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Immobilized Metal Ion Affinity Chromatography of Genetically Engineered Hirudin Variants

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Immobilized metal ion affinity chromatography (IMAC) was used to separate various types of recombinant hirudins from the culture broth. The wild type hirudin exhibited a retention in Cu(II)-chelated affinity chromatgoraphy since it contained a single exposed histidine at position 51. To obtain a stronger retention on an IDA-Cu(II) column, the hirudin variants were genetically engineered to contain one or two histidine (s) more than the wild type. While the affinity of the variants for IDA-Cu(II) ligand increased in comparison to that of the wild type, the antithrombin activities reduced to a certain degree. Cu(II), Ni(II) and Zn(II) ions were applied separately to the metal chelate column to investigate ligand specificity with respect to protein retention. As a result, the Cu(II) chelated chromatography gave the best resolution for all the hirudins tested and appeared to be the only IMAC that could be used generally for the purification of hirudins with a decreasing pH gradient.

Since Porath *et al.* (11) first introduced immobilized metal ion affinity chromatography (IMAC), it has been an important and widely-used tool in the analytical and preparative purification of proteins. The concept of IMAC is to use the ability of the functional groups on the protein surface to bind the metal ions immobilized on the solid matrices. Although IMAC holds a number of advantages over conventional biospecific affinity chromatography for protein purification (1), its use was often limited to the purification of proteins containing particular surface-accessible amino acids or coordination groups which exhibit a high affinity for immobilized metal ions. Advances in the recombinant DNA technology have made it possible to use IMAC for the purifcation of proteins or peptides without surface-accessible metal-binding groups, by engineering them so that they contain amino acids with a metal-binding property (6, 9, 13). More recently, a His-X_3-His synthetic metal-binding site has been incorporated into an α-helix of bovine somatotrophin and cytochrome c, and partitioning experiments of these variants have been carried out in metal-affinity aqueous two-phase systems (15, 16).

Hirudin, a polypeptide of 65-amino acid protein isolated from the salivary gland of the bloodsucking leech, is a potent thrombin-specific inhibitor (10). It blocks thrombin-mediated conversion of fibrinogen to fibrin in clot formation. The gene coding for hirudin has been synthesized and cloned into a yeast expression vector, YEGα-1, containing the α-mating factor pre-pro leader sequence and galactose-inducible promoter, *GAL*10 (14). Biologically active hirudin was found to be secreted into the extracellular medium by recombinant *Saccharomyces cerevisiae*.

In this study, IMAC has been applied as a tool for the purification of recombinant hirudin produced by *S. cerevisiae*. In addition, hirudin variants were engineered genetically to contain more surface histidines. Then the metal-binding affinities and the specific antithrombin activities of these variants were compared with those of the wild type.

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Key words: Immobilized metal ion affinity chromatography, hirudin variants, histidine

MATERIALS AND METHODS

Yeast Strains and Plasmids

Haploid *S. cerevisiae* 2805 (*Mat α pep4::HIS3 prb1-δ can1 his3d ura3-52*) was used as a host for the gene expression and secretion of both the wild type and the variants of hirudin. Plasmid pBα-HIR12 harboring mating factor α(MFα) pre-pro leader sequence and an original synthetic hirudin gene were used as the PCR template in the preparation of hirudin variants. Plasmid YEGα-1 containing *GAL10* promoter, MFα pre-pro leader sequence, *URA3*, 2 μm and β-lactamase gene was used as the general backbone of expression and secretion vector for the wild type hirudin and its variants (14). The genes of the wild type and its variants were subcloned to YEGα-1, and the resulting plasmids, YEGα-HIR525, YEGα-MH7, YEGα-MH9 and YEGα-MH13, were transformed to *S. cerevisiae* strain following the method described by Gietz and Schiestl (5). Individual colonies of the transformants harboring plasmids were used to prepare both the hirudin wild type and its variants.

Construction of Hirudin Variants

The hirudin variants were prepared by PCR mutagenesis as described by Horton and Pease (7). To mutagenize the wild type gene, several PCR primers synthesized by DNA synthesizer (Applied Biosystems, Model 391, USA). Two mutagenic PCR primers, PH7-19(+)(dGGTACTCCAAACCCAGAAT) for the variant MH7 and PH9-20(+)(dCTCCAAACCACGAATCTCAC) for the variant MH9, were used to replace Gly^{54} with His^{54} and Pro^{48} with His^{48}, respectively. The PCR mutagenesis consisted of two consecutive steps. In the first step, 177 bp DNA fragment in MH7 was amplified with the mutagenic primer, PH7-19(+), and the reverse primer, dGTACCAGTATCGACAA, using pBα-HIR12 as a template. In the second step, a mutagenized full-length hirudin gene was derived from 177 bp mutagenized DNA fragment from the first PCR step and the universe primer, dGTAAAACGACGGCCAGT, from the template, pBα-HIR12. The 698 bp DNA fragment was digested with *Eco*RI-*Sal*I and subcloned to pBluescript KS+. The resulting plasmid, pBKS-MH7, was used as a template for the next PCR mutagenesis. A hirudin variant MH13 was made from MH7 with PH9-20(+) as a mutagenic primer by using the same procedure described above. In the variant MH13, Pro^{48} and Gly^{54} were replaced by histidine. The DNA sequence was confirmed by the dideoxy method (12).

Media and Culture Conditions

For seed culture, the cells were cultivated at 30℃ in 100 m*l* of glucose-based medium composed of 40 g/*l* of glucose, 8.5 g/*l* of yeast nitrogen base w/o amino acid, 10 g/*l* of succinic acid, 4 g/*l* of NaOH, 5 g/*l* of casamino acid, 200 mg/*l* of adenine and 200 mg/*l* of tryptophan. After 24 hours of cultivation, 100 m*l* of the seed culture was transferred and cultivated at 30℃ for 80 hr in a 2 *l* scale jar fermentor containing 900 m*l* of galactose based medium containing 40 g galactose, 20 m*l* glycerol, 8.5 g yeast nitrogen base w/o amino acid, 10 g succincic acid, 4 g NaOH, 5 g casamino acid, 200 mg adenine and 200 mg tryptophan.

Immobilized Metal Ion Affinity Chromatography (IMAC)

Chelating Sepharose 6B(Pharmacia) was poured onto a chromatographic column (bed volume, 5 m*l*), and the column saturated with the metal ions. 20 mM solution of metal was applied to the column in the form of sulfate salt to remove unbound metal ions, the column was washed with distilled water, 20 mM sodium acetate/1 M NaCl buffer (pH 4.0) and finally with 20 mM sodium acetate/1 M NaCl buffer (pH 6.0). Before the sample was loaded onto the column, the culture broth was diafiltered with 20 mM sodium acetate/1 M NaCl buffer (pH 6.0) and concentrated with a ultrafiltration device equipped with a membrane of molecular cut-off 3,000. For the elution of proteins, the column was washed with 20 mM sodium acetate/1 M NaCl buffer (pH 5.0) and finally by a continuous pH gradient formed by the gradual mixing od 10 mM sodium acetate/1 M NaCl buffer (pH 4.0) with 20 mM sodium acetate/1 M NaCl buffer (pH 6.0). After each experiment, the column was regenerated with 0.1 M EDTA and washed with distilled water. The metal-free chelating gels were stored at 4℃ and charged with the metal ions directly before use.

Analytical Methods

Antithrombin activity was measured by a colorimetric assay using Chromozyme TH as a thrombin chromogenic substrate. The amidolytic cleavage of Chromozym TH made by thrombin was measured as a rate of increase in absorbance at 405 nm using a microtiter plate reader (BioRad, USA). Antithrombin activity was expressed in ATH: one unit represents 1 NIH unit of thrombin neutralized at 37℃. The commercial hirudin from leeches (Sigma, USA) was used as a standard.

RESULTS AND DISCUSSION

Protein retention in IMAC depends on the surface-accessible amino acids such as histidine, cysteine and tryptophan, capable of complexing with the immobilized metal ion. Among these amino acids, the exposed histidine is the amino acid which plays a major role in the interaction between proteins and metal ions. If a protein contains surface-accessible histidines, it binds the immobilized metal ions through the coordination between the free-base form of the imidazole ring and metal ion. The wild type hirudin contains a single histidine at position 51, which appears to be solvent-accessible because it

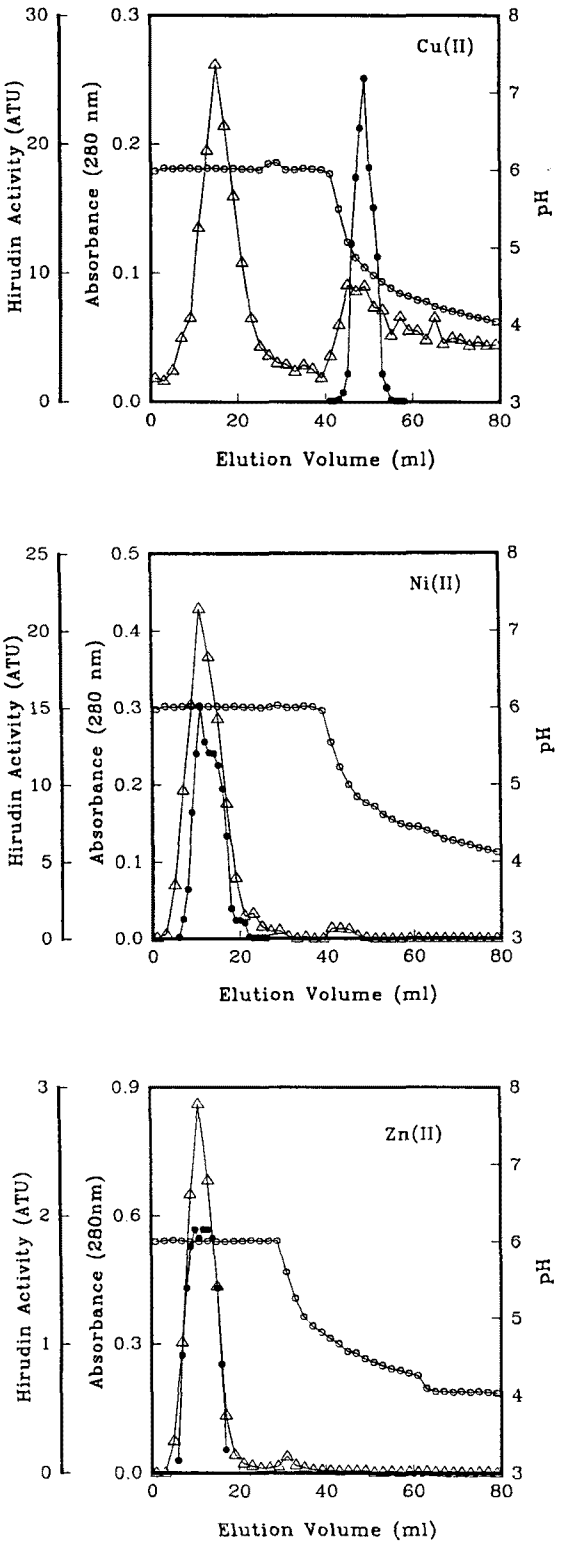
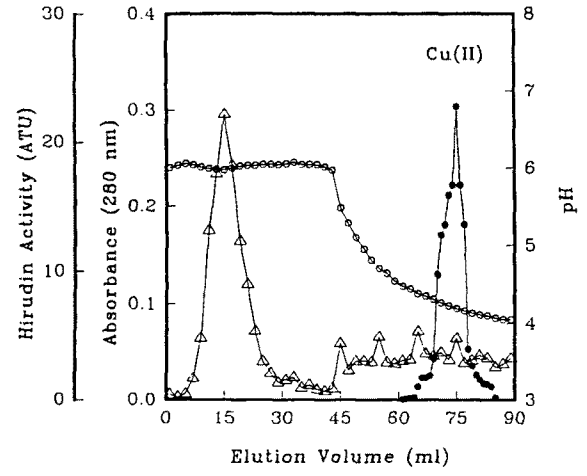


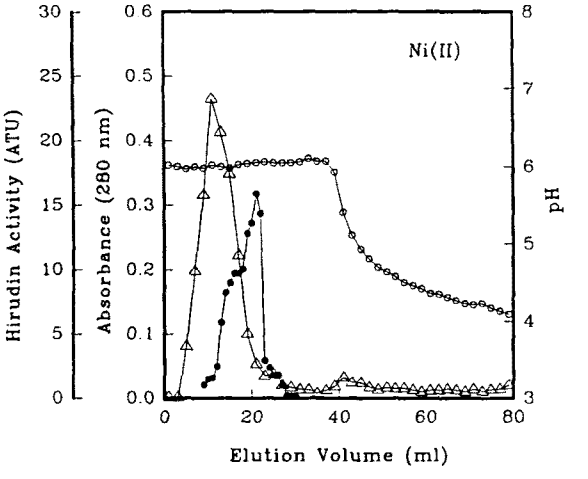
Fig. 1. Elution profiles of the culture borth containing the wild type hirudin on an IDA-Sepharose 6B gel chelate column charged with Cu(II), Ni(II) and Zn(II) ions: (△) absorbance at 280 nm; (○) pH; (●) hirudin activity.

is located in the exposed loop (residues 47-55) (4). As expected, the wild type exhibited retention in Cu(II)-chelated affinity chromatography, mainly due to the interaction between the immobilized Cu(II) ion and His^{51} (Fig. 1). Most contaminant proteins were eluted with a starting elution buffer of pH 6.0, and they indicated no interaction with the immobilized Cu(II) ion at this pH. When the buffer became increasingly more acidic, hirudin was eluted by the protonation of the imidazole ring of His^{51}. In addition to Cu(II) ion, Ni(II) and Zn(II) ions were also applied separately to the metal chelate column to investigate the ligand specificity with respect to protein retention. No retention of the wild type was observed on Ni(II)- and Zn(II)-chelated chromatography columns. The results demonstrate that IDA-Cu(II) column has a unique resolution for the wild type hirudin which conveys a single exposed histidyl residue.

Histidine is a relatively rare amino acid, accounting for only about 2% of the amino acids in globular proteins, and only about half are exposed on the protein surface (1). The scarcity of the surface-accessible histidines limited the use of IMAC in the purification of native proteins. But, recently, this problem was solved by conferring the proteins of interest by using recombinant DNA technology so that they exhibit an affinity for immobilized metal ions. As noted above, the wild type hirudin was retarded on an IDA-Cu(II) column due to the presence of a single surface-accessible histidine. However, it appears that more surface-accessible histidines are required for a stronger hirudin/metal ion interaction. In this study, therefore, the hirudin variants were genetically engineered to contain more histidines so that the retention in IMAC can be increased.

The MH7 and MH9 variants were constructed by substituting Pro^{48} and Gly^{54} with His, respectively. A single substitution of Pro^{48} with His increased dramatically the retention for the MH7 variant (Fig. 2). However, the MH9 variant which conveyed two histidines was eluted at pH 5.2 on an IDA-Cu(II) column. The MH9 retention had increased very slightly (elution pH=5.4) (Fig. 3). Although these two variants contained the same number of histidines, they showed a significant difference in their affinities for the IDA-Cu(II) ion. In general, the protein retention in IMAC is affected by the number of accessible electrondonating groups on the protein surface, the type of the metal immobilized, and the pH of the elution buffer. Besides these parameters, the degree of surface exposure and accessibility can also affect the interaction between proteins and immobilized metal ions. Therefore, His^{48} of the MH7 variant is probably more accessible to the immobilized Cu(II) ion than His^{54} of the MH9 variant.





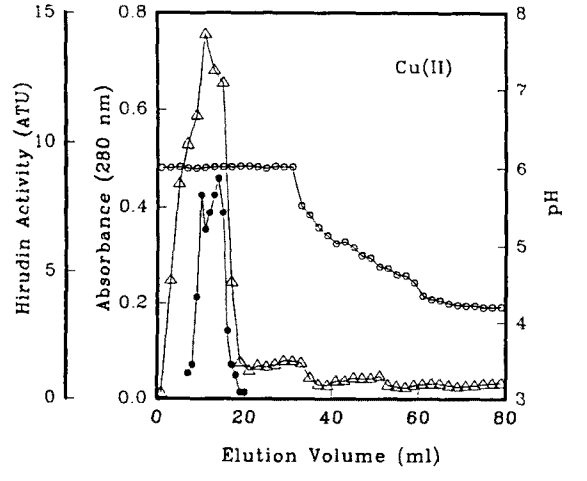
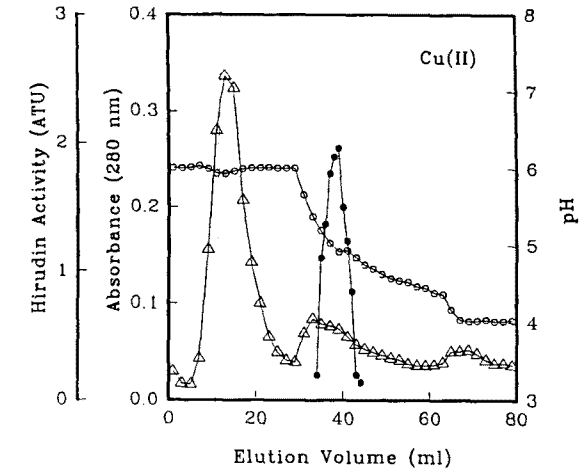
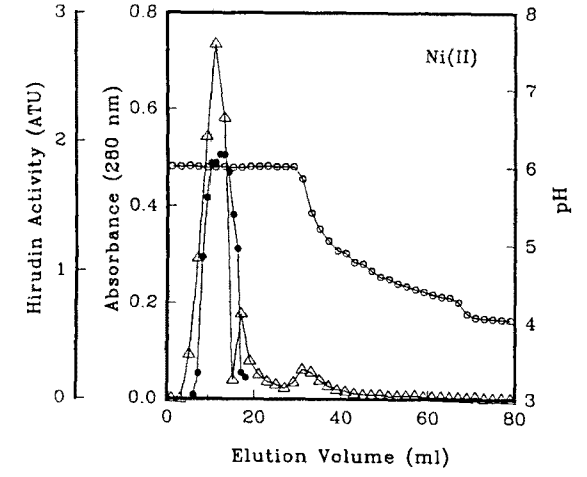


Fig. 2. Elution profile of the culture broth containing the MH7 variant on an IDA-Sepharose 6B gel chelate column charged with Cu(II), Ni(II) and Zn(II) ions: (△) absorbance at 280 nm; (○) pH; (●) hirudin activity.





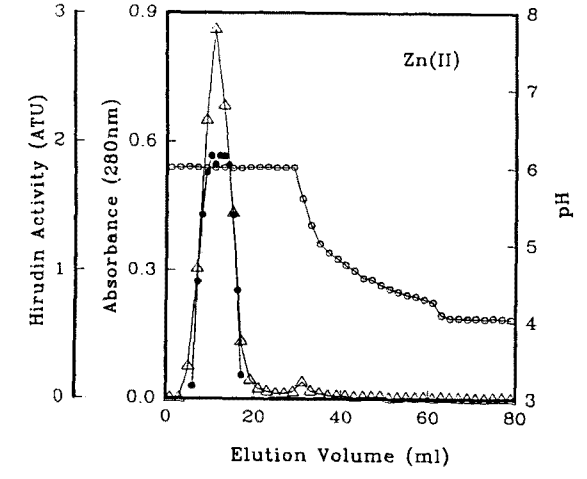


Fig. 3. Elution profiles of the culture broth containing the MH9 variant on an IDA-Sepharose 6B gel chelate column charged with Cu(II), Ni(11) and Zn(II) ions: (△) absorbance at 280 nm; (○) pH; (●) hirudin activity.

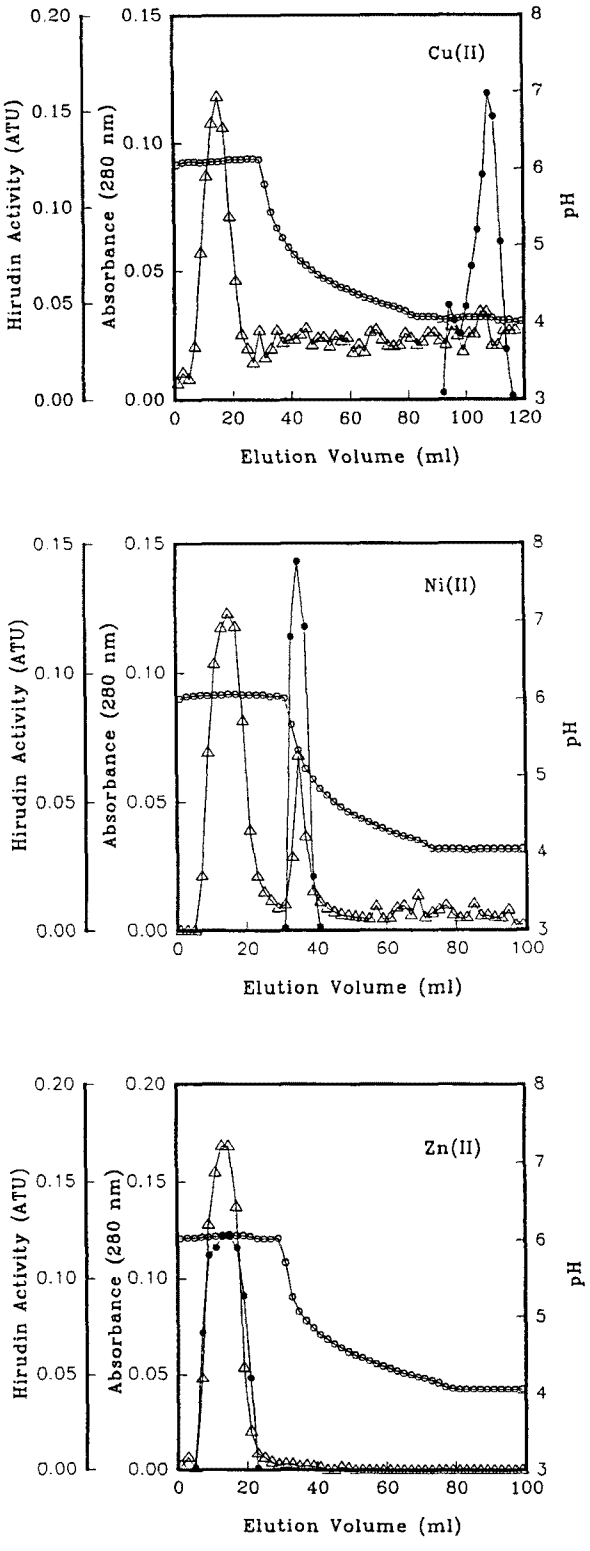


Fig. 4. Elution profile of the culture broth containing the MH13 variant on an IDA-Sepharose 6B gel chelate column charged with Cu(II), Ni(II) and Zn(II) ions: (△) absorbance at 280 nm; (○) pH; (●) hirudin activity.

The MH13 variant was engineered so as to contain two histidines more than the wild type, by substituting both Pro^{48} and Gly^{54} with His. As shown in Fig. 4, the MH13 variant was eluted when the pH of elution buffer reached 4.0, indicating the highest affinity for IDA-Cu(II) ion among the four hirudins tested. This increased retention of the MH13 variant resulted from the increased number of surface-accessible histidines. Furthermore, the MH13 variant exhibited retention on an IDA-Ni(II) column.

The MH7 and MH13 variants were retarded on a NI(II) chelated column, while no retention on a Zn(II) chelated column was observed with all the hirudins tested. Although the resolutions of the wild type, MH7 and MH9 variants on an IDA-Ni(II) column were not satisfactory, IDA-Ni(II) ligand could be used to purify the MH13 variant from the culture broth. The elution pH's of the four hirudins in Cu(II), Ni(II) and Zn(II) chelated affinity chromatography are summarized in Table 1. The separation of three sets of standard protein mixtures on a high-performance IMAC has been previously performed by Belew *et al.* (2). The affinity of the test proteins for the immobilized metal ions followed the order of Cu(II) >Ni(II)>Zn(II), which corresponded with our observation. As a result, the Cu(II) chelated chromatography gave the best resolution for all the hirudins tested and appeared to be the only IMAC that could be used generally for the purification of hirudins with a decreasing pH gradient.

As described above, the hirudin variants exhibited more retention in IMAC than the wild type, offering the potential for the large-scale application of IMAC in the purification of these variants. But because the variants have to retain biological activities equivalent or comparable to the wild type to be used practically, we investigated whether their biological activities had been affected by the site-directed mutageneses. The specific antithrom-

Table 1. Elution pHs of various types of hirudins in IMAC

Hirudin type	Metal ion	Elution pH
wild type	Cu(II)	5.5
	Ni(II)	flow-through
MH7	Cu(II)	4.4
	Ni(II)	flow-through
MH9	Cu(II)	5.2
	Ni(II)	flow through
MH13	Cu(II)	4.0
	Ni(II)	5.7

*All the hirudins exhibited no retention on an IDA-Zn(II) column.

Table 2. Structure and specific antithrombin activity of various types of hirudins

Hirudin type	Histidine sites	Specific activity
wild type	His^{51}	13,000 ATU
MH7	His^{48}-X_2-His^{51}	11,000 ATU
MH9	His^{51}-X_2-His^{54}	9,700 ATU
MH13	His^{48}-X_2-His^{51}-X_2-His^{54}	4,300 ATU

bin activity of the purified recombinant hirudin was assessed to be 13,000 ATU/mg protein on the basis of the amount of protein calculated from amino acid composition analysis (14). This agrees with the values reported for the antithroumbin of the natural and recombinant hirudins which range from 12,000 to 15,000 ATU/mg protein (8). The specific activities of the hirudin variants were determined by comparing the peak area of the wild type obtained from a HPLC chromatogram with those of the variants (3). Table 2 shows specific antithrombin activities of the wild type and its variants. The specific acitivities of MH7 and MH9 variants were 11,000 ATU/mg and 9,500 ATU/mg, respectively.

It is of interest to compare the experimental results on MH7 and MH9 variants. Replacement of a single amino acid residue of different positions resulted in significant differences in their metal-binding characteristics and biological activities. The mutation site of MH7 variant is located in the region concerning the interaction of NH_2-terminal residue of hirudin with a thrombin active site, while the mutation site of MH9 variant is not related to any active site. However, the MH7 variant exhibited higher antithrombin activity than the MH9 variant. It appears that the replacement of Pro^{48} with His affects the antithrombin activity of hirudin less than that of Gly^{54}. The MH13 variant with two mutation sites retained only 33% of the specific antithrombin activity of the wild type, and this should prevent this variant from being used as a therapeutic agent, even though it showed the strongest retention on the IDA-Cu(II) column.

On the basis of these results, attempts were made to fractionate the wild type and the MH7 variant on an IDA-Cu(II) column, and the results were compared by electrophoretic analysis of the subfractions on gradient polyacrylamide gels (Fig. 5). In both cases, the most contaminant proteins were eluted in a washing step, and the wild type and MH7 variant were purified to a considerable degree by one step IMAC. Although the MH7 variant was more purified than the wild type in IMAC, some contaminant proteins still existed in this collected fraction. Collected hirudin fractions retained over 90% of their original antithrombin activity in all cases. In terms of both the antithrombin activity and the purification

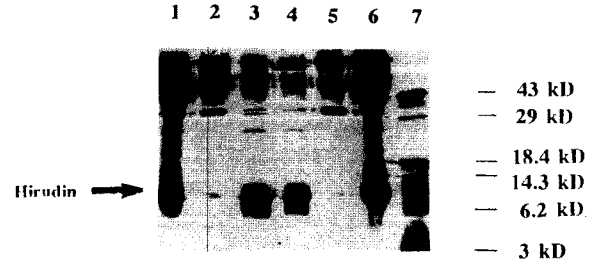


Fig. 5. Polyacrylamide gradient gel electrophoretic analysis of fractions from the experiments referred to in Fig. 1 and 2.

Lane 1: protein mixture containing the wild type before IMAC, lane 2: flow-through in Fig. 1, lane 3: fraction of the wild type, lane 4: fraction of the MH7 variant, lane 5: flow-through in Fig. 2, lane 6: protein mixture containing the MH7 variant before IMAC, lane 7: molecular weight marker proteins.

efficiency using IMAC, the MH7 variant can be recommended as the most favorable one among four hirudins. However, further efforts should be made to develop a more sophisticatedly-designed hirudin variant which is capable of retaining the native hirudin activity as well as interacting with immobilized metal ions in a more selective manner.

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