A New Metal Chelate Sorbent for Glucose Oxidase: Cu(II)- and Co(II)-Chelated Poly(*N*-vinylimidazole) Gels

BEKIR SALIH, NURSEL PEKEL, OLGUN GÜVEN

Department of Chemistry, Hacettepe University, 06532 Ankara, Turkey

Received 12 September 2000; accepted 10 December 2000

ABSTRACT: Poly(*N*-vinylimidazole) (PVIm) gels were prepared by irradiating a binary mixture of *N*-vinylimidazole (VIm)–water in a ^{60}Co-γ source having 4.5 kGy/h dose rate. In the glucose oxidase (GOx) adsorption studies, affinity gels with a swelling ratio of 1100% for PVIm and 40 and 55% for Cu(II)- and Co(II)-chelated PVIm gels, respectively, at pH 6.5 in phosphate buffer were used. FTIR spectra were taken for PVIm and Cu(II)- and Co(II)-chelated PVIm, and glucose oxidase adsorption on these gels, to characterize the nature of the interactions in each species. The results show that PVIm–glucose oxidase interaction is mainly electrostatic and metal ion–chelated PVIm gel–glucose oxidase interaction is of coordinate covalent nature. Cu(II) and Co(II) ions were chelated within the gels via amine groups on the imidazole ring of the gel. Different amounts of Cu(II) and Co(II) ions [maximum 3.64 mmol/g dry gel for Cu(II) and 1.72 mmol/g dry gel for Co(II)] were loaded on the gels by changing the initial concentration of Cu(II) and Co(II) ions at pH 7.0. GOx adsorption on these gels from aqueous solutions containing different amounts of GOx at different pH was investigated in batch reactors. GOx adsorption capacity was further increased when Cu(II) and Co(II) ions were attached [up to 0.53 g GOx/g dry Co(II)-chelated PVIm gels]. More than 90% of the adsorbed GOx was desorbed in 5 h in desorption medium containing 1.0*M* KSCN at pH 7.0 for plain gel and 0.05*M* EDTA at pH 4.9 for metal-chelated gel. Nonspecific glucose oxidase adsorption on/in the metal ion–chelated PVIm gel was investigated using 0.02*M* of phosphate buffer solution. The nonspecific GOx adsorption was determined to be about 18% for PVIm and 8% for the metal ion–chelated PVIm gels. The ionic strength effect was investigated both on PVIm and on the metal ion–chelated PVIm gels for the glucose oxidase adsorption. It was found that ionic strength was more effective on the PVIm gel because of the electrostatic interaction between protonated gel and the deprotonated glucose oxidase side chain. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 82: 446–453, 2001

Key words: glucose oxidase adsorption; affinity gels; poly(*N*-vinylimidazole); metal chelate affinity

INTRODUCTION

Chromatographic separation and purification of biomolecules such as proteins and enzymes need several steps involving methods that select on the basis of molecular size (gel permeation chromatography), electrical charge (ion-exchange chromatography), hydrophobicity (hydrophobic interaction chromatography), or biological recognition (bioaffinity chromatography). The overall procedure will be efficient if the techniques separate according to those properties that best discriminate between the material of interest and the impurities. One of the most important forms of

Correspondence to: B. Salih (bekir@hacettepe.edu.tr).

Journal of Applied Polymer Science, Vol. 82, 446–453 (2001)

chromatography used in biomolecule separations is ion-exchange chromatography.[1] In this technique, the net charge carried by a biomolecule is a function of pH, given the wide range of pH values shown by the ionizable amino acid side chains. However, it is desirable to carry out adsorption at a pH close to neutrality, to prevent denaturation of biomolecules by extremes of pH. The desirable ion exchanger is the strong ion exchanger because they retain their full charge at all biocompatible pH.[2]

The other important separation technique for biomolecules is metal chelate affinity chromatography, which introduces a new possibility for selectively interacting materials on the basis of their affinities for chelated transition metal ions. The separation is based on differential binding abilities of the proteins or enzymes to interact with chelated metal ions to a solid carrier.[3–6] Metal chelate affinity chromatography of proteins, with metal chelate linked to Sepharose, was first introduced by Porath et al.[7] They reported a model system using Zn(II) and Cu(II) columns in tandem for the fractionation of human serum proteins. Subsequent studies have shown the wide applicability of the technique and consistency of the methodology. The plasma proteins α_2-macroglobulin and α_1-proteinase inhibitor, for example, have been purified to homogeneity on zinc chelate columns.[8,9] Metal chelate affinity chromatography has also been used to provide immunologically and physicochemically pure α_2-HS glycoprotein from plasma.[10,11] Plasminogen activators from both normal tissue (human uterus) and human melanoma cells have been isolated by metal chelate affinity chromatography,[12,13] as have nucleoside diphosphatase,[14] human lactoferrin,[15] lectin,[16] interferon,[17] and carboxypeptidase B.[18]

The content and distribution of metal-coordinating residues on the biomolecule surfaces dictate separation in the metal-affinity process. When histidines and cysteine are on the biomolecules, these are the most important metal ion–coordinating ligands at neutral pH. If a sufficiently large number of such ligands are accessible, the biomolecules can be recovered efficiently from crude mixtures. In the biomolecules, the histidine amino acid residue plays an important role for the metal ion coordination.[19–21] On the other hand, a histidine-like synthetic ligand containing an imidazole ring shows similar coordination with different metal ions such as Cu(II), Ni(II), Zn(II), and Co(II).[22] Because of this reason synthetic polymers containing large numbers of imidazole ligands such as PVIm can be used for the separation in the metal-affinity process of biomolecules. When the metal ion–chelated polymeric form was used for the biomolecules, some coordination number of metal ion in the complex structure that was occupied with water molecules could be replaced by the biomolecules in the aqueous medium. In this way biomolecules could be accumulated on the solid form of the metal ion–chelated sorbents. If the metal ion–chelated form of the solid sorbents was used, then both the adsorption capacity and the activity of enzyme were increased.[23]

Poly(*N*-vinylimidazole) and its copolymer, especially with *N*-vinylpyrrolidone, are among the major synthetic polymers for biomedical, pharmaceutical, and industrial applications. These synthetic polymers are rather hydrophilic and vinylimidazole on the polymeric chains provides complexation with the transition metal ions, such as Cu(II), Co(II), Ni(II), and Zn(II), giving transition metal ion complexes in the aqueous solution. In these transition metal ion complexes, some coordination number of metal ions can be occupied with weak ligation of water molecules. Weak ligation of water molecules could be replaced with the other strong ligation of nitrogen-based ligands on the biomolecules, such as proteins, enzymes, and DNA.[24,25]

The purpose of the present study is to prepare an affinity gel containing Cu(II) and Co(II) ions (in chelated form) for metal chelate affinity separation of enzymes. Glucose oxidase (GOx) was selected as a model enzyme. In this report, we present GOx adsorption/desorption properties of Cu(II)- and Co(II)-chelated PVIm gels. In addition, we tried to find the interaction nature of glucose oxidase with the PVIm and metal ion–chelated PVIm gels. FTIR technique was used to characterize the interaction between the gels and enzyme.

EXPERIMENTAL

Materials

Glucose oxidase (lyophilized, Fraction V; Sigma, Poole, UK) was purchased from Sigma Chemical Co. (St. Louis, MO) and used as received. *N*-Vinylimidazole (VIm) was obtained from Merck AG (Darmstadt, Germany) and distilled under reduced pressure in the presence of hydroquinone and stored at 4°C until use. Copper chloride

($CuCl_2$) and cobalt chloride ($CoCl_2$) were purchased from BDH (Poole, UK). All other chemicals were of reagent grade and were purchased from Merck AG.

Methods

Preparation of PVIm Gel

VIm–water mixture (10 mL) containing 8 mL of VIm and 2 mL of water was placed in 3-mm diameter poly(vinyl chloride) (PVC) straws and irradiated in a ^{60}Co-γ source at a fixed dose rate of 4.5 kGy/h. The gel was removed from the straws (cut to 3–4 mm in length), washed several times with distilled water, and then dried under nitrogen atmosphere in a vacuum oven at 60°C. Finally, dried gels were ground and stored until use. Fine particles of hydrogels with 800 μm average size were used for the GOx adsorption. Other details related to the polymerization system and procedure were given elsewhere.[22,26]

Water Content of PVIm Gels. The swelling behavior of PVIm gels was determined in distilled water. Dry gel pieces were placed in distilled water and kept at a constant temperature of 25 ± 0.5°C. Swollen gels were periodically removed and weighed. The water content of the swollen gels was calculated by using the following expression:

$$\text{Swelling ratio } \% = [(W_s - W_o)/W_o] \times 100$$

where W_0 and W_s are weights of gel before and after swelling, respectively.

FTIR Spectra. FTIR spectra of the plain and metal-attached PVIm gels were obtained by using a FTIR spectrophotometer (Nicolet 520 Model FTIR spectrometer; Nicolet Instruments, Madison, WI). Dried PVIm and metal ion–chelated PVIm gels (0.01 g) were thoroughly mixed with KBr (0.1 g) separately and these homogeneous mixtures were pressed to form tablets, after which the spectra were recorded.

Incorporation of Cu(II) and Co(II) Ions to PVIm Gels

Chelates of PVIm gels with Cu(II) and Co(II) ions were prepared as follows: PVIm gels (0.01 g) were mixed with 20 mL of aqueous solutions containing 100–1200 ppm Cu(II) and Co(II) ions, at a constant pH of 6.5 (adjusted with NaOH and HNO_3), which was the optimum pH for Cu(II) and Co(II) chelate formation at room temperature.[26] A 1200-ppm solution of $CuCl_2$ and $CoCl_2$ was used as the source of Cu(II) and Co(II) ions. The flasks were shaken in a thermostatically controlled water bath at 30°C for 2 days (sufficient to attain equilibrium). The concentration of the metal ion in the resulting solutions was determined with an UV-Vis spectrophotometer (Philips PU 8715 Model; UK).

Metal ion leakage from the PVIm gels was investigated in a medium containing NaCl at 0.01 ionic strength and pH in the range 4.0–8.0, and also in a medium containing 1.0*M* of KSCN, at pH 7.0. The gel suspensions were stirred for 24 h at room temperature. After this period, the leakage of metal ions was determined in the supernatant using an atomic absorption spectrophotometer (GBC 932 AA; Australia).

Glucose Oxidase Adsorption

GOx was selected as a model enzyme in this study. GOx adsorption of the plain and Cu(II)- and Co(II)-chelated PVIm gels was studied at various pH values. The pH of the adsorption medium was varied between 3.0 and 8.0 by using different buffer systems (0.02*M* CH_3COONa–CH_3COOH for pH 3.0–6.0, 0.02*M* K_2HPO_4–KH_2PO_4 for pH 7.0 and 8.0). The initial GOx concentration was changed between 0.5–10.0 g/L. In a typical adsorption experiment, GOx was dissolved in 10 mL of 0.01*M* of phosphate buffer solution to which gels were added. The adsorption experiments were carried out for 2 h at 4°C in a water bath. At the end of the equilibrium period (i.e., 90 min), the gels were separated from the solution. The GOx adsorption capacity was determined spectrophotometrically, by measuring the initial and final concentrations of GOx in the adsorption medium at 276 nm, which was the maximum absorption wavelength for GOx.

Glucose Oxidase Desorption

The GOx desorption experiments were performed in a buffer solution containing 1.0*M* of KSCN at pH 7.0 or 0.05*M* EDTA at pH 4.9. The GOx-loaded gels were placed in desorption medium and stirred for 20 h at 4°C in a thermostatically controlled water bath. The final GOx concentration in desorption medium was determined by spectrophotometry. In the case of Cu(II)- and Co(II)-carrying gels, desorption of metal ions was also measured in desorption media by an atomic ab-

sorption spectrometer. It was found that there was slight metal ion leakage in the desorption condition of GOx. The ratio of desorbed metal ion was not more than 1% compared to the adsorbed GOx. The desorption ratio was calculated from the amount of GOx adsorbed on the gels and the amount of GOx desorbed.

RESULTS AND DISCUSSION

Characteristics of PVIm and Metal Ion–Chelated PVIm Gels

Preparation, characterization, and the metal ion adsorption details of PVIm gels were given in our previous work.[27] The gels prepared in this study were highly swellable (swelling ratio of 3000% for the protonated form of PVIm gel at pH 2.5). The gel showed a homogeneous and highly open pore structure, which could possibly have led to high internal surface area (meaning high adsorption capacity) with low diffusional resistance in the matrix. The swelling ratio of Cu(II)- and Co(II)-chelated gels were very low compared to that of the plain PVIm gel. In this case, the metal ion coordinated with four imidazole nitrogen groups, the gel structure shrank, and the swelling ratio reduced, all of which affected the GOx adsorption capacity of the gels. However, GOx adsorption increased when the metal ion–chelated form of the gels was used. This is the case for the metal-chelating capacity of the gels with the GOx on the exterior surface of the compact metal ion–chelated gels. The swelling ratios of the plain and metal ion–chelated PVIm are given as a function of pH in Figure 1.

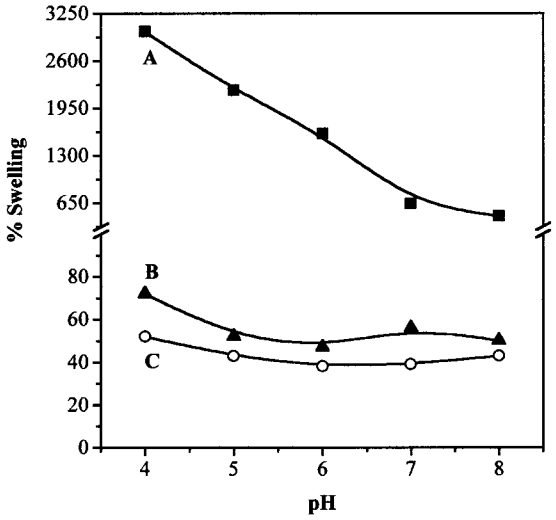

Figure 1 Swelling behavior of PVIm gels at different pH: (A) PVIm; (B) Cu(II)–PVIm; (C) Co(II)–PVIm. Temperature, 25°C; ionic strength, 0.02.

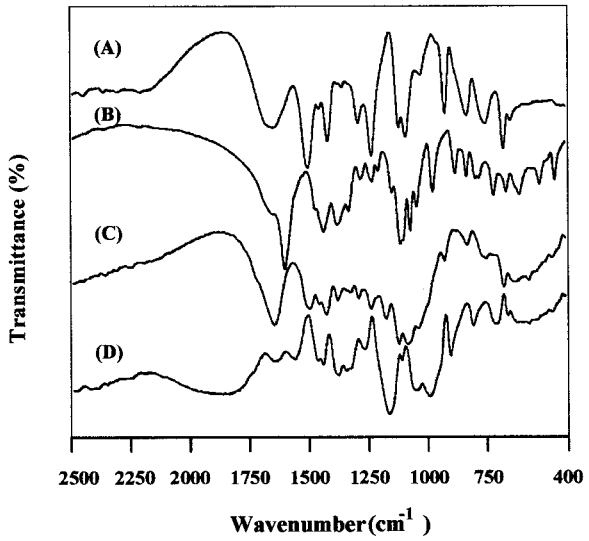

Figure 2 FTIR spectra: (A) PVIm; (B) GOx; (C) PVIm–GOx; (D) difference of (C) and (A).

To investigate the interaction between PVIm gel, metal ion, and GOx, FTIR spectra of different types of gels were recorded. In this part of the work, FTIR spectra of PVIm, GOx, PVIm–GOx, and the difference FTIR spectrum between PVIm–GOx and PVIm were recorded. These FTIR spectra are shown in Figure 2. In the difference spectrum [Fig. 2(D)], the absorption bands around 1600 cm^{-1} and broad absorption band between 850 and 1200 cm^{-1} show the same character with the FTIR spectrum of pure GOx [Fig. 2(B)]. This is the case for the high content of adsorbed GOx on the PVIm gel. The nature of the adsorption was mainly electrostatic because the absorption band around 1650 cm^{-1} was shifted to a lower wavelength and the absorption band at 1510 cm^{-1} disappeared when GOx was loaded onto the PVIm gel [Fig. 2(C)], as compared with the spectrum of the plain PVIm gel [Fig. 2(A)]. The absorption band between 920 and 1200 cm^{-1} was very broad in the spectrum of PVIm–GOx compared to the spectrum of the plain PVIm gel. The broad absorption band of the difference spectrum between 1250 and 1550 cm^{-1} shows the same characteristics with the spectrum of pure GOx. All this information shows that there is a real chemical interaction between PVIm and GOx resulting from the wavelength shift and disappearance on some absorption bands in the PVIm–

GOx spectrum when GOx adsorbed onto PVIm gel. The weak absorption bands between 2100 and 2800 cm^{-1} in the spectra of PVIm and differences between PVIm–GOx and PVIm show the protonated imidazole amine groups on the PVIm gel. This means that the interaction between PVIm and GOx is electrostatic in nature. The weakness of the absorption bands shows the interaction and the electrostatic interaction affected vibration intensity. The same behavior was shown after the metal ion incorporation to the PVIm gel, which is given in Figure 3. In this case the metal ion was first incorporated to the PVIm gel and then GOx was loaded to the metal-chelated PVIm gels. Here the difference FTIR spectrum between PVIm–Cu(II)–GOx and PVIm–Cu(II) spectra [Fig. 3(C) and (A)] showed the same characteristics with the spectrum of pure GOx [Fig. 3(B)]. All changes in the absorption bands and disappearance were similar with the FTIR spectra that were discussed in Figure 2.

Metal Ion Incorporation to the PVIm Gel

Incorporation of Cu(II) and Co(II) to the unprotonated and protonated PVIm gels is shown in Figure 4. The amount of ions adsorbed onto both forms of the PVIm gels for two metal ions increased with the initial concentration of the metal ions in solution. It reached a first plateau value of 2.06 and 0.85 mmol metal ion/g dry gel for Cu(II) and Co(II), respectively, and in the case of unprotonated gel at 400 ppm initial metal ion concentration. After the first plateau value, the amount of adsorbed metal ion increased rapidly and reached 3.64 and 1.72 mmol/g dry gel for Co(II) and Cu(II) at 1200 ppm initial metal ion concentration. In the case of protonated PVIm gel, the amount of adsorbed metal ion is 0.72 and 0.26 mmol/g dry gel for Cu(II) and Co(II), respectively, at 1200 ppm initial concentration of the metal ions. This result shows that complex formation between metal ion and the PVIm gel is less favorable when the gel has positively charged sites.

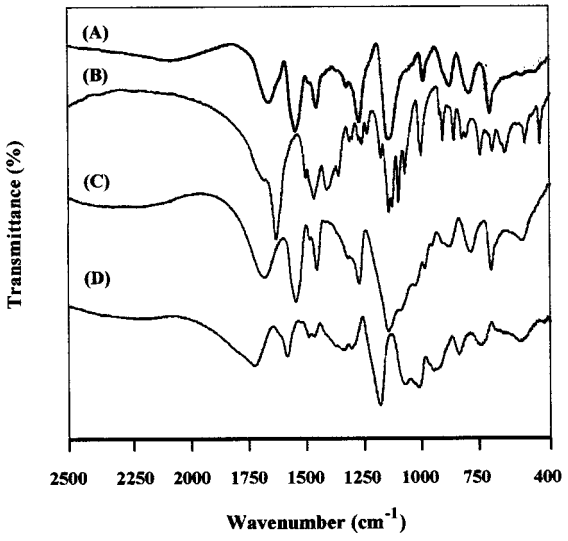

Figure 3 FTIR spectra: (A) PVIm–Cu(II); (B) GOx; (C) PVIm–Cu(II)–GOx; (D) difference of (C) and (A).

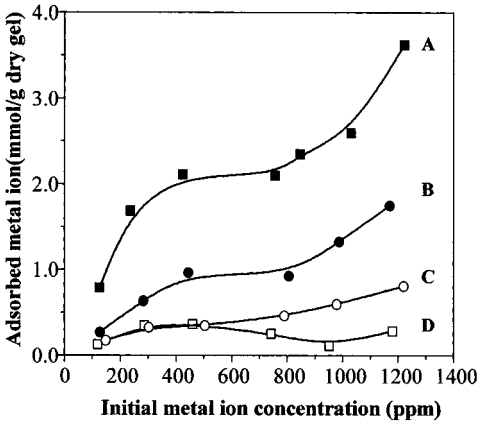

Figure 4 Metal ion adsorption capacity of the PVIm gels: Cu(II) (A) and Co(II) (B) adsorption on the deprotonated PVIm at pH 7.0. Cu(II) (C) and Co(II) (D) adsorption on the protonated PVIm at pH 2.5. Temperature, 25°C.

Glucose Oxidase Adsorption onto PVIm and Metal Ion–Chelated PVIm

pH Effect

Figure 5 shows the effect of pH on GOx adsorption onto PVIm gels. In all cases investigated, the maximum adsorption of GOx was observed at pH 7.0. Significantly lower adsorption capacities were obtained with all gels in more acidic and in more alkaline pH regions. It has been shown that enzymes have no net charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric point.[28] The isoelectric pH of GOx is 4.3. In our case, the maximum adsorption pH was not 4.3, but rather shifted to a higher pH value than the isoelectric pH of GOx. These specific interactions may result both from the ionization states of several groups on both the imidazole functional group and amino acid side chains in GOx structure, and from the conformational state

of glucose GOx at this pH, as usually discussed in the related literature.[29] At pH values higher than the isoelectric pH of GOx, the side chain of GOx is partially deprotonated. This increases the adsorption capacity of GOx on the protonated plain PVIm. In the metal-chelated form of PVIm, GOx might have completed the oxidation state of the metal ion, further increasing the adsorption capacity of GOx.

Adsorption Capacity

Figure 6 shows the effects of initial GOx concentration on adsorption. As presented in this figure, with increasing GOx concentration in solution, the amount of GOx adsorbed by the gel increases very fast at low concentrations, below about 2.0 g/L, then increases less rapidly and approaches saturation for PVIm. It becomes constant when the enzyme concentration is greater than 5 g/L. The GOx adsorption capacities of the PVIm, Cu(II)–PVIm, and Co(II)–PVIm are 228, 343, and 528 mg GOx/g gel, respectively. Chelated Co(II) and Cu(II) ions significantly increased the GOx adsorption capacity of the gel (up to 528 mg GOx/g gel), possibly because of the specific interactions between GOx and metal ions to complete the coordination number of metal ions. It is clear that this increase is attributed to chelate formation between metal ion and GOx molecule, that is, metal ions promote the adsorption of GOx. In the chelated form, the gels are very compact and swellability is very low compared to that of the pristine PVIm gel (Fig. 1). The GOx adsorption capacity for the metal ion–chelated gels is significantly higher than that of the plain gel, in spite of the rigid structure and low swellability of metal ion–chelated gels. In this manner, this chelated form of the gels can be very conveniently used for the column application. This property is not valid for the plain PVIm gels.

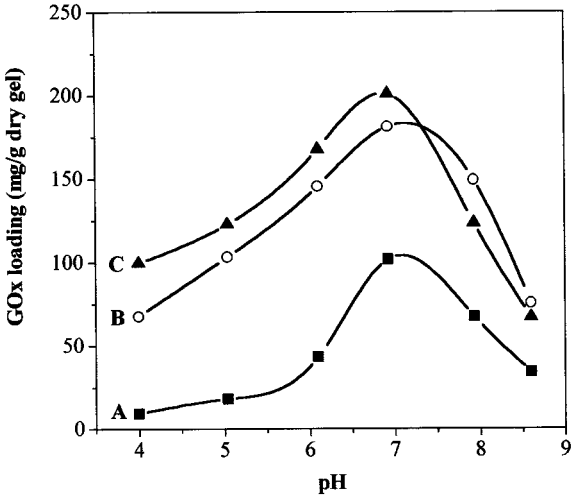

Figure 5 Effects of pH on adsorption of glucose oxidase: (A) PVIm; (B) PVIm–Cu(II); (C) PVIm–Co(II). Adsorption conditions: initial concentration of enzyme, 2 g/L; adsorption time, 2 h; temperature, 4°C. Amount of loaded metal ions: 3.64 and 1.72 mmol/g dry gel for Cu(II) and Co(II), respectively.

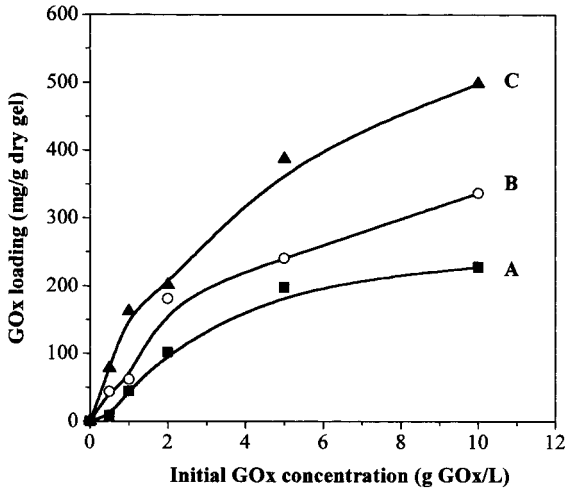

Figure 6 Effect of initial glucose oxidase concentration on glucose oxidase adsorption of the PVIm gels: (A) PVIm; (B) PVIm–Cu(II); (C) PVIm–Co(II). Cu(II) and Co(II) incorporations, 3.64 and 1.72 mmol/g dry gel, respectively; pH 7.0; temperature, 4°C.

Adsorption Kinetics

The adsorption equilibrium time of GOx molecules on the affinity gels was investigated and the results are presented in Figure 7. As seen from Figure 7, higher adsorption rates were observed at the beginning of the adsorption process; then adsorption equilibria were achieved gradually in about 90 min. Adsorption of GOx molecules was very fast, especially when the GOx concentration was very high (data not shown). It has to be noted that the GOx was adsorbed much faster onto the metal ion–chelated affinity gels than onto the plain gel because of the much higher affinity of the metal ions [i.e., Cu(II) and Co(II) ions]. The major functional groups (–NH and –SH) on enzyme contribute toward the interaction with incorporated metal ion that is weakly coordinated with water molecules in aqueous solution.

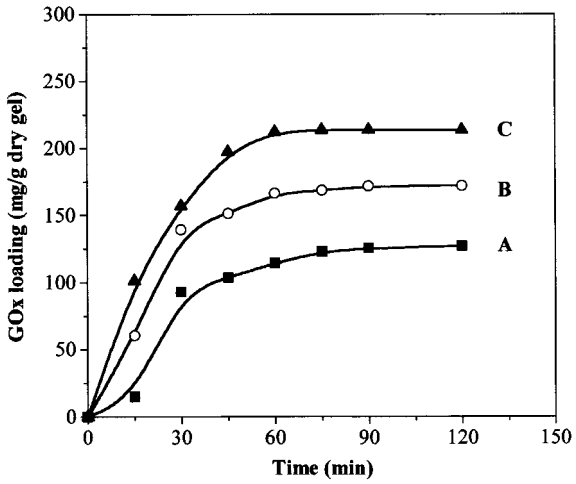

Figure 7 Adsorption kinetics of GOx on PVIm gels: (A) PVIm; (B) PVIm–Cu(II); (C) PVIm–Co(II) in pH 7.0 phosphate buffer. Temperature, 4°C; initial concentration of GOx, 2 g/L.

Glucose Oxidase Desorption from PVIm and Metal Ion–Chelated PVIm

Desorption of the adsorbed GOx from the PVIm and Cu(II)- and Co(II)-chelated PVIm gels was studied in a batch experimental setup. GOx-loaded gels were placed in desorption medium containing 1.0*M* KSCN at pH 7.0 and 0.05*M* EDTA at pH 4.9. The amount of GOx, Cu(II), and Co(II) released in 20 h was determined. The desorption ratios for both GOx and metal ions were calculated by using the following expression:

$$\text{Desorption ratio (\%)} = \frac{\text{Amount of GOx (or metal ions) released}}{\text{Amount of GOx (or metal ions) adsorbed on the gel}} \times 100$$

Table I gives the desorption data. More than 99% of the adsorbed GOx was removed when 1.0*M* of KSCN was used for desorption of GOx from plain PVIm gel. Note that there was no metal ion release in this case, which shows that metal ions are attached to imidazole molecules on the gel surface by strong forces through chelate formation. However, when EDTA was used for desorption, only 11% of GOx was removed from PVIm gel, perhaps because of a salting effect. While under the same desorption conditions, about 100% of the GOx was desorbed from the metal ions carrying PVIm gels. This means that EDTA breaks down the chelates between metal ion and imidazole amine group on the gels, releasing completely adsorbed GOx.

According to the above-described desorption data we concluded that KSCN and EDTA, especially, are suitable desorption agents for PVIm and metal ion–chelated PVIm gels, allowing repeated use of the affinity sorbents developed in this study.

CONCLUSIONS

PVIm gels were prepared by irradiating a binary mixture of *N*-vinylimidazole-water in a ^{60}Co-γ source having 4.5 kGy/h dose rate. Then, Cu(II) and Co(II) ions were incorporated with an imidazole affinity group onto the PVIm gel. The maxi mum amounts of Cu(II) and Co(II) ions chelating on the PVIm gel were obtained at 3.64 and 1.72 mmol metal ions/g dry gels, respectively. FTIR spectra showed that there was a real chemical interaction between PVIm gels and GOx. Moreover, the adsorption capacity of metal ion–chelated PVIm is reasonably high for the purification of biomolecules. The results presented in this communication showed that the GOx adsorption capacity of plain and Co(II)- and Cu(II)-chelated PVIm was 228, 343, and 528 mg/g dry gels. More than 99% of the adsorbed GOx was desorbed in 20 h in the desorption medium containing 1.0*M*

Table I Adsorption–Desorption of GOx and Metal Ions

	Adsorption of Metal Ions			Desorption of GOx (%) in		
Gels	Adsorbed Cu(II) (mmol/g dry gel)	Adsorbed Co(II) (mmol/g dry gel)	Adsorbed GOx (mg/g dry gel)	Phosphate Buffer	KSCN	EDTA
PVIm	—	—	228	18	99	11
PVIm–Cu(II)	3.64	—	343	8	40	100
PVIm–Co(II)	—	1.72	528	8	68	99

KSCN at pH 7.0 for PVIm and 0.05*M* EDTA at pH 4.9 for Cu(II)- and Co(II)-chelated PVIm gels.

REFERENCES

1. Yamamoto, S.; Nakaniski, K.; Matsuno, R. Ion-Exchange Chromatography of Proteins, Vol. 43; Marcel Dekker: New York, 1988.
2. Peterson, E. A.; Sober, H. A. J Am Chem Soc 1980, 117, 211.
3. Porath, J. J Chromatogr 1988, 443, 3.
4. Zeng, C. M.; Liao, J. L.; Nakazato, K.; Hjerten, S.; J Chromatogr A 1996, 753, 227.
5. Yip, T. T.; Nakagawa, Y.; Porath, J. Anal Biochem 1989, 183, 159.
6. Porath, J.; Olin, B. Biochemistry 1983, 22, 1621.
7. Porath, J.; Carlsson, J.; Olsson, I.; Belfrage, G. Nature 1975, 258, 598.
8. Kurecki, T.; Kress, L. F.; Laskowski, M. Anal Biochem 1979, 99, 415.
9. Sottrup–Jensen, L.; Petersen, T. E.; Magnusson, S. FEBS Lett 1980, 121, 275.
10. Lebreton, J. P. FEBS Lett 1977, 80, 351.
11. Lebreton, J. P.; Josiel, F.; Raoult, J. P. J Clin Invest 1979, 64, 1118.
12. Rijken, D. C.; Wijngaards, G.; Jong, M. Z. Biochim Biophys Acta 1979, 580, 140.
13. Rijken, D. C.; Collen, D. J Biol Chem 1981, 256, 7035.
14. Ohkubo, I.; Kondo, T.; Tanoguchi, N. Biochim Biophys Acta 1980, 616, 89.
15. Lönnerdal, B.; Carlsson, J.; Porath, J. FEBS Lett 1977, 75, 89.
16. Borrebaeck, C. A. K.; Lönnerdal, B.; Etzler, M. E. FEBS Lett 1981, 130, 194.
17. Chadha, K. J.; Grob, P. M.; Mikulski, A. J. J Gen Virol 1979, 43, 701.
18. Marquez–Mendez, M. J Biochim Biophys Methods 1992, 24, 51.
19. Masselon, C.; Salih, B.; Zenobi, R. J Am Soc Mass Spectrom 1999, 10, 19.
20. Nelson, R. W.; Hutchens, T. W. Rapid Commun Mass Spectrom 1992, 6, 4.
21. Salih, B.; Masselon, C.; Zenobi, R. J Mass Spectrom 1998, 33, 994.
22. Pekel, N.; Güven, O. Colloid Polym Sci 1999, 277, 570.
23. Arica, M. Y.; Denizli, A.; Salih, B.; Hasırcı, V.; Pişkin, E. J Membr Sci 1996, 129, 65.
24. Gold, D. H.; Gregor, H. P. J Phys Chem 1960, 64, 1464.
25. Kurimura, Y.; Abe, T.; Usui, Y. J Chem Soc Faraday Trans 1994, 90, 3563.
26. Pekel, N. Ph.D. Thesis, Hacettepe University, 1998.
27. Pekel, N.; Güven, O. Colloid Polym Sci 2001, submitted.
28. Andersson, L.; Sulkowski, E.; Porath, J. Bioseparation 1991, 2, 15.
29. Denizli, A.; Salih, B.; Pişkin, E. J Appl Polym Sci 1997, 65, 2085.