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RAPID COMMUNICATION

Simple preparation of immobilized-metal affinity chromatography media

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Abstract−Immobilized-metal affinity chromatography (IMAC) media was prepared. Iminodiacetic acid (IDA) was optimally coupled to the oxirane-activated gel at pH 13.0 and 60 °C in 0.1-0.15 g of IDA per ml of 2 M Na_2CO_3 for 5-7 hours. The amount of coupled IDA was 600-800 micromoles per gram of dried gel by determining zinc (II) ion with atomic absorption spectroscopy. Adsorption and desorption of protein sample to IDA-coupled media was made and the result is compatible to ones reported previously. The efficiency of column chromatography was discussed on partially purifying β-galactosidase from *E. coli* as the protein sample by zinc (II) ion chelate affinity column.

Key words: Iminodiacetic Acid, Zinc (II) Ion Chelate, Immobilized Metal Affinity Chromatography (IMAC)

INTRODUCTION

Immobilized-metal affinity chromatography (IMAC) is a separation technique based on the affinity of protein molecules to heavy metal ions, such as zinc (II), cupric and mercuric ions. As with other methods, IMAC is used where rapid purification and substantial purity of the product are necessary. Compared to other affinity separation it cannot be classified as highly specific, but moderately so. However, IMAC methods have several advantages over biospecific affinity chromatographic methods.

On that viewpoint, the IMAC method was used effectively in the 1970s for purifying human fibroblast interferon [1], human milk lactoferrin [2], and huma plasma α2-glycoprotein [3] after the suggestion for human serum albumin [4]. Recombinant proteins were also purified in the 1980s with new metal affinity gels [5]. More researches have been directed to membrane chromatography [6] and mechanism of adsorption on the surface of IMAC gel [7].

IDA is the most widely used chelating compound. It is commercially available from many producers such as Pierce, Amersham and Sigma. Stationary phases are also available in various forms. Soft matrix such as agarose or dextran as well as inorganic adsorbents like silica have been used as stationary phases. This research aims at assessing the conditions of coupling IDA to agarose, which is not well open in the literature.

MATERIALS AND METHODS

Sepharose 4B, butanedioldiglycidylether (oxirane), and IDA were purchased from Sigma. Zinc chloride was the product of Merck and other chemicals were of analytical reagent grade. Atomic absorption spectroscopy was by Varian Model AA575. According to the method of Sundberg and Porath [8], oxirane was attached to Sepharose 4B, and the bound oxirane was determined by titration with sodium thiosulfate [4]. Coupling IDA to the above activated gel was assessed with respect to the change of pH, temperature, time, and IDA concentrations, and they were 13.0 and 40 °C, unless stated otherwise. Coupled IDA was indirectly determined by measuring the bound zinc ion. IDA-coupled gel was soaked in $ZnCl_2$ solution (1 g/ml of distilled water) and washed extensively with water. Then, desorbing zinc ion from the washed IDA-gel with 20 ml of 0.05 M EDTA solution was measured by spectroscopy.

In IMAC, the crude β-galactosidase solution was subjected to the 0.6×8.0 cm zinc (II)-chelated column and eluted stepwise with 0.1 M tris buffer pH 8.4, 0.1 M sodium phosphate buffer (SPB) pH 7.0, and 0.1 M sodium acetate buffer (ACT) pH 4.7. The protein concentration was determined by 280 nm absorbance with conversion factor 1.0 mg/OD and the unit of the enzyme activity was defined as micromole of o-nitrophenol produced per minute from o-nitrophenyl-galactoside by 375 nm absorbance with conversion factor 1,000 μg/OD.

RESULTS AND DISCUSSION

1. Assessment of Coupling IDA to Agarose Gel

The amount of IDA coupled to the oxirane-activated gel was determined as 600-800 micromole per gram of the dried gel, and this was compatible with other results [8]. IDA coupling was increased exponentially to 75% within the first 1 h and to above 90% after 8-10 h, setting 800 micromole of IDA be 100% (Fig. 1). This result seems to be remarkably different from the other suggestions to take 16 h or 24 h [1,4].

According to the increase of pH from 8.0 to 13.0, IDA coupling was enhanced (Fig. 2). This effect implies that a non-zwitterionic form of IDA at pH 13.0 exists and there is an increase of nucleophilicity on the nitrogen atom of IDA at the strongly alkaline condition. Owing to the solubility of IDA, the reaction was not performed below pH 8.0.

By increasing temperature from 30 °C to 70 °C, IDA coupling increased from 60% to near 100%, respectively, when the reaction was performed for 1 h (Fig. 3). Thus it is considered that the coupling at a high temperature (70 °C) rather than at a lower tempera-

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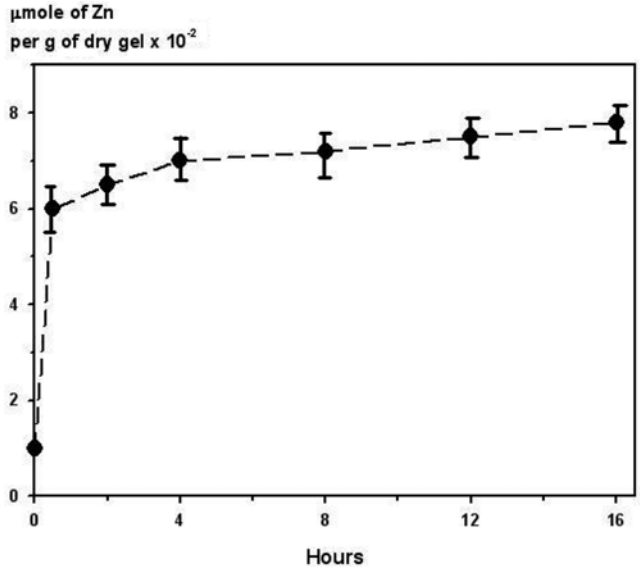

Fig. 1. Time course of IDA coupling. IDA was coupled at pH 13.0 adjusted by intermittently adding 0.6 M NaOH, 40 ºC and in 0.2 g IDA per ml mixture. Coupled IDA was determined by measuring Zn^{++} in chelation to the IDA-oxirane-sepharose gel.

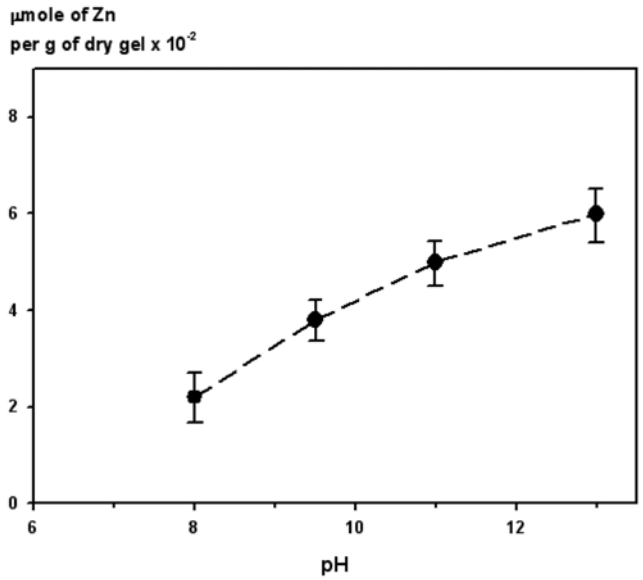

Fig. 2. pH effect on IDA coupling. At the indicated pH's, oxirane-activated gel was coupled. The IDA coupling was estimated as Fig. 1.

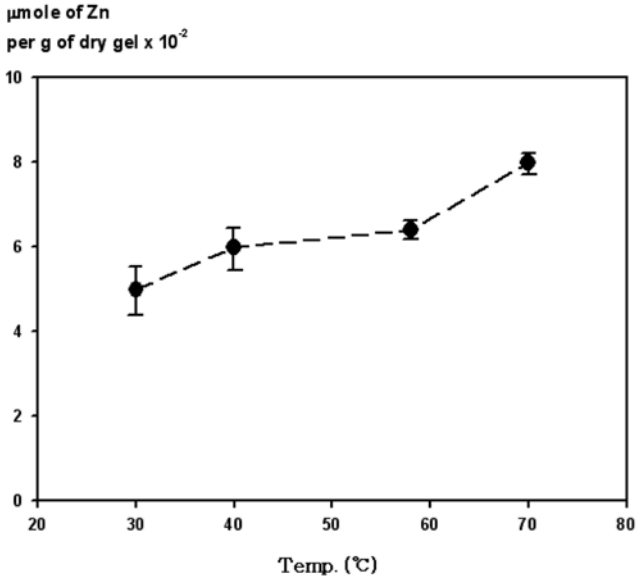

Fig. 3. Temperature effect on IDA coupling. IDA was coupled to the gel at pH 13.0 in 0.2 g IDA 1 ml mixture for 1 h. The coupled IDA was determined as Fig. 1.

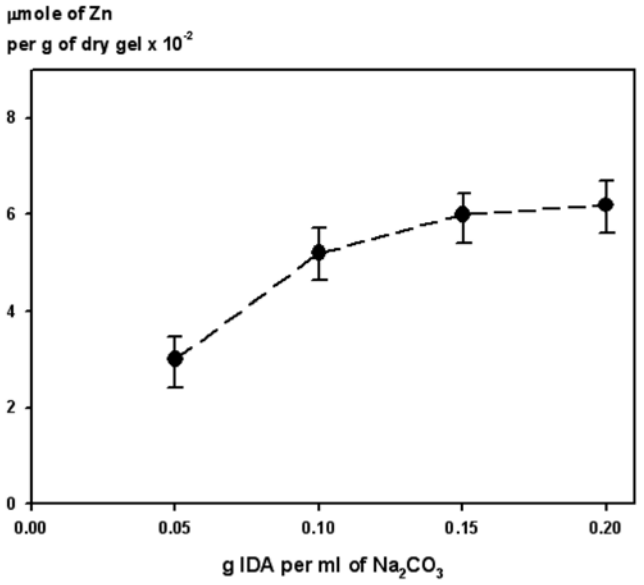

Fig. 4. Efficiency of IDA concentration in its coupling. IDA was coupled to the gel in its varying concentration in 2 M Na_2CO_3.

ture (40 ºC) is preferred as Fig. 1.

Within the limit of the solubility of IDA in 2 M Na_2CO_3, high concentration of IDA was effective in its coupling to the gel (Fig. 4), but at a concentration above 0.1 g/ml, the rate of increase of IDA coupling was not so effective only by 10% increase. Because of the solubility of IDA, the reaction in a mixture more than 0.2 g/ml was not performed.

2. IMAC of β-Galactosidase

β-Galactosidase is known not to be affected by zinc ion (slightly activated), while the inhibition by cupric or mercuric ions is remarkable [9]. When the crude fraction eluted stepwise, most of the activity occurred in the pre-elution step by 0.04 M $MgCl_2$ and 0.02 M β-mercaptoethanol (Fig. 5), showing the moderately increased purification degree (Table 1). This result was consistent with the weak binding affinity of zinc ion to the enzyme. As the control experiment, the elution pattern without zinc ion showed all of the protein and the activity in the first pre-elution step of gels (data is not shown).

Further purification by IMAC could not be carried out because

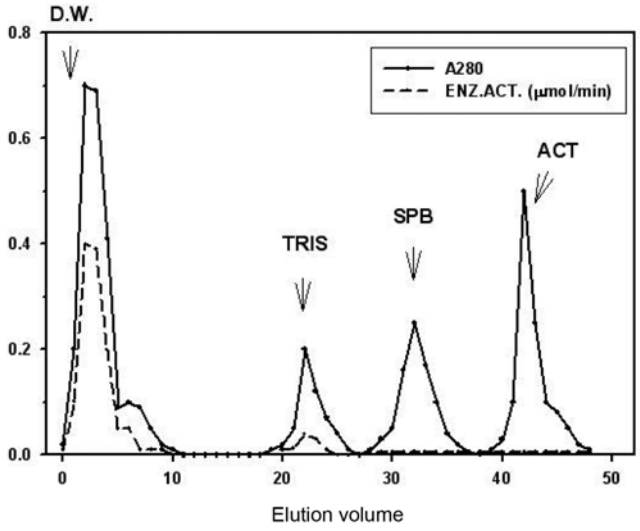

Fig. 5. Zinc (II) - chelate IMAC of β-galactosidase. Crude enzyme solution was applied to the 0.6×8.0 cm column pre-equilibrated by 0.04 M $MgCl_2$ and 0.02 M β-mercaptoethanol solution and was eluted stepwise by 0.1 M of the buffers indicated with arrows.

Table 1. Purification degree by zinc (II) - chelate IMAC

Sample	Total activity	Total protein	Specific activity	Yield (%)
Crude	21.5	5.10	4.2	100.0
Pre-elution	16.7	2.44	6.8	77.7
Tris buffer	0.44	0.35	1.3	2.0
Phosphate buffer +Acetate buffer	-	1.76	-	-

of the reactivity of β-mercaptoethanol with copper. These experiments were intended to show the efficiency, and it was reserved to test the homogeneity of enzyme eluted.

SUMMARY

Judging from the assessment of IDA-coupling to the oxirane-activated gel, it can be enough to take 5 to 7 h, not 16 h or 24 h. Furthermore, considering the solubility of IDA to 2 M Na_2CO_3, it seems advantageous to perform the coupling at a concentration of 0.1 g to 0.15 g IDA per ml of 2 M Na_2CO_3 because lower IDA concentration is more alkaline for effective coupling. IMAC was less effective for β-galactosidase than for human serum [1,4] and for others [2,3]. However, it was considered as the reason why β-mercaptoethanol for preventing the enzyme multimer from dissociation was another reactive reagent against heavy metal ions. Despite the insufficient purification, the simplicity of preparing media and the utility of the metal ligand for IMAC can be appreciated in accordance with improvements.

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