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Imidazole—A New Ligand for Metal Affinity Precipitation

Precipitation of Kunitz Soybean Trypsin Inhibitor Using Cu(II)-Loaded Copolymers of 1-Vinylimidazole with *N*-Vinylcaprolactam or *N*-Isopropylacrylamide

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ABSTRACT

Kunitz soybean trypsin inhibitor (STI) was specifically coprecipitated during precipitation of Cu(II)-loaded copolymers induced by increase in temperature and ionic strength. The copolymers used consisted of 1-vinylimidazole and *N*-vinylcaprolactam or *N*-isopropylacrylamide. The elution of STI was achieved by solubilization of the STI-Cu(II)-polymer complex in the presence of an excess of the competing ligand, imidazole, and a subsequent precipitation of the polymer with STI remaining free in solution in a purified form as judged by Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To the best of our knowledge this is the first reported successful metal affinity precipitation of protein in a heterobifunctional format.

Index Entries: Immobilized metal affinity precipitation; Kunitz soybean inhibitor; poly(*N*-isopropylacrylamide).

Abbreviations: IDA, iminodiacetic acid; IMAC, immobilized metal affinity chromatography; VI, 1-vinylimidazole; poly-VI, poly(1-vinylimi-

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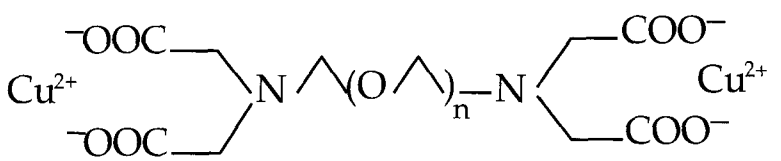
dazole); VCL, *N*-vinyl caprolactam; poly-VCL, poly(*N*-vinyl caprolactam); NIPAM, *N*-isopropylacrylamide; poly-NIPAM, poly(*N*-isopropylacrylamide); poly-VI-VCL, copolymer of VI with VCL; poly-VI-NIPAM, copolymer of VI with NIPAM; BSA, bovine serum albumin; IgG, immunoglobulin gamma; STI, Kunitz soybean trypsin inhibitor; AIBN, 2,2'-azobisisobutyronitrile; TEMED, tetraethylene methylenediamine.

INTRODUCTION

Isolation and purification of proteins still presents a challenge despite massive efforts invested and significant achievements in the field. Several new techniques have emerged recently, immobilized metal affinity chromatography (IMAC) (for review see refs. *1* and *2*) and affinity precipitation (for review see ref. *3*) among them. IMAC proved successful in purification of proteins with one or preferably several adjacent histidine residues located at the surface of the protein molecule. Imidazole moieties of these residues form a complex with a metal ion, most frequently Cu(II)-ion. The latter is fixed on a solid support by polydentate ligands like iminodiacetic acid (IDA), nitrilotriacetic acid, or tris(carboxymethyl)ethylene-diamine *(4)*. Thus the protein of interest binds to the column, whereas the other proteins with no surface histidines pass through. The bound protein can be eluted by increasing concentration of imidazole, a competing ligand for Cu(II)-ions on the support.

Affinity precipitation exploits ligands coupled to a water-soluble polymer. The ligand–polymer conjugate forms a complex with the target protein. This complex may be insoluble as it is (homobifunctional format of affinity precipitation) or phase separation of the complex may be triggered by small changes in the environment (pH, temperature, ionic strength, addition of some reagents) resulting in transition of the polymer backbone into an insoluble state (heterobifunctional format of affinity precipitation). Various ligands like triazine dyes, carbohydrates, protease inhibitors, antibodies, and nucleotides were successfully used for affinity precipitation in both formats *(3)*.

The application of immobilized metal affinity ligands in affinity precipitation is limited to only two examples. Human hemoglobin and sperm whale hemoglobin are quantitatively precipitated in model experiments with *bis*-copper chelates (Eq. 1) in a homobifunctional format *(5)*:



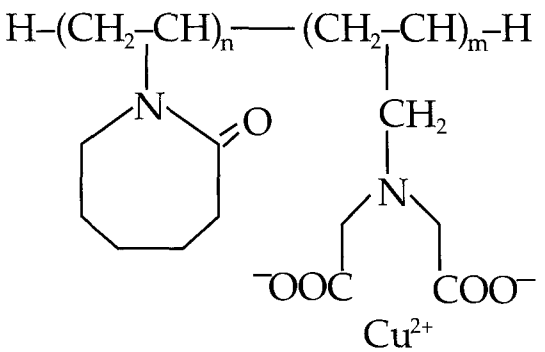
(1)

No data are presented on protein recovery from the precipitate.

Bis-zinc complex of ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetra-acetic acid (EGTA) that can be regarded as Eq. 1 with $n = 2$ was used for affinity precipitation and purification of genetically engineered carboxypeptidase A, containing a pentahistidine-affinity tail. The enzyme was purified 11-fold with the recovery in the precipitation step at least 90%, giving a final yield of 80% *(6)*.

The affinity of copper ions for histidine residues was utilized to precipitate specifically Concanavalin A, the purification fold was 4.5 *(7)*.

The heterobifunctional format was used in affinity precipitation of porcine muscle lactate dehydrogenase using thermoreactive polymer, poly(*N*-vinylcaprolactam) (poly-VCL) containing IDA-ligands Eq. 2 *(8)*. The thermoprecipitation with Cu-IDA-poly-VCL conjugate resulted in 35–45% decrease of enzyme activity in the supernatant, whereas the precipitate solubilization in the presence of the excess IDA recovered only 3–5% activity. The results were explained by the stripping off of Cu(II)-ions from Cu-IDA-poly-VCL by the enzyme. The stripped Cu(II)-ions quickly inactivated lactate dehydrogenase.



(2)

This article presents a study of affinity precipitation of Kunitz soybean trypsin inhibitor (STI) using copolymers of vinylimidazole (VI) and VCL (poly-VI-VCL) or *N*-isopropylacrylamide (poly-VI-NIPAM).

MATERIALS AND METHODS

Reagents

N-vinylcaprolactam (VCL), 1-vinylimidazole (VI) and *N*-isopropylacrylamide (NIPAM) were purchased from Aldrich (Steinheim, Germany). 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Polysciences (Eppelheim, Germany) and recrystallized with ethanol. Electrophoresis-grade ammonium persulfate and tetraethylene methylenediamine (TEMED) were purchased from Bio-Rad (Solna, Sweden). All other chemicals were from Sigma Chemical (St. Louis, MO).

Synthesis of Copolymers

Copolymer of VI with VCL (poly-VI-VCL) was synthesized by radical polymerization. VCL (2 g) and VI (0.1 mL) were dissolved in 10 mL of iso-propanol, the reaction mixture was flushed with nitrogen for 30 min. AIBN (5 mg) was added and the reaction mixture was incubated in a sealed vessel overnight at 70°C. Poly-VI-VCL was precipitated in 200 mL diethyl ether, dissolved in 20 mL iso-propanol, precipitated in 500 mL diethyl ether and dried on air giving 0.9 g of copolymer.

Copolymer of VI with NIPAM (poly-VI-NIPAM) was synthesized by radical polymerization. NIPAM (3 g) and VI (0.24 μL) were dissolved in 30 mL water, degassed under vacuum for 15 min. TEMED (20 μL) and 10% ammonium persulfate (0.2 mL) were added and the reaction was allowed to proceed overnight at room temperature. The copolymer was purified by thermoprecipitation at 50°C in the presence of 0.6 *M* NaCl, separation of the precipitate by centrifugation 10 min at 13,000*g* and dissolution of the precipitate in water at room temperature. The procedure was repeated twice. Finally the copolymer was dissolved in water to give 1% (w/w, dry weight) solution.

Cu(II)-loading

The Cu(II)-loading was carried out by addition of excess of copper sulfate (5 mL of 0.1 *M* $CuSO_4$ to 20 mL of 1% copolymer solutions) and three times reprecipitation and dissolution of copolymers to wash out unbound Cu(II)-ions. The thermoprecipitation of poly-VI-NIPAM and poly-VI-VCL was carried out as described previously. Finally the pH value of the copolymer solutions was adjusted to pH 7.0.

Affinity Precipitation

STI crude extract was prepared according to ref. *9* the pH was adjusted to pH 7.0, and the extract was dialyzed overnight against distilled water and clarified by centrifugation. Extract (1 mL) was added to 5 mL of 1% Cu(II)-poly-VI-NIPAM and the precipitation was carried out by addition of 1.5 mL 3.0 *M* NaCl. The precipitate was washed by dispersing in 5 mL 0.6 *M* NaCl and centrifuged. The elution was achieved by solubilization of the precipitate in 2 mL of 200 m*M* imidazole buffer pH 7.0 and the polymer was separated from STI by precipitation initiated by addition of solid NaCl up to a final concentration of 0.6 *M*. The copolymer precipitate was separated by centrifugation and washed by dispersing in 2 mL 200 m*M* imidazole buffer pH 7.0 containing 0.6 *M* NaCl.

Affinity precipitation of pure soybean trypsin inhibitor (STI) was carried out by addition of 3.0 *M* NaCl to the STI/Cu(II)-loaded copolymer mixture to yield up to 0.6 *M* NaCl final concentration. The temperature was 32°C in the case of Cu(II)-poly-VI-NIPAM and 50°C in the case of Cu(II)-

poly-VI-VCL. The precipitates were separated by centrifugation for 10 min at 13,000*g*. The precipitates were solubilized in 50 m*M* imidazole buffer pH 7.0.

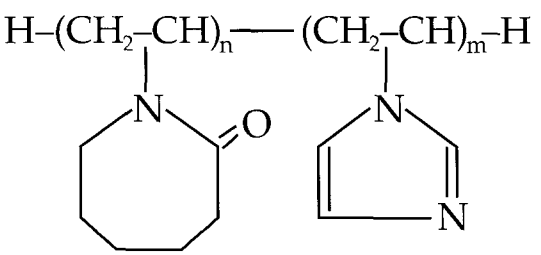
Analytical Procedures

STI activity was measured as a degree of inhibition of trypsin activity, the latter assayed according to ref. *10* using *N*-benzoyl-D,L-arginine-*p*-nitroanilide as a substrate, one unit of STI activity was determined as inhibiting 2 mg of trypsin (type IX, activity 16,000 BAEE units per mg protein, Sigma Chemical). In a separate experiment it was shown that poly-VI-VCL, poly-VI-NIPAM, Cu(II)-poly-VI-VCL, and Cu(II)-polyVI-NIPAM had no effect on trypsin activity. Protein concentration was determined according to ref. *11* using BSA as standard. The polymer spectra were recorded on DU-640 spectrophotometer (Beckman, Fullerton, CA) using water as a reference. The precipitation curves were obtained by measuring optical density of 0.1% (w/w) polymer solutions at 470 nm at different temperatures in thermostated cuvet. The polymer solutions were equilibrated at a given temperature for 5 min.

SDS-polyacrylamide gel electrophoresis with 12% gel was performed according to ref. *12*.

RESULTS AND DISCUSSION

Poly-VCL and poly-NIPAM are well-known thermoreactive polymers that precipitate from aqueous solutions on heating. Poly-VI-VCL Eq. 3 and poly-VI-NIPAM Eq. 4 were synthesized by radical polymerization procedures in organic and aqueous media, respectively.



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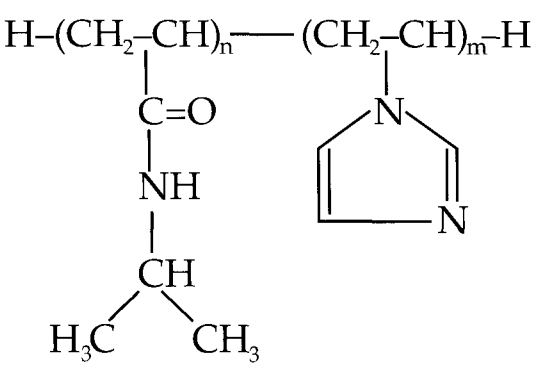

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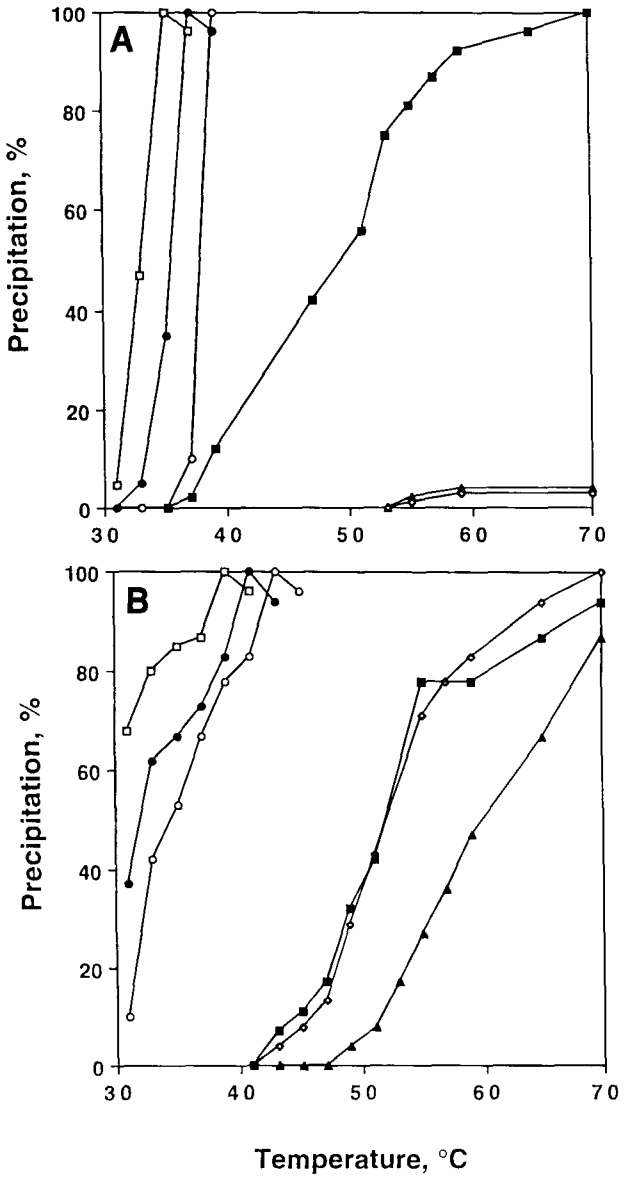

Fig. 1. Precipitation curves of poly-VI-NIPAM **(A)** and poly-VI-VCL **(B)** at pH 4.0 (▲), 6.0 (◇), and 8.0 (■), and in the presence of 0.1 (○), 0.2 (●), and 0.5 (□) M NaCl at pH 6.0. Turbidity was read at 470 nm. The maximum turbidity obtained was taken as 100% precipitation. In this case, 100% precipitation implies total precipitation of the polymer.

Both unmodified poly-VCL and poly-NIPAM precipitate from aqueous solutions at 30–35°C owing to the progressive increase in intra- and intermolecular hydrophobic interactions with elevating temperature *(13,14)*. The incorporation of relatively hydrophilic imidazole moieties into the polymer molecule hindered these hydrophobic interactions and resulted in a drastic increase in the precipitation temperature (Fig. 1). The

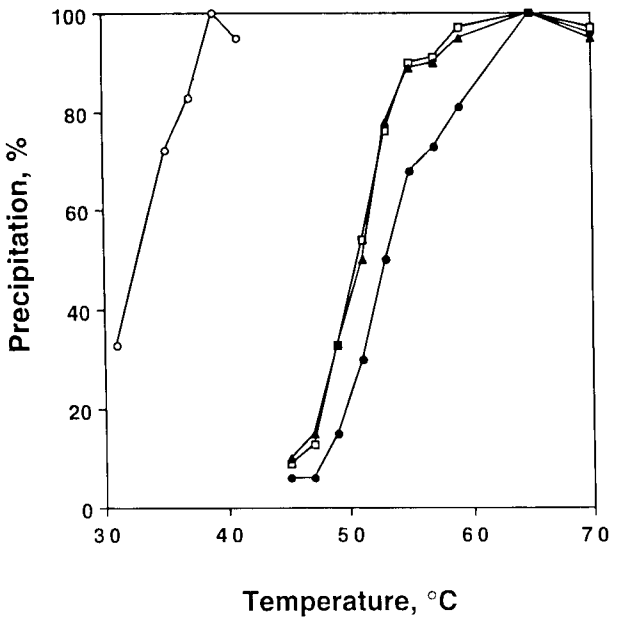

Fig. 2. Precipitation curves of Cu(II)-poly-VI-VCL at pH 4.0 (●), 6.0 (□), and 8.0 (▲) and in the presence of 0.1 *M* NaCl (○) at pH 6.0. Turbidity was read at 470 nm. The maximum turbidity obtained was taken as 100% precipitation. In this case 100% precipitation does not mean total precipitation of the polymer.

effect was more pronounced at lower pH values in which imidazole moieties were protonated and hence rendered more hydrophilicity to the polymer molecule. Poly-VI-NIPAM, for instance, did not precipitate at all up to 70°C at pH 4.0 and 6.0 (Fig. 1A). The effect of pH was less expressed in the case of poly-VI-VCL (Fig. 1B). The increase in ionic strength promoted precipitation of both copolymers.

When loaded with Cu(II), poly-VI-NIPAM did not precipitate at all on heating up to 70°C, while precipitation of poly-VI-VCL was only slightly affected by Cu(II)-loading (Fig. 2). The increase in ionic strength has a dramatic effect on precipitation of Cu(II)-loaded copolymers, especially Cu(II)-poly-VI-NIPAM. In the presence of 0.1 *M* NaCl at 32°C the pellet of flocculated material was immediately formed with some residual turbidity of the supernatant. When NaCl was added up to a final concentration 0.5 *M*, all the polymer was instantaneously precipitated and flocculated in a clump, the remaining solution being transparent. The flocculation took place also on addition of 0.2 and 0.5 *M* NaCl to Cu(II)-poly-VI-VCL, making it impossible to obtain precipitation curves at these conditions. The efficient precipitation of Cu(II)-poly-VI-NIPAM by high salt concentrations at mild temperature is very convenient for metal affinity precipitation. High salt concentration does not interfere with protein-metal ion-chelate interaction *(15)* and on the other hand it reduces the possibility of non-

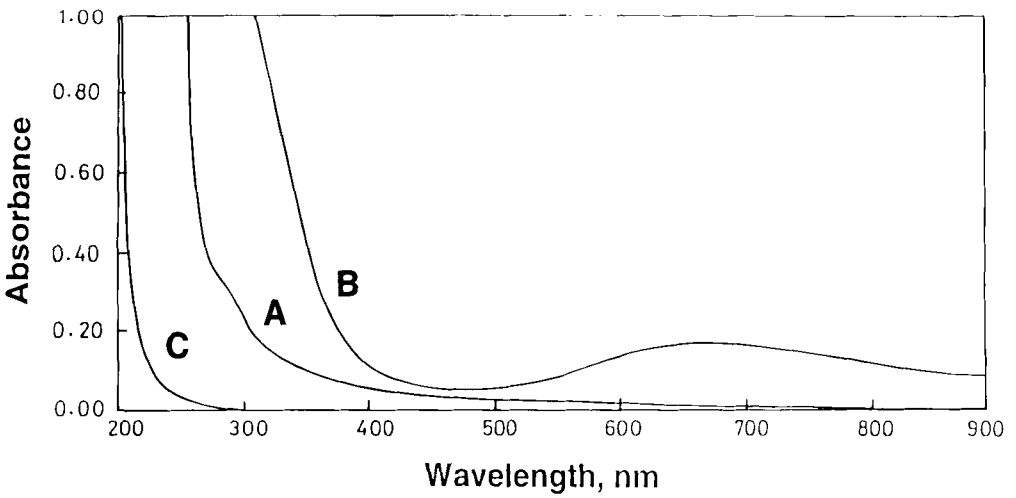

Fig. 3. Spectra of 1% (w/v) poly-VI-NIPAM **(A)**, 1% (w/v) Cu(II)-poly-VI-NIPAM **(B)** and of the supernatant after precipitation of the Cu(II)-poly-VI-NIPAM (**C**). Experimental details are given in the text.

specific binding of foreign proteins to the polymer both in solution and when precipitated.

Figure 3 presents spectra of poly-VI-NIPAM, Cu(II)-poly-VI-NIPAM and the supernatant after polymer precipitation with 0.6 *M* NaCl. It is obvious that only traces of polymer remained in solution after precipitation of Cu(II)-poly-VI-NIPAM with 0.6 *M* NaCl at 32°C.

Figure 4 presents the efficiency of precipitation of pure STI with Cu(II)-poly-VI-NIPAM and Cu(II)-poly-VI-VCL. STI was quantitatively precipitated with high enough concentration of Cu(II)-poly-VI-NIPAM. Some decrease in recovery of the target protein was displayed with high copolymer concentrations as it was observed also in affinity precipitation using pH-sensitive copolymer of methyl methacrylate and methacrylic acid, Eudragit S-100 *(16)*. The STI precipitation with Cu(II)-poly-VI-VCL was at maximum 83%. Most probably incomplete STI precipitation was because of the incomplete precipitation of the Cu(II)-poly-VI-VCL at these conditions.

Thus, Cu(II)-poly-VI-NIPAM was a more promising polymer for metal affinity precipitation than Cu-poly-VI-VCL. Quantitative precipitation of STI with Cu(II)-poly-VI-NIPAM proceeded at mild conditions (pH 7.0, 32°C, 0.6 M NaCl) and STI was eluted from the precipitate with more than 95% recovery. This polymer, Cu(II)-poly-VI-NIPAM, was chosen for further studies of metal affinity precipitation of STI from crude soybean extract.

Only 46% of trypsin-inhibiting activity was precipitated from crude extract with the excess of Cu(II)-poly-VI-NIPAM (160 mg of polymer per mg of protein). Soybean extract contains different trypsin inhibitors *(9)*. The metal-affinity technique presumably separates only Kunitz STI. The part of total trypsin-inhibiting activity precipitated with Cu(II)-poly-VI-

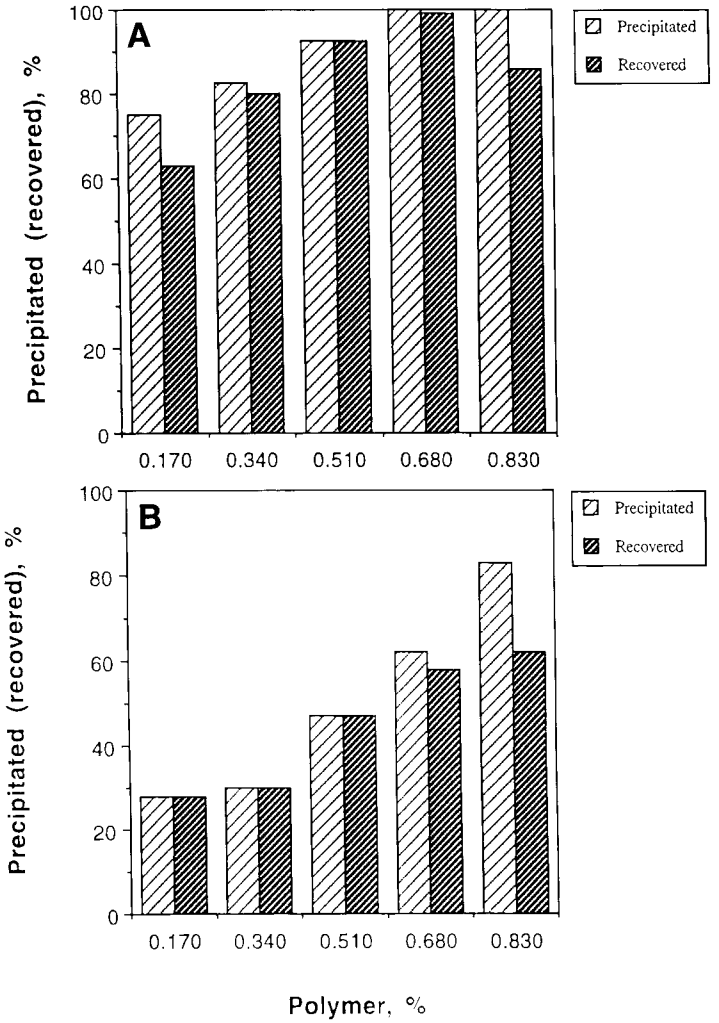

Fig. 4. Precipitation and recovery of STI after metal affinity precipitation with Cu(II)-poly-VI-NIPAM **(A)** and Cu(II)-poly-VI-VCL **(B)**. Experimental details are given in the text.

NIPAM was in a good agreement with the literature data that about 40% (exact value varies depending upon genetic variant, [*17*]) of the total trypsin-inhibitory activity is owing to Kunitz-type inhibitor *(18)*.

The crude STI extract was prepared from defatted soybean meal, a very cheap product. It was reasonable to carry out metal-affinity precipitation in overload conditions of crude extract. The results are presented in Table 1. The precipitation of about one third of STI-activity presumably represents about 60% precipitation of the Kunitz-type inhibitor. The washing of the precipitate with 0.6 *M* NaCl removed loosely bound activity (about 6% of total activity). About 82% of total bound activity could be recovered by dissolving the precipitate in imidazole-containing buffer and

Table 1
Affinity Precipitation of STI from Crude Extract

	Volume mL	Trypsin-inhibiting activity, U/mL	Total trypsin-inhibiting activity, U	Protein concentration, mg/mL	Total protein, mg
Binding stage					2.0
Initial	7.5	0.47	3.5	0.27	
Supernatant after polymer precipitation	7.0	0.33	2.3	0.25	1.7
Supernatant after first washing of the precipitate	5	0.04	0.2	0.002	0.01
Elution stage					
Supernatant after polymer precipitation	2	0.41	0.82	0.17	0.34[a]
Supernatant after washing of precipitate with elution buffer	2	0.05	0.1	0.003	0.006

[a] The measured amount of eluted protein was slightly higher than the amount of bound protein due to some interference of Cu(II) ions leached from the polymer by elution buffer containing imidazole.

reprecipitating the Cu-loaded polymer alone with 0.6 *M* NaCl. Another 10% of bound activity (3% of total activity) can be further recovered by simply washing the precipitate with imidazole-containing buffer. Thus, Kunitz inhibitor bound to the Cu-loaded polymer could be recovered with a yield of 92% in a significantly purified form as judged by SDS-PAGE (Fig. 5).

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 *(19)*. 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, *(20)*]. On the other hand, when the Cu(II)-ion forms a complex with four imidazole ligands the combined binding constant log K = log K_1 + log K_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 = 10.64–14.72 *(19,21)* and comparable with the binding of tridentate IDA

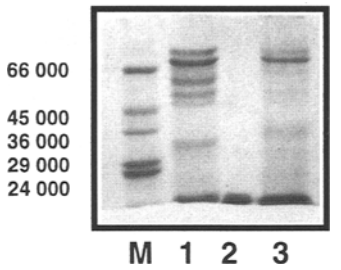

Fig. 5. SDS-PAGE pattern. Lane M: marker proteins, Lane 1: crude extract, Lane 2: commercially available purified soybean trypsin inhibitor (Sigma), Lane 3: soybean trypsin inhibitor isolated by metal-affinity precipitation.

ligand. Thus the flexible polymer like poly-VI can adopt in solution a conformation when four imidazole ligands are close enough to form a complex with the same Cu(II)-ion providing significant strength of interaction.

Coupling of imidazole ligands to a solid support makes the proper orientation of the ligands forming a complex with the same Cu(II)-ion unlikely. Predominantly 1:1 complexes are formed *(22)*. Thus, imidazole ligands are spatially separated on a solid support and bind metal ions too weakly to be used in IMAC. Nevertheless, such matrices proved to be efficient for purification of gamma immunoglobulin (IgG). IgG is bound via an ion-pairing mechanism with the involvement of hydrogen binding *(23,24)*.

To overcome the rigidity of the support and allow several imidazole ligands to interact with the same Cu(II)-ion, silica surface was covered with the flexible polymer, poly-VI. Silica-poly-VI-Cu(II) supports bind bovine serum albumin (BSA) which is eluted by increasing imidazole concentration *(25)*. This support was used for the fractionation of the three main genetic variants of desialylated human α_1-acid glycoprotein but the chromatographic behaviour was complicated by ionic and hydrophobic interactions between the proteins and the surrounding of the metal ion *(26)*.

Another way to tackle the problem is to immobilize on the support a prearranged structure, containing several adjacent imidazole ligands, for instance peptide (Gly-His-His-Pro-His)n-Gly, where n = 1–3. The immobilized 11-residue peptide was loaded with Cu(II)-ions and used to demonstrate selective adsorption and isolation of proteins from human plasma *(27, 28)*.

The successful use of imidazole ligands in metal-affinity precipitation contrary to IMAC is, in our opinion, explained by the flexibility of the water-soluble polymer as compared to the rigidity of the IMAC-matrix. Several imidazole ligands on a polymer molecule in solution can come close enough to interact with the same Cu(II)-ion and thus provide sufficient strength of polymer–Cu(II) interactions. To the best of our knowledge, this

is the first reported use of imidazole as a ligand for metal-affinity precipitation and the first reported use of metal-affinity precipitation in a heterobifunctional format for purification of a target protein from a crude extract.

Affinity precipitation procedures are one-stage procedures in contrast to chromatographic procedures. It has been claimed that the resolving power using affinity chromatography is far higher than what is obtainable in affinity precipitation when using weak ligands *(8)*. However, this statement may not be true when multipoint of attachment is a prerequisite for the protein retention. In this case, the flexibility of the soluble polymer makes it possible prior to precipitation to form strong multivalent-affinity complexes. Recently emerged technique, tentacle biochromatography, with its flexible polymer tails bearing protein binding ligands, may provide an alternative design of multipoint protein interaction with weak ligands *(29)*.

Affinity precipitation is a promising technique that can be easily scaled up, because it is based on a separation technology, centrifugation, that is operated on a large industrial scale already. The use of immobilized metal-affinity ligands and imidazole ligands in particular gives a new dimension to affinity precipitation of proteins.

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