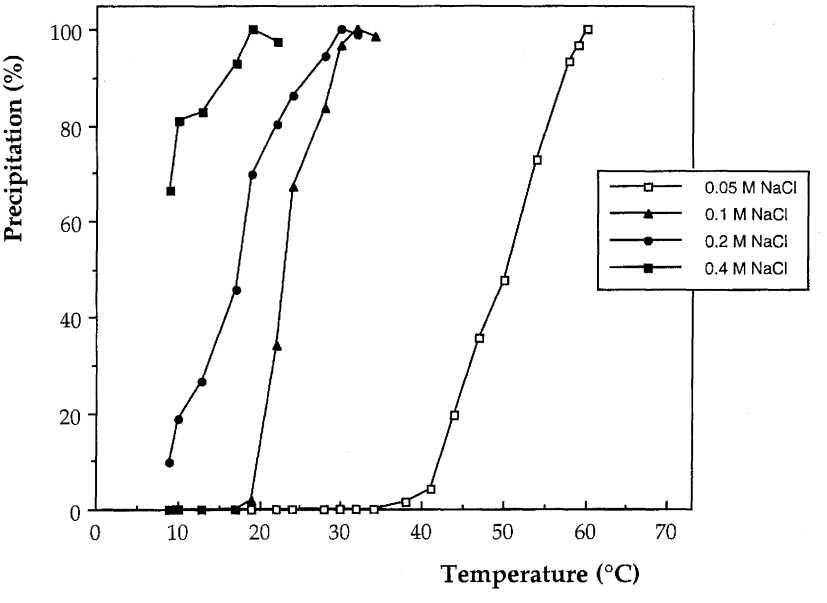

Figure 5. Thermoprecipitation of Cu(II)-poly(VI/NIPAM) (15.6 mol% VI) at various concentrations of NaCl at pH 6.0 monitored as turbidity at 470 nm. Polymer concentration 1.0 mg/ml.

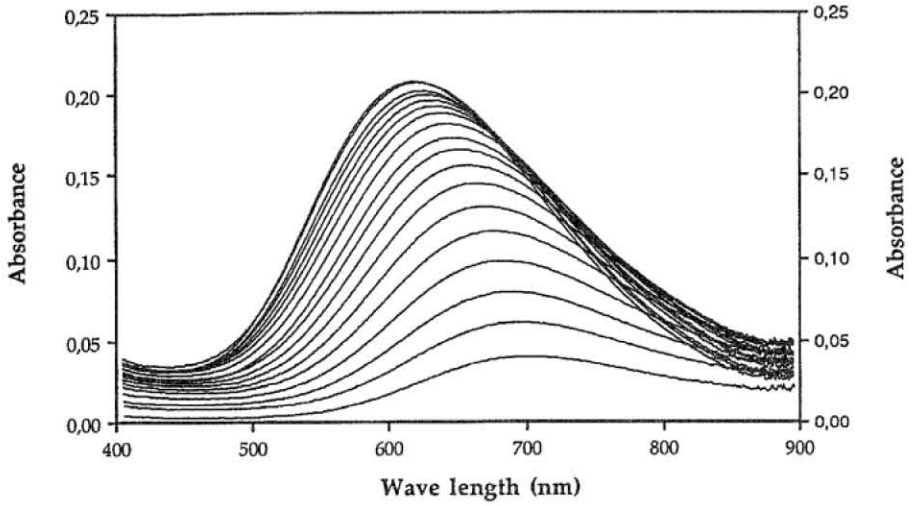

Figure 6. Difference spectra obtained when Cu(II) was titrated with increasing concentration of poly(VI/NIPAM) copolymer. Both the sample and reference cuvettes contained 1 ml of 4 mM $CuSo_4$ solution in water (pH 6.0). Same volumes of copolymer solution [1% poly(VI/NIPAM) in water, pH 6.0] and water were added to the respective sample and reference cuvette. The final concentration of VI in the sample cuvette was from 1.6 to 12.4 mM.

ed in complex formation with absorption maximum around 620-680 nm (Figure 6). The absorption maximum wavelength decreased with an increase in the complex formation. The complex formation reached a saturated stage at higher polymer amounts because of the higher imidazole concentrations and having Cu(II) concentration constant. The nature of Cu(II)-polymer complex is changing with a degree of saturation of polymer with Cu(II). As the spectra does not show the isobestic point of the complex formation, this method of determination of binding constant for vinylimidazole with Cu(II) becomes difficult. Imidazole is a monodentate ligand in Cu-complexes. Up to four imidazoles bind to one Cu(II)-ion, the log K (where K is association constant, M^{-1}) for each imidazole ligand is decreasing from log K_1 = 3.76 for binding the first imidazole ligand to log K_4 = 2.66 for binding the fourth imidazole ligand [15]. The binding of a single imidazole ligand to the Cu(II)-ion in solution is much weaker compared to the binding of tridentate IDA (log K= 11, [16]). On the other hand, when Cu(II)-ion forms a complex with four imidazole ligands the combined binding constant log K = log K_1 + logK_2 + log K_3 + log K_4 = 12.6 - 12.7. The strength of this complex is close to that of Cu(II) - ion complex with poly(1-vinylimidazole) (poly - VI), log K = 10.64 - 14.21 [15, 17] and comparable with the binding of tridentate IDA ligand.

Our studies showed that a number of metal ions bound by the polymer is coordinated by about two to three imidazole groups in the copolymers according to direct analytical measurements and NMR analysis [13]. Thus, the flexible copolymer like poly(VI/NIPAM) can adopt in solution a conformation where two to three imidazole ligands are close enough to form a complex with the same metal-ion. With about two to three imidazole ligands bound to the metal ion e.g., Cu(II) one could expect binding strength of log K= 6.0-9.0 providing a significant strength of interaction. It is clear that not all available coordination sites of the metal ion are occupied by imidazole ligands of the polymer. The unoccupied coordination sites of the metal ion could be used for complex formation with the protein molecule via histidine residues on its surface.

The vinylimidazole copolymer showed a good binding capacity of metal ions and was also quite fast. Cu(II) and Ni(II) binding to poly(VI/NIPAM) increases initially during the first 45-60 minutes and then levels off towards the equilibrium level (Figure 7). The kinetics of the metal ion adsorption will also

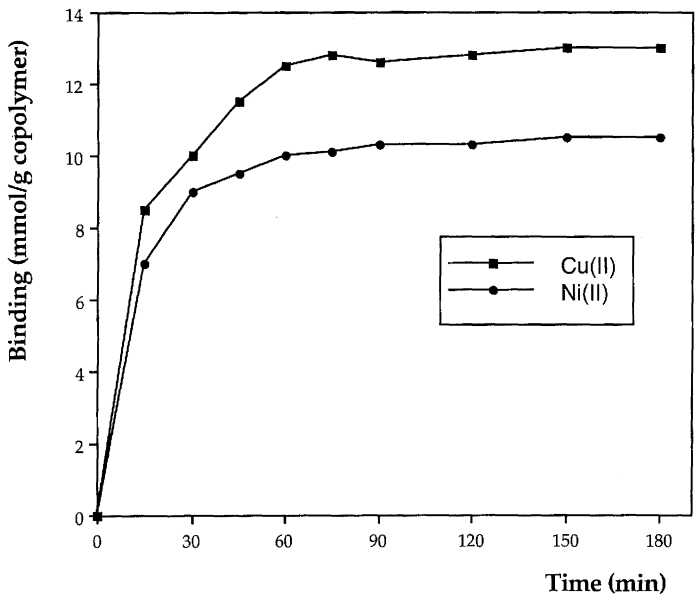

Figure 7. Binding of Cu(II) and Ni(II) by Poly(VI/NIPAM) as a function of time. The binding of metal ions to copolymer solution was carried out by adding excess of metal salts (1 ml each of 0.2 M $CuSo_4$ and 0.2 M $NiSo_4$, added separately to 4 ml of 1% copolymer solution, pH 6.0) and incubated at room temperature (20°C) for different time periods with shaking. The metal copolymer complexes were three times precipitated and dissolved with 0.5 M NaCl to wash out unbound metal ions.

depend on the vinylyimidazole content in the copolymer and adsorption pH. Such studies have been extensively carried out by other workers with metal binding properties of poly(N-vinylimidazole) [18].

Application of Cu(II) Loaded Poly(VI/NIPAM) in Protein Purification

The efficient precipitation of such copolymers using moderate concentration of salt at mild temperatures makes them favorable for application in metal chelate affinity precipitation of proteins. With Cu(II) loaded copolymer of VI and NIPAM, we have been successful in developing metal chelate affinity precipitation protocols for purifying some target proteins (Table 1).

A Kunitz type of soybean trypsin inhibitor was selectively precipitated from crude extract of soybean meal which constitutes around 46% of the trypsin inhibitory activity. The remaining activity was contributed by another class of trypsin inhibitor [19], which remained unbound in the supernatant. The bound inhibitor could be recovered with a yield of 92% in a significantly purified form [9]. When the same trypsin inhibitor was precipitated using Cu(II) loaded copolymer of VI with vinylcaprolactam, lower precipitation efficiency of the inhibitor was observed. This was primarily because of the incomplete precipitation of the vinylcaprolactam copolymer [9].

Similarly, we have been able to purify a single family of α-amylase inhibitors from wheat meal and also could separate two α-amylase inhibitors (I-1 and I-2) from seeds of ragi (Indian finger millet, *Eleusine coracana*). Cu(II)-

TABLE 1. Purification of Proteins by Metal Chelate Affinity Precipitation Using Cu(II) Loaded Thermosensitive Copolymer of Poly(VI/NIPAM) [13]

Target protein	purification	yield factor (%)
Kunitz trypsin inhibitor from soybean	3	92
α-Amylase inhibitor from wheat meal	4	89
α-Amylase/trypsin inhibitor from ragi seeds	13	84
α-Amylase inhibitor from ragi seeds	4	85

poly(VI/NIPAM) precipitated around 91% of α-amylase inhibitor activity from wheat meal and the protein inhibitor was recovered from the polymer with 89% yield and about 4-fold purification. In another case, a bifunctional inhibitor (I-1) of α-amylase and trypsin containg His and Trp residues on the surface of the protein, could be selectively precipitated by Cu(II)-poly(VI/NIPAM) from ragi extract [20]. These residues offer relevant groups for metal binding and thus could bind well to the Cu(II) chelated copolymer. The inhibitor was purified 13-fold with a yield of 84%. On the other hand, α-amylase inhibitor I-2, which is devoid of any such groups, remained unprecipitated in the supernatant. This single step separation method offers significant advantages over the multichromatographic purification protocol traditionally used for the purification of these two proteins [21]. The binding of the protein to the metal chelated copolymer was shown to be strongly pH dependent. Optimum binding could occur in the pH range of 6.0-7.0. It is known that in this pH range, imidazole groups in histidine residues remain fully unprotonated and thus coordinate well with the metal ion [22, 23].

The significant advantage using such polymer systems in affinity precipitation has been that the recovered polymer could be reused a number of times without impairing the efficiency of the polymer. The Cu(II)-poly(VI/NIPAM) recovered after first use of affinity precipitation of the α-amylase inhibitor still precipitated the protein with about 50% efficiency upto three cycles of reuse. The repeated use of the polymer did not affect the recovery of the protein from the polymer [10]. Marginal decreases in the precipitation efficiency after each reuse cycle were explained by the fact that some metal ions were stripped off of the polymer during the elution step of each recycle. However, recharging the polymer with Cu(II) after every reuse, led to higher precipitation efficiency (Figure 8).

CONCLUSION

Metal chelate interactions constitute a potentially very attractive mode of operation when designing protein purification protocols. The interaction is less sensitive to nonspecific interactions than conventionally used affinity interactions. This fact is especially important when dealing with affinity precipitation, since there it is more tedious to deal with co-precipitated irrelevent proteins as compared to chromatographic procedures.

The flexibility of the poly(VI/NIPAM) soluble-insoluble copolymer facilitates the formation of multipoint interactions, thereby improving the sepa-

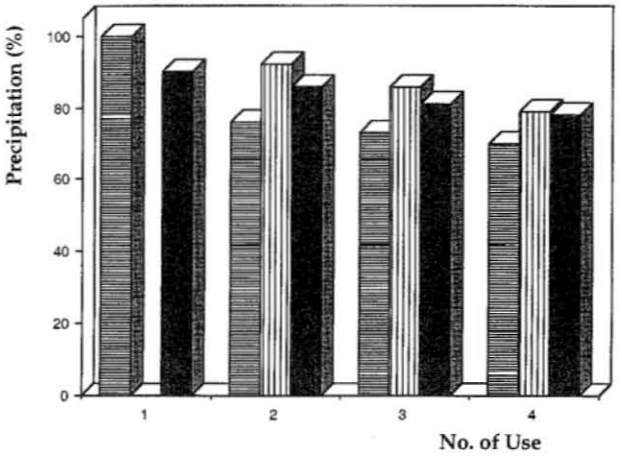

Figure 8. Recycling of the polymer after reloading with the same amount of Cu(II) lost in each recycle use. (▤) Cu(II) concentration on the polymer initially and after each recycle; (▥) Cu(II) concentration after reloading Cu(II) in each cycle and (■) precipitation of α-amylase inhibitor.

ration power of the system based on ligands with weak binding in monomeric form.

ACKNOWLEDGEMENT

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REFERENCES

[1] B. George, V. N. Rajasekharan Pillai, and B. Mathew, *J. Macromol. Sci., Pure & Appl. Chem., A35,* 495 (1998).

[2] J. Porath, *Protein Expr. Purif., 3,* 263 (1992).

[3] I. Yu. Galaev, M. N. Gupta, and B. Mattiasson, *CHEMTEC, Dec.,* 19 (1996).

[4] G. Chen and A. S. Hoffman, *Macromol. Rapid Commun., 16,* 175 (1995).

[5] C. Boutris, E. G. Chaltzi, and C. Kiparissides, *Polymer, 38,* 2567 (1997).
[6] H. G. Schild, *Prog. Polym. Sci., 17,* 163 (1992).
[7] R. Langer, *Nature, 392,* 5 (1998).
[8] M. N. Gupta and B. Mattiasson, in: *Highly Selective Separations in Biotechnology,* G. Street, Ed., Blackie Academic & Professional, London, 1994, pp. 7 -33.
[9] I. Yu. Galaev, A. Kumar, R. Agarwal, M. N. Gupta, and B. Mattiasson, *Appl. Biochem. Biotechnol., 68,* 121 (1997).
[10] A. Kumar, I. Yu. Galaev, and B. Mattiasson, *Biotechnol. Bioeng., 59,* 695 (1998).
[11] B. Mattiasson, A. Kumar, and I. Yu. Galaev, *J. Mol. Recog.* (1998) in press.
[12] J. Porath, J. Carlsson, J. Olsson, and G. Belfrage, *Nature, 258,* 598 (1975).
[13] A. Kumar, I. Yu. Galaev, and B. Mattiasson, *Bioseparation* (1998) in press.
[14] D. Guoqiang, R. Batra, R. Kaul, M. N. Gupta, and B. Mattiasson, *Biposeparation, 5,* 339 (1995).
[15] K. J. Liu and H. P. Gregor, *J. Phys. Chem., 69,* 1252 (1965).
[16] R. J. Todd, R. D. Johnson, and F. H. Arnold, *J. Chromatogr., 662,* 13 (1994).
[17] D. H. Gold and H. P. Gregor, *J. Phys. Chem., 64,* 1464 (1960).
[18] B. L. Rivas, H. A. Maturana, M. J. Molina, M. R. Gomez-Anton, and I. F. Pierola, *J. Appl. Poly. Sci., 67,* 1109 (1998).
[19] V. Fratalli and R. F. Steiner, *Biochemistry, 7,* 521 (1968).
[20] A. Kumar, I. Yu. Galaev, and B. Mattiasson, *Bioseparation* (1998) in press.
[21] B. Shivaraj and T. N. Pattabiraman, *Biochem. J., 193,* 29 (1981).
[22] E. Sulkowski, *Trends Biotechnol., 3,* 1 (1985).
[23] F. H. Arnold, *Bio/Technology, 9,* 151 (1991).